CHARACTERISATION OF T CELLS IN RATS THAT DEVELOP INDEPENDENTLY OF THE THYMUS: LYMPHOCYTES WITH POTENTIAL REGULATORY ROLES

by

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A dissertation submitted to the University of Adelaide
in candidature for the degree of
Doctor of Philosophy in the Faculty of Science
June 1999
DECLARATION

This thesis contains no material which has been accepted as full or part requirement for the award of any other degree or diploma in any university or tertiary institution.

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ABSTRACT

It is becoming accepted that there exist sites, other than the thymus, that support T cell differentiation. Proposed sites of thymus-independent T cell differentiation include the liver and the gut epithelium. Previous work in this laboratory has identified a previously uncharacterised structure within the rat small intestine as another candidate site for extra-thymic T cell development. These structures are present in both euthymic and athymic rats and take the form of modified villi that contain closely packed lymphocytes and dendritic cells. It was shown that the majority of the lymphocytes in these lymphocyte-filled villi (LFV) did not express markers of mature B cells and T cells but they did express CD25 and CD43. Furthermore, lymphocytes within LFV were shown to undergo cell division, they were excluded from the recirculating pool and they included a minor population of cells which expressed markers of mature T cells (which include the α/β TCR, CD3, CD2, CD5 and CD4). It is shown herein that the major population of CD25+ CD43+ cells also express CD44 and CD161, a phenotype similar to that expressed by immature thymocytes at the time of commitment to the T cells lineage. These observations, together with the detection of RAG-1 protein in LFV, indicate that LFV have similarities to the thymus and are likely sites of thymus-independent T cell development. It is shown that with increasing age, cells expressing the α/β TCR increase in number in the tissues of athymic rats. Cells expressing the α/β TCR are detected in the TDL of young adult athymic rats before they are found in significant numbers in lymphoid organs. This suggests that the gut may be the source of these cells and raises the possibility that T cells developing at mucosal sites, in particular in LFV, may seed the peripheral lymphoid organs of athymic rats. Furthermore, the presence of LFV in euthymic rats indicates that they may contribute a corresponding thymus-independent T cell population in normal animals. Previous
studies have shown that the α/β T cells found in athymic rats cannot mediate rejection of allografts and hence are not the functional equivalents of conventional thymus-derived T cells. Phenotypic comparisons presented in this study show that the α/β T cells present in athymic rats are distinct from the majority of α/β T cells found in euthymic littermates. They have a larger mean size, exhibit a lower level of surface TCR expression, a higher proportion express activation markers and the majority express a pattern of adhesion molecules consistent with previous antigenic stimulation. Furthermore, additional work showed that the α/β T cells found in athymic rats share a number of features with NKT cells, a recently described subset of T cells with unique functional properties. These differences may be reflected in the observations reported in this thesis that show that as a whole, the α/β T cells found in athymic rats recirculate in reduced numbers and show a different pattern of tissue distribution following adoptive transfer. Moreover, examination of the cytokines produced by T cells following in vitro stimulation showed that NKT cells and α/β T cells from athymic rats produce large amounts of IFN-γ. Collectively, the information detailed in this thesis show that the NKT cells and the thymus-independent α/β T cells present in athymic rats are phenotypically and functionally related. This raises the possibility that thymus-independent α/β T cells are distinct from conventional T cells and that their functions in normal individuals are regulatory, as has been suggested for NKT cells.
Abstracts and Conference Presentations

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ABBREVIATIONS

ATXBM  adult thymectomised, irradiated and bone marrow reconstituted mice
APC  antigen presenting cell
Az  azide
bp  base pair(s)
CD  cluster of differentiation
cDNA  complementary deoxyribonucleic acid
CFSE  5- and 6-carboxyfluorescein diacetate succinimidyl ester
CMI  cell mediated immunity
Con A  concanavalin A
DC  dendritic cell
DN  double negative
DNA  deoxyribonucleic acid
DP  double positive
FACS  fluorescence activated cell sorting
FITC  fluorescein isothiocyante
FL  logarithmic/linear fluorescence intensity
FCS  fetal calf serum
FSC  forward scatter of light
G  gauge
³H-TdR  tritiated thymidine
HEV  high endothelial venules
ICAM-1  intracellular adhesion molecule-1
IDDM  insulin-dependent diabetes mellitus
IFN-γ  interferon-gamma
IEL  intraepithelial lymphocytes
Ig  immunoglobulin
IgG₁  immunoglobulin gamma-1 isotype
IgG₂a  immunoglobulin gamma-2a isotype
IL  interleukin
IPTG  isopropyl-thiogalactoside
i.v.  intravenously
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>k/o</td>
<td>knockout</td>
</tr>
<tr>
<td>LFA-1</td>
<td>leukocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LFV</td>
<td>lymphocyte-filled villi</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mLN</td>
<td>mesenteric lymph nodes</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction</td>
</tr>
<tr>
<td>MNC</td>
<td>mononuclear cells</td>
</tr>
<tr>
<td>mφ</td>
<td>monocytes and macrophages</td>
</tr>
<tr>
<td>MPC</td>
<td>magnetic particle concentrator</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NMS</td>
<td>normal mouse serum</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rat serum</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>post capillary venules</td>
</tr>
<tr>
<td>PE</td>
<td>R-phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PLN</td>
<td>peripheral lymph nodes</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristateacetate</td>
</tr>
<tr>
<td>pTα</td>
<td>pre-T cell α-chain</td>
</tr>
<tr>
<td>PWM</td>
<td>pokeweek mitogen</td>
</tr>
<tr>
<td>R</td>
<td>receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
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</table>
rNu  Rowett mutation
RTE  recent thymic emigrant
RT-PCR reverse transcription-polymerase chain reaction
RTE  recent thymic emigrant
SCID  severe combined immunodeficiency
SD  standard deviation
SEM  standard error of the mean
SPF  specific pathogen free
SSC  side scatter of light
TAP  transporter associated with antigen processing
TCR  T cell receptor
TCR\textsubscript{intermediate}  \(\alpha/\beta\) T cells which express intermediate levels of TCR
TDL  thoracic duct lymph
TdT  terminal deoxynucleotidyl transferase
TN  triple negative
TNP  trinitrophenol
v/v  volume per volume
VLA  very late antigen
w/o  week old
w/v  weight per volume
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AMENDMENTS

p6  3rd last line, “theses” change to - these

p12  line 9, “thymus independent T” should read - thymus independent T cells

p17  line 14, “αβ T cells” should read - αβ T cells

p23  line 15, “of B7.1” omit - of

p40  line 2, “rearranged” change to - rearrange

p40  line 9, “show clearly shows” omit - shows

p60  line 3, “seconding” change to - secondary

p76  line 14, “μ/ml heparin” change to - U/ml heparin

p98  line 3, “determined the via” omit - the

p123  3rd last line, “continuos” change to - continuous

p129  4th last line, “αβ TCR+ cells contributed” should read - αβ TCR+ cells

from rNu/rNu rats contributed

p134  line 13, “γδ T cells rats” should read - γδ T cells in rats

p153  line 9, “lower that seen” should read - lower than that seen

p161  line 1, “larger mean” should read - larger mean size

p169  last line, “CD11b+ cell” should read - CD11b+ cells

p176  line 10, “ion” change to - in

p203  line 9, “CD161 (10/78” should read - CD161 (10/78)

p215  line 8, “sub-population to” should read - sub-population of

p219  line 18, “CD62low” change to - CD62Llo

Figure 6.2.3 legend, “(E-H)” should read - (E and F)
Chapter 1

Introduction
1.1 General Introduction

Lymphoid tissues are comprised of primary and secondary lymphoid organs. Secondary lymphoid organs, which include the spleen, lymph nodes and Peyer’s patches, are structures through which lymphocytes recirculate and where, in the event of antigen presentation, differentiation into effector cells may occur. In contrast, primary lymphoid organs are sites in which committed lymphoid stem cells undergo differentiation and maturation into functional lymphocytes. These processes in primary lymphoid tissues are independent of exogenous antigen. Classical primary lymphoid organs such as the thymus and the bursa of Fabricius are not sites of lymphoid recirculation. B cell differentiation, in mammals, occurs in the liver in foetal life and later in the bone marrow. T cell differentiation takes place primarily in the thymus, although recent research has shown that extra-thymic sites may also support T cell development. In particular, the gut appears to be a major site of thymus-independent T cell development.

1.2 Definition of T cells

The term T cell was coined to describe a cell belonging to a subset of lymphocytes that develops only in the presence of a functional thymus. T cells are essential in cell-mediated immunity (CMI) and although they are involved in antibody production in response to some antigens, they are distinct from antibody producing B cells.

The essential role of the thymus in the development of CMI was recognised from observations on congenital thymus deficiency (DiGeorge syndrome) in children (reviewed by Good, 1991). Contemporaneously, support for the vital role of the thymus was obtained from studies on the effects of neonatal thymectomy in mice (reviewed by Miller, 1991) and later, further support came from observations on congenitally athymic
(nude) animals (discussed in subsection 1.4.1). Before the discovery of the T cell receptor (TCR) the most definitive evidence that T cells were involved in an immunological response was the demonstration that such functions were absent in athymic individuals and that they could be restored by thymus engraftment. Later, with the development of antibodies that recognised quasi-lineage-specific surface antigens, identification of function by adoptive transfer of peripheral cells provided further evidence of T cell-dependence of CMI.

The definition of T cells changed after the identification of the antigen-specific T cell receptors α/β (Allison et al., 1982) and γ/δ (Brenner et al., 1986), and of the intimately associated CD3 complex (Reinherz et al., 1983). T cells expressing these receptors will be referred to herein as α/β T cells and γ/δ T cells respectively. Subsequent to the discovery of these uniquely lineage-specific antigen receptors, T cells could be redefined as cells that express a T cell receptor-CD3 complex (TCR-CD3). Importantly, this definition does not insist on the thymus as an essential site of T cell development.

1.3 T cell differentiation

1.3.1 T cell development in the thymus

The thymus gland is the main site of development for the great majority of T cells that are found at peripheral sites. Precursor cells expressing the CD3⁺CD4⁻CD8⁻ phenotype migrate from either the foetal liver or the bone marrow to the thymus, where they have the potential to develop into mature T cells (Wu et al., 1991). Some cells which migrate from the bone marrow have been shown to have TCR genes in germline configuration (Shortman et al., 1991) and they have the potential to give rise to either the α/β or the γ/δ T cell lineages. The mechanisms which regulate expression of the alternative TCR
lineages are unknown. However, the commitment to either type occurs before the co-expression of the CD4 and CD8 accessory molecules (Petrie et al., 1992).

The precursor cells that colonise the thymus are not restricted to differentiation in the T cell lineage. Recently, foetal blood haematopoietic progenitor populations have been characterised using the Thy1 (CD90) and c-kit (CD117) antigens. A population of cells that expresses the phenotype CD117+ CD90- contains multipotent cells, whereas cells with phenotype CD117lo CD90+ are T lineage restricted (Rodewald, 1995). Both populations can seed the thymus and it is not surprising, therefore, that some T cell progenitors in the thymus have the capacity to also generate cells of other lineages. Early thymic T cell progenitors have the potential to give rise to B cells, natural killer (NK) cells and dendritic cells (DC) (reviewed by Shortman and Wu, 1996 and Zúñiga-Pflücker and Lenardo, 1996).

Within the thymic environment, the CD4lo progenitors give rise to cells that express CD44 but are negative for CD3, CD4 and CD8 (triple negative [TN] cells) (reviewed by Godfrey and Zlotnik, 1993). Expression of CD44 is significant, as there is evidence to suggest that CD44 interactions are important during early thymic development (Patel et al., 1995). Godfrey and Zlotnik (1993) propose that commitment to the T cell lineage proceeds soon after with the expression of CD25 (IL-2 R-α chain) by this CD44+ population.

This model has been supported and extended by a recent study detailing commitment and differentiation events in the mouse foetal thymus (Carlyle et al., 1997). Upon entry into the thymus, there is rapid commitment of the multipotent progenitors to the
lymphoid lineages. These committed cells express CD117 and CD44. The thymic microenvironment induces expression of NK1.1 (CD161) by these cells and this step marks a commitment to the T and NK lineages, with the loss of the potential to differentiate into B cells. A second thymus-induced differentiation step is proposed in which cells acquire CD25, lose NK1.1 and become committed to the T cell lineage. Those cells that do not undergo this second step cease to express CD117 and become committed precursors of the NK lineage. Although research has focused on the murine model, it appears that this developmental pathway may be conserved across species, as immature human thymocytes have also been shown to express CD161 (Poggi et al., 1996).

As T-lineage committed cells continue to differentiate, they down-regulate expression of CD44. This transition from the CD44+ CD25+ TN stage to the CD44−CD25+ stage is accompanied by rearrangement of the TCR-β and TCR-γ loci (Godfrey et al., 1993). It is proposed that the branching point of the α/β and γ/δ TCR developmental pathways occurs soon after this. Support for this is derived from findings that many α/β T cells carry evidence of attempted rearrangements of TCR-γ genes (reviewed by Godfrey and Zlotnik, 1993). In the development of α/β T cells, those thymocytes that rearrange TCR-β genes productively in association with the recently identified invariant pre-TCR α (pTα) chain (Groettrup et al., 1993) are selected to continue maturation (reviewed by Groettrup and von Boehmer, 1993; Saint-Ruf et al., 1994 and Fehling et al., 1995). The pTα chain (or gp33) is expressed exclusively by T-lineage specific cells in both mice (Bruno et al., 1995) and humans (Ramiro et al., 1996).

The next stage of thymocyte development (reviewed by Godfrey and Zlotnik, 1993)
involves reduction in the expression of CD25, accompanied by the expression of CD4 and CD8 co-receptors. This transition also involves the first round of TCR-α gene rearrangement and expression, leading eventually to expression of low levels of CD3/TCR and high levels of CD4 and CD8. This phase of differentiation is referred to as the double positive (DP) stage. The thymic microenvironment is essential for this differentiation step to occur as isolated CD25+ TN cells fail to progress to DP cells in vitro.

Two recombination activating genes (RAG) are essential for recombination events leading to rearrangement of both immunoglobulin (Ig) and TCR genes in developing B and T cells respectively. As a consequence of this, inactivation of either the rag-1 or the rag-2 gene in mice results in the complete absence of both mature T and B cells. Differentiation of T cells in rag-1 and rag-2 mutant mice does not progress to the DP stage, remaining blocked at the CD117+ CD25+ CD4− CD8− (DN) stage (reviewed by Chen et al., 1994).

After reaching the DP stage of differentiation, thymocytes undergo positive and negative selection (Blackman et al., 1990, and reviewed by von Boehmer, 1990; Kisielow and von Boehmer, 1990). Negative selection inactivates cells with potentially autoreactive TCRs while during positive selection, TCR interaction with self-histocompatibility complex (MHC) molecules appears to avert programmed cell death (apoptosis). Less than 10 percent of cells survive the selection criteria and enter the periphery as self MHC-restricted immunocompetent cells (Egerton et al., 1990). Positive selection appears to be dependent on epithelial cells in the microenvironment of the thymus, while
negative selection appears to involve bone marrow-derived (possibly dendritic) cells (reviewed by Anderson and Jenkinson, 1997; Farr and Rudensky, 1998).

The process of T cell maturation in the thymus can be traced from the outer thymic cortex towards the cortico-medullary junction, where the processes of selection are believed to take place. Cells then enter the medulla, the site where the most mature T cells are found in the thymus (Blackman et al., 1990).

1.3.2 Thymus-independent T cell development

The near absence of T cells and the loss of classical T cell function in young neonatally thymectomized mice, adult-thymectomized irradiated bone marrow-reconstituted (ATXBM) mice and congenitally athymic mice and rats highlights the important role of the thymus in immunity. Clearly, thymic differentiation is the source of the majority of T cells found in peripheral sites within normal, euthymic animals and it is necessary for development of the classical functions of T cells.

However, cells expressing T cell receptors exist in congenitally athymic (nude) animals, in particular in mature animals. The existence of such cells raises the possibility that alternative pathways of T cell differentiation are used in these animals and that similar thymus-independent pathways of T cell differentiation may also exist in euthymic animals. In the particular instance of intraepithelial lymphocytes (IEL), there is strong evidence that a subpopulation of thymus-independent T cells that is present in nude animals is also present in euthymic animals and that these cells develop locally in the gut epithelium. These cells are discussed further in section 1.8. In vitro studies have shown that T cells can be cultured from human bone marrow cells (Adibzadeh et al.,
suggesting that thymus-independent T cell differentiation is also possible in humans, provided that appropriate signals are available in the extra-thymic environment (McCune et al., 1988).

As discussed above, expression of the recombination activating genes \textit{rag-l} and \textit{rag-2} are essential for TCR rearrangement and subsequent T cell development. Furthermore, expression of the pT\(\alpha\) chain is essential during the differentiation of \(\alpha/\beta\) T cells, although a functional gene encoding pT\(\alpha\) is not essential for development of \(\gamma/\delta\) T cells (Fehling \textit{et al.}, 1995). In tissues where B cells are not produced, identification of \textit{rag} gene products and/or pT\(\alpha\) gene products in lymphoid cells would provide convincing evidence that such cells were committed to differentiate into T cells. It is noteworthy, therefore, that recent studies have identified expression of the \textit{rag-1}, \textit{rag-2} and \textit{pT\(\alpha\)} genes at extra-thymic sites (see below). These findings provide strong evidence for thymus-independent T cell development.

1.4 Thymus-independent T cells

1.4.1 Congenitally athymic animals

Thymus-deficiency, in association with hairlessness, was first reported in mice in 1968 (Pantelouris, 1968). A similar genetic disorder was reported in rats in 1978 (Festing \textit{et al.}, 1978). In these athymic (nude) animals, the thymus does not develop fully and in postnatal life it is present only as a rudiment composed of undifferentiated epithelium and fat (Pantelouris, 1968; Festing \textit{et al.}, 1978; Blackburn \textit{et al.}, 1996). Recently, the cause of this defect has been linked to a mutation of the \textit{whn} gene, a member of the winged-helix protein family (Nehls \textit{et al.}, 1994; Blackburn \textit{et al.}, 1996). As mentioned earlier, the thymic epithelium and stromal cells provide the unique microenvironment
necessary for intra-thymic T cell differentiation (reviewed by Anderson and Jenkinson, 1997; Farr and Rudensky, 1998). As a result of the whn gene mutation, there is a defect in the differentiation of thymic epithelial progenitors (Blackburn et al., 1996). Also, the thymic rudiment of nude mice does not express MHC class II molecules (Jenkinson et al., 1981). The thymic rudiment in nude animals contains few lymphoid cells (Vos et al., 1980) and these do not appear to progress to establish a functionally significant pool of peripheral T cells.

Because they lack a functional thymus, congenitally athymic animals appear to provide an ideal model in which to study the development and function of thymus-independent T cells. However, despite the absence of key cell-mediated functions in nude mice and rats (Thomas et al., 1991), it has been proposed in one report that the thymic rudiment in nude mice is a source of functional T cells (Ikehara et al., 1987). Nevertheless, more recent studies (see below) have shown that the T cells which develop in nude mice are distinct from the majority of T cells present in normal mice phenotypically. In addition, the presence of cells that have a similar phenotype in ATXBM mice (Sato et al., 1995) shows convincingly that the thymic rudiment is very unlikely to be the source of T cells in these animals.

Another potential problem of the nude animal model is the possibility that thymus-derived T cells in euthymic animals might affect the development and function of thymus-independent T cells indirectly. Indeed, re-examination of the work of Bell and colleagues (1989) indicates that the number of thymus-independent T cells in nude rats is reduced by the presence of thymus-derived T cells. As another example, it has been postulated that thymus-dependent cells in euthymic animals control the differentiation of
NK cells into cytotoxic CD8* α/β TCR* cells (Kikly and Dennert, 1992). The observation that the levels of NK activity in peripheral tissues of nude mice and nude rats are higher than in normal littermates (De Jong et al., 1980) supports this suggestion. Because T cells and NK cells appear to arise from a common progenitor (see section 1.3.1), it is possible that thymus-derived T cells might exert a regulatory effect not only on NK cells but also on non-classical T cell subsets such as NKT cells (see section 1.7), IEL (see section 1.8) and on other thymus-independent T cells (see section 1.9).

### 1.4.2 Immunological characteristics of athymic animals

Athymic mice and rats have normal numbers of B cells and these cells respond in vitro to stimulation with pokeweed mitogen (PWM) in a manner comparable to the responses seen with lymphocytes from euthymic animals. Nude animals also produce antibody responses after immunisation with T-independent antigens such as lipopolysaccharide (LPS). In contrast, responses to T-dependent antigens such as tetanus toxoid and ovalbumin are poor or absent (Vos et al., 1980) and these animals fail to develop either germinal centres, memory B cells, or immunoglobulin isotype switching (Jacobsen et al., 1974).

With respect to cell mediated immunity (CMI), nude mice fail to reject either xenografts or allografts, while nude rats accept allografts. However, nude rats have the capacity to reject xenografts (Thomas et al., 1991) and to eliminate allogeneic lymphocytes rapidly (Rolstad and Ford, 1983) by a mechanism which may be initiated by NK cells (Rolstad and Fossum, 1987; Rolstad and Ford, 1983; De Jong et al., 1980). Nevertheless, congenitally athymic animals of both species lack classical CMI. Furthermore, the importance of thymus-derived T cells in this form of immunity can be demonstrated in
nude animals by the restoration of CMI by either adoptive transfer of T cells from normal animals or by engraftment with foetal thymi. For example, young nude rats grafted with a thymus are able to exhibit both the humoral and the cellular immune responses characteristic of euthymic animals (Hougan and Klausen, 1987). Nude rats are able also to reject allografts after reconstitution with syngeneic CD4+ T cells (Bradley et al., 1992; Dallman et al., 1982) or CD8+ T cells (Dallman et al., 1982) from normal syngeneic donors.

It remains a paradox, therefore, that nude animals have T cells (cells expressing surface TCR) and yet they fail to demonstrate classically T cell-mediated immune responses. The function(s), if any, of these thymus-independent T cells remains unknown. It is possible that these unique T cells are activated by non-classical antigens or via non-classical pathways and that they exhibit non-classical functions which are not detected by the usual in vivo measurements of CMI or by measurement of proliferative responses to antigens in vitro.

1.4.3 Characteristics of T cells found in nude animals

Lymphocytes from young athymic animals fail to respond to the T cell mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA). However, there is an increase in the responsiveness to these mitogens with age. This increased responsiveness correlates with increasing numbers of cells expressing T cell markers such as Thy-1 (CD90), α/β TCR, γ/δ TCR, CD5, CD4 and CD8 in the spleens and lymph nodes in nude mice (Kennedy et al., 1992, Lawetzky and Hündig, 1988) and with cells expressing the α/β TCR, CD5, CD4 and CD8 in the spleens, lymph nodes and thoracic duct lymph (TDL) of nude rats (Vaessen et al., 1986; Sarawar et al., 1991). In addition, T cells
expressing the α/β TCR purified from nude rats respond in mixed lymphocyte reactions (MLRs). An interesting addendum to this study of responsiveness to alloantigens was the observation that T cells from nude rats but not T cells purified from normal littermates, exhibited an MLR response to MHC class I molecules (Sarawar et al., 1991). The significance of this observation with respect to the antigen recognition mechanisms of NK T cells will be discussed later (section 1.7).

It appears, therefore, that T cells present in nude animals can indeed be stimulated by mitogens and that they may also respond to some foreign antigens. As discussed earlier, it is paradoxical that nude rat T cells respond in MLR but do not mediate allograft rejection. These findings can be interpreted in one of two ways. Either, the thymus-independent T cells that are present in nude animals represent an aberrant "dead-end" pathway of T cell development and have no useful function in vivo, or they could be cells which have specialised requirements for activation and/or functions which have not been recognised previously.

The α/β TCR+ cells that develop in athymic nude mice exhibit a number of phenotypic differences which distinguish them from the majority of α/β T cells present in euthymic animals. These differences will be summarised here briefly and discussed in more detail in later sections. Firstly, T cells from nude mice express lower levels of surface TCR when compared to normal littermates (Iiai et al., 1992) and the TCR repertoire is oligoclonal and contains potentially autoreactive clones (Emoto et al., 1997; Rocha, 1990; MacDonald et al., 1987). Whereas the thymus eliminates potentially autoreactive cells during T cell development, this does not appear to occur in nude mice. The thymus-independent α/β T cells in these animals express Vβ genes which encode...
potentially self-reactive TCRs. For example, in mice that express Mls-1 or I-E, T cells with the $\alpha/\beta$ TCR consisting of rearranged gene products of V$\beta$6 (anti-Mls 1) or V$\beta$8.1 and V$\beta$11 (anti-I-E) are deleted during maturation in the thymus (Rocha, 1990). It is plausible, therefore, to expect that thymus-independent T cells might not undergo negative selection and hence might contain potentially autoreactive cells. The repertoire of $\alpha/\beta$ TCR$^+$ cells in nude mice is often dominated by expression of genes of the V$\beta$8 family. Furthermore, in contrast to euthymic mice in Mls-1$^+$ or I-E$^+$ strains, $\alpha/\beta$ T cells that express V$\beta$6 (Mls-I reactive) and V$\beta$11 (I-E reactive) are present (Rocha, 1990). This observation supports the theory that thymus-independent T do not undergo the same selective pressures that occur in the thymus.

More recently, examination of the TCR $\alpha$ chains expressed by T cells in nude mice revealed that the majority of the cells expressed the V$\alpha$14 and J$\alpha$281 genes (Makino et al., 1993). Interestingly, both NKT cells and those T cells which are proposed to be of extra-thymic origin in the murine gut and liver, also display the same bias in usage of the V$\alpha$14 and J$\alpha$281 genes. In contrast, only a small proportion of $\alpha/\beta$ T cells in euthymic mice express TCR $\alpha$ chains that utilise these genes.

In addition to differences in TCR expression by $\alpha/\beta$ T cells from athymic and euthymic animals, other surface antigens are also expressed at different levels. A characteristic shared by $\alpha/\beta$ TCR$^+$ cells in the livers, spleens and lymph nodes of nude mice and by NK cells is the constitutive expression of the IL-2 receptor $\beta$ chain (CD122) in the absence of the IL-2 receptor $\alpha$ chain (CD25) (Ilii et al., 1992). Furthermore, the $\alpha/\beta$ T cells from the livers and spleens of nude mice also were shown to express high levels of
the adhesion molecules CD44 and LFA-1 (CD11a), while very few expressed L-selectin (CD62L) (Emoto et al., 1997). In comparison, only activated α/β T cells in euthymic mice express CD122 and in this case it is associated with CD25. In addition, the majority of circulating conventional α/β T cells express lower levels of CD44 and CD11a, and most of them express CD62L.

1.5 Differentiation states of mature T cells

1.5.1 Peripheral development of T cells

The hallmark of protective adaptive immunity is the body’s ability to resolve rapidly and effectively infections that involve agents which express antigens that it has encountered previously. Immunological memory is carried by both T and B cells and it reflects both the expansion of specifically reactive clones of lymphocytes and the generation of specialised cells with heightened responses against the antigen(s) concerned.

Naive T cells comprise the major population of the total T cell pool and can be defined as post-thymic resting T cells that have never encountered receptor-specific antigens in the periphery. Individual T cells bear unique antigen-specific receptors that are clonally distributed in the population. During primary immune responses, small numbers of antigen-specific T cells undergo marked clonal expansion and differentiate into effector cells. Effector cells are involved actively in the immune response. When the specific antigen has been cleared and they are no longer required, their removal preserves the primary TCR repertoire, thus maintaining a maximal response to new pathogens. However, complete elimination of all responsive cells would sacrifice the advantage gained by contact with antigen and would lead to future tolerance rather than immunity. The generation of memory cells overcomes this. Memory cells are long-lived, antigen-
specific cells which enable the immune system to facilitate rapid responses against antigens when they are re-encountered (reviewed by Sprent, 1997).

1.5.2 Surface phenotypes of naive, effector and memory T cells

Although unique markers that accompany transition from naive to effector or memory cells have not been identified, the level of expression of a number of markers can be used to assess the functional state of a T cell (reviewed by Sprent, 1997; Swain et al., 1996; Mackay, 1991). Prior to activation, unprimed small resting ‘naive’ T cells express low levels of a number of activation markers and adhesion molecules. They express low to moderate levels of hyaluronate receptor (CD44), LFA-1 (CD11a), ICAM-1 (CD54), VLA-4 (α4β1) and α4β7. In contrast, they express high levels of the lymph node homing receptor, L-selectin (CD62L). Before activation, they express few receptors for cytokines such as IL-2 (IL-2R) and they are in the G0 state of the cell cycle. After activation, the adhesion molecule profile of T cells is reversed. Expression of CD62L is down-regulated and there is up-regulation of CD44, CD11a, CD54, α4β1 and α4β7. Expression of cytokine receptors is upregulated and the cells become larger and progress through the cell cycle. A further important distinction between naive and activated/memory CD4+ T cells is in the isoforms of CD45 that they express. Naive CD4+ T cells express a high molecular weight form of CD45 (CD45RA in humans, CD45RB in mice, CD45RC in rats), while activated CD4+ T cells express the low molecular weight isoform (CD45RO in humans).

Effector cells are generally large and they express high levels of the adhesion molecules CD2, CD44, CD11a, CD54 and α4β1. Some of these markers, such as CD44, remain at elevated levels indefinitely, whereas others such as CD69, CD40L and CD25 (IL-2Rα)
are maintained at maximum levels for only a short time following activation and then decline in the absence of further stimulation (reviewed by Swain et al., 1996). The molecules expressed by activated cells aid in cell function and division. The transferrin receptor (CD71) is expressed by dividing cells, including T cells, highlighting the essential role of iron in cell division (Trowbridge and Omary, 1981). The IL-2R α-chain (CD25) is a component of the high-affinity IL-2R complex and is restricted largely to activated T cells. This reflects the role of interleukin-2 (IL-2) in T cell growth (Miyawaki et al., 1984) and the expression of this molecule can be used as a marker of activation in T cells in a variety of species.

However, activation-induced expression of some molecules is species-specific. Activated α/β T cells from humans (Evans et al., 1978; Engleman et al., 1980) and rats (Seddon and Mason, 1996; Reizis et al., 1994; Broeren et al., 1995) synthesise and express MHC class II molecules but this does not appear to occur in mice (Seddon and Mason, 1996). Rat CD4+ α/β T cells express the OX40-antigen (CD134), a member of the tumour necrosis factor receptor (TNFR) family believed to be important in maintaining a state of activation in CD4+ T cells (Paterson et al., 1987) and its homologue is expressed also by activated human CD4+ T cells (Latza et al., 1994). In addition to the surface markers described above, some effector cells express high levels of Fas and FasL, while naive cells express only low levels of these molecules (Suda et al., 1995). The regulation of effector cells is likely to be through their expression of pro-apoptotic molecules such as Fas, combined with the reduced expression of anti-apoptotic molecules, such as those of the Bcl-2 family (reviewed in Sprent, 1997).

Following clonal expansion, memory CD4+ cells revert to a resting G0 state, where in
mice they show only limited expression of the acute activation markers such as CD69, CD40L and CD25. These cells maintain essentially the CD62L\(^{lo}\)CD44\(^{hi}\) phenotype and they are not thought generally to express the high molecular weight forms of CD45 (reviewed by Sprent, 1997, and Swain \textit{et al.}, 1996). Hence, as is the case for effector cells, those memory T cells that express this pattern of markers can be distinguished readily from naive T cells. However, this distinction is not clear-cut. In humans, the down-regulation of the CD45RA and CD45RB isoforms is associated with the up-regulation of CD45RO (a low molecular weight CD45 isoform) on memory CD4\(^{+}\) T cells but these cells may be either CD62L\(^{-}\) or CD62L\(^{+}\) (Kanegane \textit{et al.}, 1996). Furthermore, there is circumstantial evidence that CD45RO memory CD4\(^{+}\) T cells in humans can revert to a CD45RA phenotype \textit{in vivo} (Michie \textit{et al.}, 1992). Although there is no known CD45RO counterpart in mouse, down-regulation of CD45RB on mouse memory/activated CD4\(^{+}\) T cells is paralleled by the up-regulation of a sialylated variant of CD45RB, CZ-1 (reviewed by Sprent, 1997). Memory and effector CD4\(^{+}\) T cells in rats were distinguished from their naive counterparts on the basis of their loss of CD45RC expression (Powrie and Mason, 1989). However, there is compelling evidence that rat CD4\(^{+}\) memory \(\alpha/\beta\) T cells can revert to a CD45RC\(^{+}\) phenotype when transferred adoptively in the absence of the relevant stimulating antigen. However, memory cells transferred with antigen maintain the CD45RC\(^{-}\) phenotype (Bunce and Bell, 1997), suggesting that re-stimulation by antigen is necessary to maintain the CD45 phenotype associated commonly with memory cells.

Collectively, these data suggest that late memory T cells can re-acquire some surface features of naive cells and that this reversion probably occurs in the absence of continued contact with residual antigen. However, the extent to which this phenotypic
reversion is accompanied by return to the functional state of naïve cells is uncertain. It is a common assumption that memory T cells generically are functionally distinct from naïve T cells.

### 1.5.3 Functional characteristics of naïve, effector and memory T cells

As discussed above, memory cells are responsible for the rapid generation of antigen-specific T cells after challenge with antigen. Recent studies suggest that memory T cells are hyper-reactive to antigen (reviewed by Sprent, 1997). This phenomenon is important theoretically. Hyper-responsive memory T cells would react readily to small amounts of antigen, giving rise to large numbers of progeny sharing this enhanced reactivity. From a relatively small repertoire in the peripheral T cell pool, this process would lead to the rapid production of specific effector cells and removal of the invading pathogen, whilst maintaining the overall diversity of the TCR repertoire.

The production of cytokines is a very important component of T cell function. This is especially the case for CD4⁺\(\alpha/\beta\) TCR⁺ T cells. In fact, the functional activities of this subset of T cells can be defined largely by the quantities and types of cytokines that are produced by the cells when they are activated by specific antigen. At different states of differentiation, peripheral T cells display distinct cytokine-producing capacities which mirror their functions (reviewed by Swain et al., 1996).

Naïve CD4⁺ T cells can produce only IL-2 after stimulation (Croft et al., 1992). The IL-2 produced is thought to support only autocrine growth initially. Naïve T cells are, therefore, unable to ‘help’ cells other than T cells as this function depends on the production of other cytokines. *In vitro* stimulation of resting T cells has shown that
expression of the IL-2 cytokine gene takes 12-18 hours, whereas expression of other cytokines requires further differentiation and usually occurs over a period of 4-5 days. During this time the T cells have acquired phenotypic characteristics, such as the expression of surface activation markers, that are associated with effector cells.

In contrast to naive CD4$^+$ T cells, effector T cells can produce a range of cytokines. Under certain conditions *in vitro*, it is possible to generate CD4$^+$ T cell populations whose profiles of cytokine production are polarised strongly. T-helper 1 (T$_{H1}$) cells are characterised by the production of IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), whereas T-helper 2 (T$_{H2}$) cells produce IL-4, IL-5 and IL-6 (Mosmann and Coffman, 1989). In addition, some populations of CD4$^+$ T cells are capable of producing most of the T cell cytokines and are referred to as T$_{H0}$ cells. Studies on individual cells have shown that they can produce either a wide range of cytokines, or display a polarised pattern of cytokine expression (Kelso, 1995). Given this, it is fair to say that populations of T cells may display biased cytokine profiles characteristic of either T$_{H1}$ or T$_{H2}$ but that within these populations, individual cells exhibit considerable heterogeneity. CD8$^+$ T cells are typically thought to secrete T$_{H1}$-type cytokines such as IFN-γ but some secrete IL-4 (Carter and Dutton, 1996). However, others (Kelso and Gough, 1988) have reported that both CD4$^+$ and CD8$^+$ T cell clones secrete a similar range of cytokines (T$_{H1}$- and T$_{H2}$-type cytokines) but that CD4$^+$ cells typically secrete higher levels of these cytokines.

Cytokines are only produced for a short time following TCR engagement (reviewed by Swain *et al.*, 1996). Messenger RNA (mRNA) is detectable within 3-5 hours of stimulation and cytokine protein is present soon afterwards. Peak levels of cytokine
mRNA are reached within 6-12 hours and they are then downregulated rapidly. Cytokine production is, therefore, a rapid response but it is short-lived in the absence of continued activation through the TCR. Upon \textit{in vitro} activation, effector CD4$^+$ T cells (generated from purified memory CD4$^+$ cells, which were stimulated \textit{in vitro} for 3 days) produce large quantities of cytokines compared to stimulated naive CD4$^+$ T cells. Comparisons using TCR-transgenic mice have shown that on a per cell basis following re-stimulation, effector cells produce approximately 10 fold more IL-2 than stimulated naive cells, and the titres of other cytokines are 100-1000 fold higher (Swain \textit{et al.}, 1996). Using these transgenic mice, Swain and colleagues have been able to compare directly the cytokine titres produced by effector CD4$^+$ and memory CD4$^+$ T cells that have been derived from naive CD4$^+$ T cells, all of which bear an identical TCR. Memory CD4$^+$ T cells do not produce high titres of cytokines. However, they do produce low levels of IL-2 and IFN-$\gamma$ in the case of T$_{\text{H}1}$ cells or IL-4 in the case of T$_{\text{H}2}$ cells.

Another distinction between memory and naive CD4$^+$ T cells is the rate at which they produce cytokines after stimulation. As discussed above, naive CD4$^+$ T cells differentiate into cytokine-secreting effector cells \textit{in vivo} over a period of 4-5 days. In contrast, resting memory CD4$^+$ T cells require only 2-3 days to differentiate into effectors producing even higher titres of cytokines (reviewed by Swain \textit{et al.}, 1996). The significance of this is that effector cells can be produced rapidly when antigen is re-encountered. Furthermore, if the primary immune response to an infective agent has led to a bias in the cytokines produced, the relevant memory CD4$^+$ T cells will produce the patterns of cytokines appropriate for effective immunity on subsequent encounters.
The isoform of CD45 expressed by CD4\(^+\) T cells depends on the history of the activation of the cell. In rats, the monoclonal antibody OX22 (Spickett et al., 1983) recognises an epitope that identifies the CD45RC isoform. In comparison with CD45RC\(^-\) CD4\(^+\) T cells, CD45RC\(^+\) CD4\(^+\) cells produce more IL-2 after stimulation and respond well to alloantigens and to mitogens (Arthur and Mason, 1986). CD45RC\(^-\) CD4\(^+\) T cells provide help for B cells in secondary antibody responses (Arthur and Mason, 1986) but they are poor mediators of allograft rejection (Yang and Bell, 1992). In the rat, CD45RC expression appears to correlate with a naive and/or T\(_{H1}\) phenotype for CD4\(^+\) T cells, whereas the CD45RC\(^+\) phenotype is indicative of a memory and/or T\(_{H2}\) phenotype. As alluded to above (section 1.5.2), this simplified statement is not without challenge, because there are reports that isoforms can be co-expressed and that reversion of isoform phenotypes can occur in both rat CD4\(^+\) T cells (Sarawar et al.; 1993, Bunce and Bell; 1997) and human CD4\(^+\) T cells (Michie et al., 1992). CD45 isoforms could well represent states of activation in CD4\(^+\) T cells and the expression of the isoforms may well correlate with the functions performed by these cells.

Expression of activation markers, adhesion molecules, (discussed in section 1.5.2), cytokine production profiles and CD45 isoforms, therefore, are tools used commonly to assess the functions of CD4\(^+\) T cells and the history of their contact with antigen.

### 1.5.4 Requirements for activation of naive, effector and memory T cells

Activation of T cells requires recognition of processed antigen in association with an MHC molecule followed by signalling through the TCR/CD3 complex. However, activation also requires signalling from other receptors on T cells, following their interaction with their specific-ligands on antigen presenting cells (APCs). These other
receptors are known as costimulatory receptors. Their engagement controls the immune response, because a T cell-mediated immune response can occur only when the antigen is presented with appropriate costimulation and the ligands for T cell costimulatory factors are expressed by activated APCs.

The first signal of T cell activation is provided by the binding of the MHC-associated antigen to the TCR and it is transduced by the CD3γ-chain of the CD3 complex. The second or costimulatory signal may involve one of a number of specific cell surface interactions. These include engagement of either CD28 or CTLA-4 by B7 molecules expressed by APCs, or stimulation by soluble factors such as IL-1 or IL-6. The costimulatory interaction between CD28 on resting T cells and the B7 molecules (B7.1 and/or B7.2) on APCs has been studied widely (reviewed by Chambers and Allison, 1997; Allison, 1994; Jenkins and Johnson, 1993). Expression of the B7 molecules is up-regulated by macrophages and B cells after stimulation and antigen presentation by these cells is, therefore, only efficient after activation (reviewed by Allison, 1994).

Other cell surface molecules that play a role in costimulation are CD45, CD54, CD11a, CD2 and CD5. The CD45 molecule has tyrosine phosphatase activity associated with its cytoplasmic domain and there is some evidence that this molecule may regulate aspects of T cell activation (reviewed by Alexander et al., 1992; Mackay, 1992). CD45 is believed also to associate with a range of other molecules, which include Thy-1 (CD90), CD2, CD7, TCR-CD3ζ, CD4/CD8, CD11a and CD58. These interactions are believed to increase cell-cell contact and as a result, enhance activation through the TCR (reviewed by Altin and Sloan, 1997). CD2, which associates with CD58, enhances adhesion between T cells and cytotoxic targets and APCs. However, some anti-CD2
monoclonal antibodies can co-activate T cells, suggesting that it may provide an independent pathway for costimulation under some circumstances (Driscoll et al., 1991). More recently, it has been proposed that CD2 enhances antigen recognition and, therefore, the T cell response by concentrating the TCR in the regions of cell-cell contact (reviewed by Davis et al., 1998). Antibodies against CD5 also enhance T cell proliferation in response to either lectins or anti-CD3 antibodies and this is perhaps associated with increased levels of cytoplasmic calcium within the antibody-treated T cells (Van de Velde et al., 1991). Further evidence that links CD5 to T cell activation (McAteer et al., 1988) is the observation that CD5 and the TCR-CD3 complex co-precipitate in immuno-precipitation studies (Beyers et al., 1992). In this respect, it is interesting to note that most extra-thymically-derived IEL in the mouse (reviewed by Rocha et al., 1995) and rat (Helgeland et al., 1996; Fangmann et al., 1991) do not express CD5. However, the relevance of this observation to the mechanisms of activation of these cells and to their function is unknown. Interactions with a number of adhesion molecules expressed by naive or activated CD4+ T cells have been shown to enhance T cell activation (reviewed in Allison, 1994; Collins et al., 1994). For example, interactions between CD11a and CD54 have been shown to enhance CD4+ T cell responses by enhancing the adhesion between the T cell and APC (reviewed by Collins et al., 1994; Jenkins and Johnson, 1993).

Studies by Swain and colleagues (reviewed by Swain, 1994) utilising TCR transgenic mice have identified different activation requirements for naive (freshly isolated cells), effector (naive cells stimulated in vitro for 3 days) and memory T cells (descendants of effector cells recovered 8 weeks after adoptive transfer). T cells in different states of differentiation have different requirements for costimulation and adhesion molecule
interactions. Naive CD4\(^+\) transgenic T cells are absolutely dependent on costimulation for activation. Presentation of specific antigen by APCs which do not express B7 or ICAM-1, or stimulation with plate-bound anti-CD3 antibodies, does not induce either IL-2 synthesis or cell proliferation (Dubey et al., 1995; Sagerstrom et al., 1993). However, culture of these cells with APCs expressing B7.1 and ICAM-1 induced proliferation and IL-2 production (Dubey et al., 1995). These results indicate that signalling roles mediated through CD28 (B7.1 interaction) and LFA-1 (ICAM-1 interaction) are important during antigen presentation to naive CD4\(^+\) T cells.

In contrast, stimulation of effector T cells by plate-bound anti-CD3 antibodies resulted in rapid DNA and cytokine synthesis. The addition of APCs expressing B7.1 and ICAM-1 did not greatly enhance the cytokine production by effector T cells in response to anti-CD3 antibodies (McKnight et al., 1994). This result indicates that a strong signal through the TCR overrides the need for costimulatory signalling in effector T cells. However, when stimulation of the TCR is provided by TCR-specific antigenic peptide presented by of B7.1\(^-\) ICAM-1\(^-\) APCs, the response by effector T cells is weak (Swain et al., 1996). In this case, addition of APCs expressing B7.1 and ICAM-1 results in a response equivalent to that obtained with plate-bound anti-CD3. It was concluded that the main roles of the B7:CD28 and ICAM-1:LFA-1 interactions were to increase the avidity or longevity of the T cell:APC interaction, resulting in a strengthened TCR-mediated signal.

Another difference between naive and effector T cells is the amount of peptide required to elicit a response. In comparison with naive T cells, effector T cells produce more cytokines and proliferate more readily at lower antigen concentrations (Swain et al.,
The use of TCR transgenic animals has demonstrated that the requirement of effector T cells for antigen can be up to 100 fold less than is the case for naive T cells. It is worth noting that while a strong TCR stimulus (potentially by non-professional APCs) can lead to the production of cytokines such as IL-4 and IFN-γ by effector T cells, the presence of accessory molecules (expressed by professional APCs) is essential for their continued growth and production of IL-2 (Swain et al., 1996). As discussed above, T cells must produce IL-2 to maintain viability and proliferation. Effector T cells that do not produce IL-2 undergo programmed cell death and this may provide a regulatory mechanism whereby effector T cells can carry out their functions and are then eliminated unless they are in an environment that provides co-stimulation.

Resting memory T cells differ from naive T cells in that they, like effector T cells, can respond to anti-CD3 in the absence costimulation via B7.1 and ICAM-1 (Croft et al., 1994 and reviewed by Croft, 1994). However, whereas there was little enhancement of the effector T cell response to plate-bound CD3 by the addition of APCs expressing B7.1 (Swain, 1994), addition of anti-CD28 (which mimics the B7 interaction) enhanced the response of memory T cells to anti-CD3 stimulation considerably. Swain (1994) has reported that similar results are obtained using specific peptide in their model that employs T cells from TCR-transgenic mice. These findings indicate that memory T cells have a costimulatory requirement which is less than that required by naive T cells, yet greater than that needed by effector T cells.

These differences in co-stimulation requirements are also evident when different types of APCs are examined (reviewed by Bradley et al., 1993). Using cells from TCR-transgenic mice, it has been shown that naive T cells respond only to antigen presented...
by either dendritic cells (DC) or activated B cells. This finding could be attributed to the fact that these cells express high levels of costimulatory molecules such as B7.1, B7.2 and ICAM-1. Effector T cells and memory T cells are able to respond to a wider range of APCs, including DC, B cells and macrophages. As a consequence, memory T cells and effector T cells can respond rapidly to antigen presented in vivo by a range of APCs and this ability is very important in terms of the heightened secondary immune response (Swain, 1994).

1.6 Recirculation and homing of lymphocytes

In order for a T cell-mediated immune response to occur, T cells must be able to migrate to the site of action. Naive T cells must be able to circulate through tissues such as lymph nodes, where they are able to sample antigen presented by APCs. Following activation, effector T cells must then be able to relocate to wherever their function is required. Ideally, memory T cells should recirculate, or be maintained in the areas where subsequent immune responses may be required. Differentiating T cells regulate expression of adhesion molecules which are responsible for their differing recirculation and homing characteristics.

1.6.1 Lymphocyte recirculation

The recirculation of lymphocytes was demonstrated by Gowans and Knight (1964), by the use of isotopic labelling procedures. It was observed that the great majority of lymphocytes migrate through the body by moving from the blood to secondary lymphoid organs. In lymph nodes and Peyer’s patches, this occurs via specialised post capillary vessels, termed high endothelial venules (HEV), that are characteristic of the thymus-dependent areas of these tissues. This migration process is mediated by homing
receptors and adhesion molecules that are comprised of immunoglobulin super-family members, selectins and integrins (Springer, 1994). From the lymph nodes and Peyer's patches, lymphocytes return to the blood via the efferent lymphatics and major lymphatic trunks such as the thoracic duct. In the spleen, lymphocytes leave the blood via the marginal sinuses of the white pulp by an unknown mechanism, and after a short residence in the white pulp, return to the blood.

Morphologically, lymphocytes in the thoracic duct can be described as either small or large lymphocytes. The large lymphocyte population consists of blasts and activated cells and most of these cells do not recirculate (Ford, 1975). Studies in which dividing cells are identified by incorporation of isotypically-labelled deoxyribonucleotides have shown that large lymphocytes demonstrate a tropism for the gut in normal animals (Gowans and Knight, 1964). Activated T lymphoblasts appear to traffic mainly to sites of inflammation (Ford, 1975) and some may reappear in afferent lymph (reviewed by Mackay, 1993).

Recent work has demonstrated that lymphocyte subsets demonstrate distinct recirculation patterns. In the rat, CD4+ CD45RC+ T cells are found predominantly in the early-mobilised population of thoracic duct lymphocytes. B cells, with increased numbers of CD4+ CD45RC− T cells, are dominant in the late mobilised population following thoracic duct cannulation (Westermann et al., 1994). This suggests that CD4+ CD45RC+ T cells recirculate more quickly than CD4+ CD45RC− T cells and B cells. Alternatively, these latter cell types may have different tissue retention characteristics than CD45RC+ CD4+ T cells.
1.6.2 Comparison of the recruitment of naive, effector and memory T cells to various tissues

As discussed above, naive CD4$^+$ T cells express L-selectin (CD62L), the lymph node homing receptor. Antibody blocking studies have shown that CD62L expression by these cells is very important for their entry not only into lymph nodes (Bradley et al., 1994) but also into the gut associated lymphoid tissues (GALT) including the mesenteric lymph nodes and Peyer’s patches. Entry into these latter tissues is mediated mainly by the $\alpha_4\beta_7$-integrin, which is a counter-receptor for the mucosal vascular addressin MadCAM-1. MadCAM-1 is expressed on the HEV of Peyer’s patches and mesenteric lymph nodes, and on the endothelium of venules in the mucosa of the small intestine (Berlin et al., 1993; Briskin et al., 1993; Schweighoffer et al., 1993). Studies by Swain and colleagues (reviewed by Swain et al., 1996) have shown that antibodies against either CD62L or LFA-1 (CD11a) block the entry of naive T cells into lymph nodes and as a consequence, primary immune responses fail to develop. Studies in the recently generated CD62L knockout mouse further highlight the importance of CD62L expression. Adoptive transfer studies showed that after 48 hours very few lymphocytes from knockout mice had localised in the lymph nodes and Peyer’s patches of recipient animals (Tang et al., 1998). It would appear therefore, that the recirculation of naive T cells through lymph nodes (largely mediated by CD62L) and their subsequent encounter with antigen presented by DC is an essential process in the initiation of an antigen-specific immune response.

As outlined above, phenotypic changes following activation accompany the transition from a naive T cell to an effector cell. In particular, effector cells express reduced levels of CD62L but there is increased expression of other adhesion molecules such as CD11a,
CD54, CD44 and CD49d. These changes enable effector T cells to distribute to the anatomical sites where their functions are required. Furthermore, the expression of molecules such as CD44 and CD49d may be important in the tissue localisation and retention of activated T cells in their responses to cognate antigen locally. Memory T cells that have been generated during immune responses maintain many of the surface characteristics of the effector population (reviewed by Sprent, 1997; Swain et al., 1996; Mackay, 1993; Bradley et al., 1993) and this suggests that these cells maintain the capacity to recirculate, probably via peripheral tissues and afferent lymphatics (Mackay et al., 1990 and reviewed in Mackay, 1991; Mackay, 1993). However, recent studies in the rat have shown that comparable numbers of CD4+ CD45RC+ ('naive') and CD4+ CD45RC− ('memory') cells enter lymph nodes and Peyer's patches via HEV (Westermann et al., 1997). Interestingly, it was noted that CD45RC− cells migrated through the T-dependent regions of the lymphoid tissues more quickly than CD45RC+ cells.

Recent studies in TCR-transgenic mice have shown clearly that naive and memory T cells are recruited to different tissues (reviewed by Swain et al., 1996; Mackay, 1993). Swain and colleagues (reviewed by Swain et al., 1996) have shown that naive CD4+ T cells distribute rapidly to the lymph nodes and Peyer's patches, whereas 12 hours after adoptive transfer, memory CD4+ T cells are found in extremely low numbers in these sites. Memory T cells do, however, accumulate in significant numbers in the spleen and are present in high numbers in the peripheral blood after this time. In addition, they are also more numerous than naive T cells in non-lymphoid tissues such as the liver, small intestine and lungs. However, within a few days, memory CD4+ T cells are found in the lymph nodes. Because memory cells are essentially L-selectin negative, it was
concluded that they probably enter lymph nodes via afferent lymphatics. However, as mentioned above, studies in the rat by Westermann et al. (1997) have shown that both CD45RC+ and CD45RC- T cells can transverse HEV in lymph nodes. At least a portion of the memory population recirculates, as the TDL and the blood contain CD4+ T cells with the CD45RC- phenotype in rats (Westermann et al., 1994) and CD4+ cells with memory phenotype can be found in the TDL of TCR transgenic mice for months after initial antigen exposure (Swain et al., 1996).

With respect to tropism for the gut mucosa, it would be expected that those CD4+ T cells that are recruited would express the low molecular weight isoform of CD45. It has been shown in sheep that CD45RO+ (low molecular weight isoform) T cells, both small (memory) and large (blast/activated) cells, home to the gut (Mackay et al., 1992). As discussed above, cells in humans and mice that are recruited to the gut express α4β7 (Briskin et al., 1993). T cells in humans which express α4β7 have a memory phenotype (Schweighoffer et al., 1993) and interestingly, the β7 integrin is upregulated on T cells following in vitro activation. This may account for the localisation of many activated T cells in the gut.

Recent studies have suggested that there may be a correlation not only between surface antigen phenotype at tissue localisation, but also between the characteristics and the functions of activated cells. As discussed above, upon activation CD4+ T cells can differentiate into two main subsets of T\(_H\) cells. T\(_H1\) cells are pro-inflammatory and they secrete a range of cytokines including IFN-γ. In contrast, T\(_H2\) cells induce humoral and allergic responses, down-regulate local T\(_H1\)-mediated inflammation and they secrete a number of cytokines including IL-4. Recent work has shown that T\(_H1\) cells are able to
bind to P-selectin (CD62P) but not E-selectin (CD62E), whereas Th2 cells bind E-selectin but not P-selectin (Austrup et al., 1997). In models of Th1-mediated inflammation, such as contact hypersensitivity and experimental polyarthritis, Th1 cells generated in vitro entered the inflamed sites. Conversely, Th1 cells did not gain access to sites of Th2-mediated inflammation. It appears, therefore, that expression of CD62P and CD62E at sites of inflammation, coupled with the differential expression of ligands for these selectins on activated CD4+ T cells, may be responsible for the differential trafficking of the Th1 and Th2 subsets. A mechanism such as this would ensure that effector T cells generated in regional lymphoid tissues are distributed by the circulation to sites that are appropriate for the effector functions of those cells.

In the case of memory T cells, it is important that they recirculate as this enables them to be mobilised rapidly to sites of antigenic challenge. Moreover, it is thought that the distinct profile of adhesion molecules expressed by memory T cells enables them to survey non-lymphoid tissues via both normal and inflamed endothelium. From the tissues, they can enter the draining lymph nodes via the afferent lymphatics (reviewed by Springer, 1994). Thus memory T cells distribute and recirculate in such a way as to maximise a potential re-encounter with the original immunising antigen. With the extensive information now available on the adhesion molecules expressed by T cells, the identification of these molecules on lymphocytes can provide important clues about their migration patterns and their functions.

1.7 NKT cells

1.7.1 Distribution and phenotypic markers of NKT cells

Recent studies have identified a unique T cell population, characterised by co-expression
of the α/β TCR and NKR-P1 (CD161). In the mouse, these cells have been termed
NK1^+ T cells, NK1.1^+ T cells, natural T cells (NT cells) or simply NKT cells (reviewed
by Taniguchi *et al.*, 1996; Vicari and Zlotnik, 1996; MacDonald, 1995; Bendelac, 1995;
Bix and Locksley, 1995). Hereafter, these NKR-P1^+, α/β TCR^+ cells will be referred to
as NKT cells because these unique lymphocytes have also been identified in rats
(Brissette-Storkus *et al.*, 1994) and humans (Porcelli *et al.*, 1993; Lanier *et al.*, 1994;
Davodeau *et al.*, 1997).

In the late 1980s, an α/β TCR^+ thymocyte population was described that was negative
for the CD4 and CD8 co-receptors (reviewed by Vicari and Zlotnik, 1996; Bix and
Locksley, 1995). Later, these cells were shown to include a population expressing
NK1.1 (Ballas and Rasmussen, 1990), an allele of the murine NKR-P1 homologue.
Subsequent studies have revealed that mouse NKT cells are also present at extra-thymic
sites (Watanabe *et al.*, 1995 and reviewed by Vicari and Zlotnik, 1996; Taniguchi *et al.*, 1996; MacDonald, 1995). The tissue distribution of these cells (expressed as a
percentage of mature α/β T cells) is as follows; thymus, 2-3%; spleen, 2-5%; lymph
nodes, 2%; bone marrow, 40%; liver, 20-30%. In addition to these sites, some
intraepithelial lymphocyte (IEL) subpopulations express NK1.1 (Guy-Grand *et al.*, 1996),
and NKT cells have also been identified in the lamina propria of the small
intestine (Ohteki *et al.*, 1992).

Apart from expressing NKR-P1, NKT cells express a number of surface markers which
are usually associated with effector or memory function in T cells (reviewed by Vicari
and Zlotnik, 1996; Taniguchi *et al.*, 1996; MacDonald, 1995). NKT cells express high
levels of CD44, low levels of CD45RB, and low levels of L-selectin (CD62L).
However, unlike most memory T cells, some NKT cells express the activation marker Ly-6C and the B cell markers B220 and CD38. Expression of Mac-1 (CD11b), an adhesion molecule found on NK cells, has also been reported on NKT cells. This molecule has been reported on a small subset of activated T cells in humans (Baars et al., 1995; de Jong et al., 1992; Rotteveel et al., 1988) but the relationship of these cells to NK cells and NKT cells has not been investigated.

The most striking feature of NKT cells, as their name suggests, is the expression of a variety of NK cell markers, including CD16 (IgG Fc receptor), CD56, CD57, Ly-49A, Ly-49C and CD122 (β-chain of the IL-2 receptor). Indeed, electron microscopy reveals that NKT cells are intermediate between NK cells and T cells morphologically (Watanabe et al., 1995). In respect to size and granularity, as well as their expression of adhesion molecules, NKT cells are phenotypically similar to the α/β T cells present in nude mice (liai et al., 1992), to hepatic T cell populations in normal mice (Emoto et al., 1997) and to thymus-independent murine IEL (Ohtsuka et al., 1994). In particular, all of these T cell populations express CD122 (IL-2Rβ) constitutively.

Unlike the majority of conventional T cells which express either the CD4 or CD8 co-receptors, mouse NKT cells are almost exclusively CD4+ or double negative (DN) (reviewed by Taniguchi et al., 1996; Vicari and Zlotnik, 1996; MacDonald, 1995; Bendelac, 1995). Similarly, human NKT cells are also either DN or CD4+ (Davodeau et al., 1997; Porcelli et al., 1993). Intriguingly, rat NKT cells are predominantly CD8α+ (Brissette-Storkus et al., 1994). In addition, mature murine CD4-CD8- α/β T cells can express CD8α upon activation (Bean et al., 1995). Future work is required, therefore, to determine whether rat NKT cells express the CD8αα homodimer, or the CD8αβ
heterodimer. For comparison, rat NK cells express CD8α but not the CD8β chain (Torres-Nagel et al., 1992). This is an important distinction to make, because rat CD4+ T cells can express CD8α chains but not CD8β chains after activation (Torres-Nagel et al., 1992; Ramirez et al., 1992). There is a distinct possibility, therefore, that expression of CD8α by rat NKT cells represents an activated phenotype and that rat NKT cells may share with those from mice and humans a basal phenotype that is either CD4+ or DN.

Expression of CD8αα homodimers by some murine NKT cells is a feature they share with some murine, human and rat IEL. These thymus-independent T cells in mice include characteristically a population which utilises the FcεRIγ chain, in place of the CD3ζ chain, as a component of the CD3-TCR complex (Guy-Grand et al., 1994; Simpson et al., 1995). It is of interest, therefore, that some splenic NKT cells also express FcεRIγ as part of their CD3-TCR complex (reviewed by Vicari and Zlotnik, 1996).

Another interesting feature of murine NKT cells relates to their expression of the TCR. NKT cells express a surface density of α/β TCR that is approximately threefold lower than that expressed by classical T cells. This is a feature that they share with the α/β T cells present in nude mice and with a population of hepatic T cells found in normal mice. T cells exhibiting this low level of TCR expression are referred to in the literature as TCR ‘intermediate’ (TCRintermediate) cells (reviewed by Vicari and Zlotnik, 1996; MacDonald, 1995). Although the majority of T cells in nude mice are NK1.1+, the lower levels of TCR expressed by T cells in these animals suggests that the TCRintermediate cells in nude mice and the NKT cells in normal mice may be related in lineage and/or
function. The TCR_{intermediate} cells in the livers of normal mice may also be related to NKT cells.

A further point of similarity between the two cell types is in the usage of TCR genes. TCR usage by mouse NKT cells is biased heavily towards certain α and β chains (reviewed by Vicari and Zlotnik, 1996; Taniguchi et al., 1996; MacDonald, 1995; Bendelac, 1995; Bix and Locksley, 1995). Most NKT cells use one of only three Vβ genes. These are Vβ8 (predominantly Vβ8.2), which is used by up to 50% of NKT cells, while the rest use either Vβ7 or Vβ2. In addition, all NKT cells that have been cloned express mRNA for an invariant α chain, Vα14-J281 and most express Vα14 at the cell surface (Makino et al., 1995). Utilisation of Vα14 is significant because its usage is almost undetectable among thymus-derived peripheral T cells. In contrast, high levels of mRNA for Vα14 have been detected among T cells isolated from extra-thymic sites such as the liver and the intestinal epithelium in normal mice and from the spleen in nude mice (Makino et al., 1993). Human NKT cells also express an invariant Vα chain, Vα24JαQ, which has very close sequence similarity to murine Vα14-J281 (Porcelli et al., 1993).

It appears, therefore, that the TCRs of NKT cells have been conserved across species. In addition, it has been shown recently that human and mouse NKT cells share close phenotypic and functional characteristics (Davodeau et al., 1997). As discussed below, these observations suggest that NKT cells have evolved to recognise a restricted range of antigens and/or restriction elements and that these are conserved across a number of species. Alternatively, they may be selected by a limited range of antigens that are also
important in the selection process in a number of species. These possibilities are not mutually exclusive.

Finally, it has been shown recently that expression of NK1.1 by NKT cells is not constitutive. Purified mouse CD4⁺ NKT cells ceased expression of NK1.1 after activation and some ceased expression of CD4 also. The cells acquire, therefore, a double negative phenotype (Chen et al., 1997). This raises the possibility that T cells expressing intermediate levels of surface TCR and an NK1.1⁺ DN phenotype in vivo (see section 1.11) could represent activated NKT cells. In particular, the thymus-independent T cells present in nude mice, which express intermediate levels of surface TCR, could be related to NKT cells phenotypically.

1.7.2 Developmental requirements and origin of NKT cells

As discussed above (section 1.3.1), development of conventional T cells in the thymus is dependent on the expression of MHC molecules by the thymic epithelium. Classically, MHC class II and MHC class I molecules mediate the positive selection of CD4⁺ α/β T cells and CD8⁺ α/β T cells respectively. As a consequence, it is thought that expression of MHC molecules is essential for the development of conventional α/β T cells and this expectation has been confirmed in β2-microglobulin knockout (k/o) mice and in MHC class II k/o mice (Chen et al., 1997). β2-microglobulin associates non-covalently with both classical and non-classical MHC class I molecules and is essential for their expression at the cell surface. Studies by Bendelac and colleagues (Bendelac et al., 1994) have shown that NKT cells fail to develop in β2-microglobulin-deficient mice. As expected, classical CD8⁺ T cells were not selected positively and interestingly, the predominantly CD4⁺ and DN NKT cells were not selected also. In contrast, NKT cells
were found in normal numbers in MHC class II-deficient mice. These findings led to the conclusion that NKT cells, irrespective of expression of CD4 and CD8 co-receptors, were selected by MHC class I molecules. Subsequent studies have disproved this hypothesis with respect to classical MHC class I molecules. However, the conclusion from this study that β2-microglobulin expression is essential for NKT cell development appears to be correct.

CD1 molecules are trans-membrane cell surface glycoproteins that are related structurally to MHC class I molecules. However, unlike classical MHC class I molecules, they are non-polymorphic. It has been shown that CD1 molecules, like MHC class I, require β2-microglobulin for their synthesis and cell-surface expression (Bauer et al., 1997). Recently, it has been shown that CD1 molecules are expressed by APCs and exhibit the ability to present antigen (reviewed by Porcelli et al., 1998; Brossay et al., 1998). Although NKT cells fail to develop in β2-microglobulin-deficient mice, this is not due to the lack of MHC class I but to the concurrent absence of CD1, which appears to be essential for NKT cell development (Ohteki and MacDonald, 1994; Adachi et al., 1995). Indeed, mouse NKT cells have been shown to recognise CD1 antigens (Bendelac et al., 1995). Recently these findings have been confirmed in CD1-deficient mice, which are deficient in NKT cells (Chen et al., 1997; Mendiratta et al., 1997). Whereas thymic epithelium expresses classical MHC molecules, which are essential for the positive selection of conventional T cells, positive selection of NKT cells that express the invariant Vα14 α-chain in the mouse is mediated by cells that express CD1 (Adachi et al., 1995). This finding reinforces the need for CD1-expressing cells in the selection of NKT cells and suggests that bone marrow-derived (CD1-expressing) cells rather than thymic epithelium are essential for the development of these cells. The thymus may not
be essential, therefore, for NKT cell development and this raises the possibility that NKT cells can develop at extra-thymic sites. It is of interest that CD1 expression has been reported in a variety of tissues in addition to the thymus. These sites include the intestinal epithelium of mice (Bleicher et al., 1990), rats (Kasai et al., 1997; Burke et al., 1994) and humans (Blumberg et al., 1991), and also the liver of mice (reviewed by Porcelli et al., 1998; Brossay et al., 1998). Interestingly, both the liver and intestinal epithelium have been proposed as sites that support extra-thymic T cell development (discussed below).

A feature of CD1 which distinguishes it from MHC class I is that it is not reliant on the transporter associated with antigen processing (TAP) molecule for stable expression at the cell surface (reviewed by Brossay et al., 1998). The TAP molecule is a heterodimer produced by two MHC-encoded genes, TAP-1 and TAP-2. Cells which lack expression of either of these genes are unable to express MHC class I molecules stably at the cell surface. This observation is consistent with the finding that CD1-reactive NKT cells are present in expanded numbers in TAP-1 deficient mice, whereas conventional T cells do not develop in such mice (Joyce et al., 1996). It also suggests that NKT cells may pre-date classical T cells in evolution. They are produced and they recognise novel ligands, without the need for the highly evolved peptide transport system that is necessary to present peptide antigens to conventional T cells via classical MHC molecules.

The TCR-CD3 complex is very important in NKT cell development. The generation of Vα14 transgenic (Tg) mice on a TCRα-deficient background (TCRα+/Vα14Tg mice) results in the expression of only Vα14 α-chains. These can associate with the full range
of endogenous Vβs. However, only Vα14+ NKT cells developed in these mice and there was a complete block in the development of conventional α/β T cells (Taniguchi et al., 1996). These results indicate that the expression of the invariant Vα14 TCR in some as yet undefined way leads preferentially to the development of NKT cells. In another study, utilising Vα14-Jα281α transgenic mice, the T cells that were generated were DN and CD4+ NKT cells (Bendelac et al., 1996). The Vβ usage in the TCRs formed by these transgenic mice resembled NKT cells in normal mice in that Vβ8 and to a lesser extent Vβ7 usage was prominent. The authors argue that Vβ8, Vβ7 and Vβ2 TCR β-chains, coupled with Vα14-Jα281, allows for high affinity binding to CD1. They propose that the additional binding strength provided by the CD8 co-receptor results in the deletion of CD8+ cells. Indeed, as discussed above, mouse NKT cells are essentially CD8- and this proposal is supported by the finding that in transgenic mice that express CD8 constitutively, there is no development of Vα14-Jα281+ NKT cells (Bendelac et al., 1994).

Clearly, expression of Vα14 as the TCR-α chain is important in the development of NKT cells. The question arises to whether or not expression of the Vα14-J281α chain is genetically programmed to occur in a specific cell lineage committed to NKT cell development. Recently, this question has been addressed and it appears that it is ligand selection, rather than a directed mechanism, which creates the restricted TCR repertoire exhibited by NKT cells (Shimamura et al., 1997). In this study, a panel of T cell hybrids, which had been derived from NKT cells, were examined for the rearrangement of the Vα14 gene by polymerase chain reaction (PCR) and Southern blot. Seventeen out of the twenty hybrids that were examined had rearrangement of the Vα14 gene to the
Ja281 gene. However, these rearrangements had occurred only on one chromosome of the pair and were accompanied by other productive Vα-Jα rearrangements on the other chromosome. It was concluded on this evidence that selection through CD1 was responsible for the selection of TCR in NKT cells. However, the question remains as to how selection of this particular TCR directs the cell down the lineage of NKT cells.

Controversy surrounds the origin of NKT cells and whether they are all part of a single lineage. As discussed above, DN α/β T cells were first described in the thymus and some were shown subsequently to be NKT cells. It has also been shown that NKT cells can originate from foetal thymic organ cultures established from d14 mouse embryos (Bendelac et al., 1994). However, the presence of functional NKT cells in nude mice (Kikly and Dennert, 1992; Ohteki et al., 1992; Hashimoto et al., 1995) suggests that these cells can also develop in the absence of a thymus. Further to this, T cells that develop in athymic nude mice have been shown to exhibit the same dominance of expression of TCRs containing Vα14 (Makino et al., 1993) and circular DNA amplified by PCR from extra-thymic tissues such as the liver and intestine are rich in Vα14-Jα281-mediated signal sequences (Makino et al., 1995). These results imply that Vα14+ NKT cells can develop extra-thymically and without thymic influence. In addition, NKT cells have also been shown to develop selectively in the livers of ATXBM mice (Sato et al., 1995). In contrast, in irradiated euthymic mice both TCRintermediate NKT cells and conventional TCRhigh cells develop. It is also worth noting that the development of normal T cells was delayed relative to the appearance of NKT cells in these reconstituted euthymic animals.

It has become apparent recently that Vα14+ NKT cells develop early in embryogenesis,
before the development of the thymus. They are present, therefore, far earlier than conventional T cells, which do not rearranged functional TCR until late in gestation (day 17) (Strominger, 1989). Vα14 TCR transcripts and Vα14 gene-containing circular DNA have been detected at days 9.5 to 13.5 of gestation in the mouse foetus, before colonisation of the developing thymus by haematopoietic cells (Makino et al., 1996). Other TCRs which are re-arranged in the thymus, such as Vα1, were not present at this time. By day 13.5, NKT cells expressing Vα14 TCRs in association with Vβ8 were detectable in the foetal liver. These α/β T cells expressed NK1.1 and were mainly DN in phenotype. The findings show clearly shows that NKT cells are present before the development of conventional T cells but whether the liver remains a site of extra-thymic T cell differentiation in post-natal life remains controversial.

1.7.3 Antigen recognition and functions of NKT cells

1.7.3.1 Cytolytic activities

As discussed above (section 1.7.2), CD1 molecules appear to be responsible for the selection of the restricted TCR repertoire of mouse NKT cells. Furthermore, the restriction element for antigen recognition by NKT cells in vitro has been shown to be CD1 (Bendelac et al., 1995). Bendelac et al. (1995) showed that after stimulation by cells that expressed CD1, Vα14-Jα281⁺ NKT cell hybridomas produced cytokines and that the response was inhibited by anti-CD1 mAbs. Human DN α/β T cells have been shown to recognise microbial antigens in the context of CD1b (Porcelli et al., 1992; Thomssen et al., 1995) and importantly, the antigens presented by CD1 have been found to be glycolipids. This finding led Kawano and colleagues (Kawano et al., 1997) to propose glycolipids as candidate ligands for the Vα14 TCR of NKT cells. These
workers identified a synthetic glycosylceramide as a ligand for the Vα14-Jα281 TCR of NKT cells and showed that monoclonal antibodies against CD1 abolished ligand recognition. Interestingly, glycolipids similar to the one used by Kawano et al. have been detected in certain bacteria and also in mammalian foetal tissues, cancer cells, kidney and intestine. These authors propose that a natural ligand, similar to the one used in their study, may exist in certain mammalian tissues or that it could be expressed on stressed cells or during malignancy.

Thus, NKT cells could contribute potentially to non-specific immune defences by recognising damaged cells or they could play a more direct role by recognising unique bacterial antigens. Their response to infected or stressed cells could be cytolyltic and it is of interest that NKT cells have been reported to display cytolytic functions in vivo. Hashimoto et al., (1995) have shown that administration of IL-12 intravenously to mice with hepatic metastases induces NKT cell-mediated destruction of tumour cells in the liver. A recent study also highlights the essential requirement of NKT cells in the IL-12-mediated rejection of liver tumours (Cui et al., 1997). This study showed that administration of IL-12 led to a four-fold increase in NKT cell numbers in the liver and that these cells were activated and able to destroy tumour cells by an NK cell-like cytolytic process.

Although recognition of CD1 by the TCR appears to be essential for the activation of NKT cells, a role for NKR-P1 (CD161) in ligand recognition cannot be discounted. Indeed NKR-P1, which is a member of the C-type lectins, has been shown to interact with oligosaccharides present on the surfaces of target cells that are recognised by NK cells. In NK cells, this interaction induces a signalling pathway that activates lytic
function (Bezouska et al., 1994). It is of interest, therefore, that both NK cells and activated mouse NKT cells are able lyse Fc-receptor-bearing target cells in the presence of anti-NK1.1 antibodies (reviewed by MacDonald, 1995). Similarly, rat NKT cells, after incubation with IL-2, exhibit MHC-unrestricted cytotoxicity and the capacity for re-directed antibody-dependent cellular cytotoxicity mediated via NKR-P1. This feature of NK cells is shared with NKT cells but not with NKR-P1− conventional α/β T cells (Brissette-Storkus et al., 1994). These findings suggest that expression of NKR-P1 contributes to cytolytic function. It is possible, therefore, that NKR-P1 might interact with an oligosaccharide on target cells, or perhaps with a carbohydrate expressed by the CD1 molecule.

Another mechanism by which NKT cells can mediate cytotoxicity has been documented. NKT cells have been shown to express Fas ligand (FasL), a molecule that is also expressed by activated T cells (Suda et al., 1995). This conclusion is based on the finding that NKT cells can kill cells that express Fas (CD95), such as CD4+CD8+ thymocytes and certain tumour cell lines. NKT cells from FasL-deficient gld mice did not kill Fas expressing targets, thus indicating that FasL expression by NKT cells in normal mice could mediate the killing of target cells via the Fas apoptotic signalling pathway (Arase et al., 1994). Suda and colleagues (Suda et al., 1995) also propose that FasL expressing NKT cells could regulate the generation of conventional T cells in the thymus by the elimination of thymocytes that express Fas.

1.7.3.2 Role in regulation of haematopoiesis and lymphopoiesis

As discussed in section 1.7.3.1, thymic NKT cells have the capacity to kill immature CD4+CD8+ thymocytes (Arase et al., 1994). It is of interest that NKT cells are present
also in high frequency in the bone marrow and in sites such as the liver and the small intestine (see sections 1.8.1 and 1.9.2) that have been proposed to support extra-thymic T cell development. These observations have given rise to the speculation that NKT cells could play a regulatory role in haematopoiesis and lymphopoiesis (reviewed by Vicari and Zlotnik, 1996). In support of this proposal is the finding that NKT cells are present during the early stages of embryogenesis (Makino et al., 1996). It is feasible, therefore, that NKT cells seed the bone marrow, thymus and other sites during embryonic development and that they can carry out regulatory functions during the establishment of haematopoiesis and lymphopoiesis.

1.7.3.3 Regulatory functions in immune responses

A well documented functional characteristic of mouse NKT cells is their ability to produce large quantities of cytokines rapidly following stimulation (reviewed by Taniguchi et al., 1996; Vicari and Zlotnik, 1996; MacDonald, 1995; Bendelac, 1995; Bix and Locksley, 1995). Mouse NKT cells have been reported to produce IL-4, IFN-γ, IL-5, IL-10 and IL-13. Activated NKT cells have been reported also to produce the chemotactic chemokines macrophage inflammatory protein 1α (MIP-1α), MIP-1β and lymphotactin. After activation, human NKT cells exhibit a cytokine secretory profile similar to their mouse counterparts, producing large quantities of IL-4 and IFN-γ (Davodeau et al., 1997). Cloned rat NKT cells also produce a range of cytokines following activation, in particular producing large amounts of IFN-γ (Knudsen et al., 1997).

Because mouse NKT cells produce large quantities of IL-4 rapidly after in vitrō
stimulation, it has been proposed that they may play an important role in biasing immune responses *in vivo*. The production of IL-4, a cytokine which is known to enhance the induction of Th2-type cells (Chen *et al.*, 1997; Chen and Paul, 1997), could bias developing immune responses towards antibody production rather than CMI. This effect of NKT cells has been demonstrated *in vivo* following activation with anti-CD3 mAb (Yoshimoto and Paul, 1994). Furthermore, Vα14 TCR transgenic mice with increased numbers of NKT cells exhibit elevated levels of serum IL-4 and raised levels of immunoglobulin E (IgE), the major immunoglobulin associated with a Th2 response (Bendelac *et al.*, 1996). In support of the role of NKT cells in IgE production, SJL mice, which are deficient in NKT cells, are defective in the production of IgE (Yoshimoto *et al.*, 1995). Furthermore, CD1-mutant mice, which exhibit impaired NKT cell development, are deficient in early production of IL-4 (Chen *et al.*, 1997; Mendiratta *et al.*, 1997).

As discussed above, IL-12 induces the activation and cytolytic activity of NKT cells (Cui *et al.*, 1997; Hashimoto *et al.*, 1995). However, it has been shown recently that the *in vivo* administration of IL-12 biases NKT cells towards the production of IFN-γ (Leite-De-Moraes *et al.*, 1998). Previous studies showed that the presence of IL-7 was essential for the production of IL-4 by NKT cells *in vitro* (Leite-De-Moraes *et al.*, 1997) and while NKT cells from IL-7-deficient mice cannot produce IL-4, this capacity can be restored after reconstitution with exogenous IL-7 (Vicari *et al.*, 1996). These findings demonstrate that the functional characteristics of NKT cells are dependent on the cytokines present in their microenvironment. The presence of IL-7 is essential for IL-4 production, while the presence of IL-12 leads to an increase in IFN-γ production. Thus
it can be seen that NKT cells have the potential to be involved in both Th1, and Th2 immune responses and to regulate these responses.

1.7.3.4 Role in autoimmunity

Recent work has indicated that NKT cells could play an important role in preventing the onset of autoimmune diseases. The lpr mouse (which has a mutation in the gene encoding Fas) and the gld mouse (which has a mutation in the gene encoding FasL) are both susceptible to certain autoimmune diseases. Mutations in either Fas or FasL prevent Fas-mediated apoptotic cell death and it is postulated that this results in the accumulation of autoreactive cells. (NZB\times NZW)F1 mice represent another model of autoimmune disease, where female mice produce autoantibodies against nuclear antigens. Interestingly, in each of these genetically based models of autoimmunity, there is a correlation between reduced numbers of Vα14+ NKT cells during post-natal development and the onset of autoimmune disease (Mieza et al., 1996). Furthermore, treatment of neonatal lpr mice with anti-Vα14 mAbs results in an earlier onset and enhanced development of autoimmune disease. These observations suggest that Vα14+ NKT cells can prevent or modify the development of autoimmune disease.

The non-obese diabetic (NOD) mouse develops T cell-mediated insulin-dependent diabetes mellitus (IDDM) spontaneously. The disease, which resembles human diabetes closely, results from the autoimmune destruction of insulin-producing β cells in the islets of Langerhans (Makino et al., 1980). As discussed in section 1.7.1, the majority of murine NKT cells have the CD4+CD8− (DN) or CD4+ phenotype. Indeed, mature αβ T cells expressing the DN phenotype and having the capacity to produce large quantities of IL-4 have been identified as NKT cells (see section 1.7.3.3). NOD mice and some other
species do not carry the NK1.1 allele, but because NKT cells have a number of other identifying characteristics (see section 1.7.1) they can be detected in these strains. However, these 'NKT cells' are rare in NOD mice (Gombert et al., 1996; Godfrey et al., 1997) and given the potential significance of NKT cells in immunoregulation, it has been proposed that the deficiency of these cells could contribute to the pathogenesis of IDDM. This proposal is supported by the observation that mice which received enriched NKT-like cells did not develop diabetes (Baxter et al., 1997). Thus, it would appear that the presence of functional NKT cells can prevent the onset of disease in the mouse model of type 1 diabetes.

Susceptibility to a number of autoimmune diseases, including diabetes, is influenced by the allelic compositions of the genes encoding MHC molecules. However, there is incomplete concordance in expression of a number of autoimmune diseases between identical twins, where some individuals can carry autoreactive T cells, without development of disease. Thus, it would appear that factors other than MHC background also influence the onset of disease. Recently, a study by Wilson and colleagues (Wilson et al., 1998) examined individuals prone to diabetes genetically. The results of the study showed that diabetics had lower frequencies of NKR-P1+ CD4-CD8- Vα24JαQ+ T cells than their non-diabetic siblings. Furthermore, the NKT cells in the diabetic individuals produced IFN-γ but unlike the NKT cells from normal siblings, they did not produce IL-4. The findings from this study support a model for IDDM in which NKT cells, through their capacity to produce IL-4, regulate the onset of disease in both humans and mice.

1.8 Intraepithelial lymphocytes (IEL)

The presence of small round cells located between epithelial cells of the small intestine
has been recognised for 150 years (Weber, E., (1847) Arch. Anat. Physiol. Wiss. Med. cited in Poussier and Julius, 1994). Intraepithelial lymphocytes (IEL) constitute a large pool of T cells, comparable in number to the T cells present in the spleen and lymph nodes in mice (Rocha et al., 1991). However, the functions of these cells have remained a mystery until recently. Current studies have indicated that IEL comprise a distinct and unique lymphoid population. Some IEL have an origin and function which is different from the majority of T cells found in other lymphoid tissues.

1.8.1 Surface markers and origins of IEL

The intestinal epithelium and a component of the thymic epithelium are both derived from endoderm. It is interesting, therefore, that both of these tissues appear to support T cell development. Most of the T cells that appear initially in the intestinal epithelium of mice express the \(\gamma/\delta\) TCR, a sequence also seen in T cell ontogeny in the thymus (Havran and Allison, 1988). With increasing age, both in the thymus and in the intestinal epithelium, there is a dramatic increase in the proportions of T cells expressing the \(\alpha/\beta\) TCR. However, given that the absolute number of IEL increases five- to ten-fold between the ages of 6 and 20 weeks, it is interesting to note that the absolute number that expresses the \(\gamma/\delta\) TCR varies little during this period (reviewed Poussier and Julius, 1994). The frequency of \(\gamma/\delta\) T cells varies between species. Only a very small proportion of IEL in rats (Fangmann et al., 1991) and humans (Trejdosiewicz et al., 1989) expresses the \(\gamma/\delta\) TCR. The great majority of IEL in these species express the \(\alpha/\beta\) TCR and indeed the majority of IEL in adult mice also express the \(\alpha/\beta\) TCR (Lefrançois, 1990).

As discussed in sections 1.3.2 and 1.4.1, lymphocytes that express the CD3-TCR
complex are present in athymic animals. IEL comprise a large proportion of the thymus-independent T cells in athymic mice, where most of them express the γ/δ TCR+(Guy-Grand et al., 1991; Bandeira et al., 1991). In athymic rats, the predominant IEL expresses the α/β TCR, although the proportion of γ/δ TCR+ T cells is greater than in normal rats (reviewed Rocha et al., 1992 and Mayrhofer, unpublished observations).

Given that IEL are present in athymic animals, it is of interest that studies have shown that IEL can arise from donor bone marrow in ATXBM rats (Mayrhofer and Whatley, 1983) and mice (Bandeira et al., 1991; Poussier et al., 1992; Lefrançois et al., 1990).

Furthermore, earlier studies had shown that neonatal thymectomy in mice abrogated the production of peripheral T cells, yet it had little effect on the frequency of IEL in the small intestine (Ferguson and Parrott, 1972). Collectively, these findings show that some IEL can develop by a thymus-independent means and taken together with the observation that T cell development can occur in athymic mice after the peripheral engraftment of foetal intestine (Mosley and Klein, 1992), the intestinal epithelium has been proposed to support thymus-independent T cell development.

Because the IEL present in athymic mice express the γ/δ TCR predominantly (Guy-Grand et al., 1991; Bandeira et al., 1991), it is not unreasonable to assume that this population is thymus-independent. In addition, the great majority of IEL in athymic mice do not express the Thy-1 (CD90) antigen but they express the CD8 co-receptor.

Expression of CD8 is unusual however, in that CD8α chains are expressed in the absence of CD8β chains. It is proposed that these CD8α chains are expressed as a homodimer (CD8αα), whereas the CD8 expressed by peripheral CD8+ T cells is in the form of a heterodimer (CD8αβ). Interestingly, the few α/β TCR+ T cells present in
athymic mice express CD8αα, suggesting that IEL expressing this unusual form of CD8 are extra-thymically derived.

IEL in mice have a heterogeneous surface phenotype (reviewed by Rocha et al., 1995; Poussier and Julius, 1994; Poussier and Julius, 1994a; Rocha et al., 1992). In euthymic mice, approximately half of the IEL exhibit a phenotype which is comparable to that of peripheral T cells. These cells express both the α/β TCR and Thy-1, most express the CD8αβ co-receptor and a few express CD4. However of the CD4+ cells, some display a CD4+CD8αα+ phenotype, a characteristic shared with some activated CD4+ T cells (see section 1.7.1). In addition to these similarities, this subset of IEL, like peripheral T cells, does not contain potentially autoreactive clones and it appears to be subjected to negative selection (Rocha et al., 1991). The remaining half of murine IEL consists mostly of cells which resemble those IEL that are found in athymic mice and those which are generated in ATXBM chimeras (Mosley et al., 1990). Most express CD8αα homodimers, although a small number are CD4'CD8-. This group of IEL contains Thy-1+ and Thy-1- sub-populations and the cells may express either the α/β TCR or the γ/δ TCR. With respect to the expression of the CD8αα homodimer, it is worth noting that cells expressing this molecule are also numerous among IEL in rats (Helgeland et al., 1996; Torres-Nagel et al., 1992) and humans (Latthe et al., 1994; Jarry et al., 1990).

IEL expressing the α/β TCR and CD8αα in mice exhibit a limited TCR repertoire (oligoclonal) and they express TCR Vβ genes that in some circumstances are potentially autoreactive (Regnault et al., 1996; Regnault et al., 1994; Rocha et al., 1992; Guy-Grand et al., 1992; Rocha et al., 1991; Guy-Grand et al., 1991). Similarly, IEL in humans display a skewed Vβ repertoire and oligoclonality of the TCR (Koningsberger et al.,
1997; Pluschke et al., 1994; Van Kerckhove et al., 1992). Furthermore, there is variation from individual to individual in the clones that are dominant. The explanation for this limited repertoire is unknown. It is possible that the TCR genes expressed by this lineage are the result of positive selection by dominant epitopes (possibly presented in association with novel restriction molecules).

The presence in normal mice of IEL which resemble those seen in athymic mice and in ATXBM chimeras has resulted in the proposal that there exists a dual origin for IEL (Rocha et al., 1994; Guy-Grand et al., 1991). IEL expressing the \( \alpha/\beta \) TCR in association with either CD8\( \alpha \beta \) or CD4 are thought to be thymus-dependent and as a consequence they resemble peripheral T cells. The remaining IEL, which express either the \( \alpha/\beta \) TCR or the \( \gamma/\delta \) TCR and are dominated by co-expression of CD8\( \alpha \alpha \), have been proposed to differentiate locally within the gut itself. These thymus-independent IEL have a number of other phenotypic differences which distinguish them from thymus-dependent IEL. As discussed above, their expression of Thy-1 is variable. In addition, they lack the peripheral T cell markers CD5 and CD28 (Ohteki and MacDonald, 1993).

IEL in rats also exhibit an unusual and related phenotype, where most are CD2\(^{-}\) and a large proportion are CD5\(^{+}\) or CD5\(^{lo}\) (Helgeland et al., 1996; Fangmann et al., 1991). Similarly in humans the proportion of CD8\(^{+}\) IEL that expresses CD5 (Lundqvist et al., 1995; Deusch et al., 1991; Trejdosiewicz et al., 1989) or the co-stimulatory molecule CD28 (Lundqvist et al., 1995) is low, compared to peripheral blood lymphocytes.

However, almost all IEL in mice express the \( \alpha_\beta \gamma \) integrin. The ligand for this integrin has been identified recently as E-cadherin, a member of the immunoglobulin gene superfamily expressed by gut epithelial cells (reviewed by Rocha et al., 1995). The
integrin is expressed in a similar pattern by IEL in humans (Lundqvist et al., 1995; Parker et al., 1992; Cerf-Bensussan et al., 1987) and in rats (Brenan and Rees, 1997). Another feature of IEL is that they express a surface antigen phenotype consistent with memory in classical T cells, although many in mice express also the activation marker CD69 (reviewed by Rocha et al., 1995). IEL in mice do not express CD45RB, while those in rats are CD45RC⁺ (including the dominant CD8⁺ cells which in the case of classical T cells are typically CD45RC⁺, see section 1.5.3) and CD62L⁻ (Kearsey and Stadnyk, 1996). Most IEL in humans express the CD45RO isoform (Lundqvist et al., 1995).

During thymocyte development, precursors with the phenotype TCR⁺CD3⁻CD4⁻CD8⁻CD25⁻ express the CD3 ζ/η chains and also the γ chain of the FceRI (Guy-Grand et al., 1994). The FceRI γ chain is similar in structure to CD3 ζ/η and it can replace the latter functionally in the CD3 complex (Malissen et al., 1993). At the next stage of thymic differentiation (TCR-CD3⁺CD4⁺CD8⁺CD25⁺), expression of the FceRI γ chain ceases and as a consequence, all TCR⁺ expressing cells in the thymus and subsequently in the periphery express CD3 ζ/η chains exclusively in their TCR-CD3 complex. In this respect, thymus-dependent IEL resemble peripheral T cells (Guy-Grand et al., 1994). In contrast, thymus-independent IEL express both ζ/η and FceRIγ chains and these are associated with the TCR as either homodimers or heterodimers. Guy-Grand et al., (1994) noted that thymus-independent T cells from lymph nodes in aged athymic mice also exhibited the same characteristic. These findings, together with the observation that T cells fail to develop in the thymus of CD3-ζ/η gene-knockout mice although thymus-independent IEL are present in normal numbers (Malissen et al., 1993), suggest that utilisation of the FceRI γ chain in the CD3 complex is a characteristic of thymus-
independent T cell development. It has been shown subsequently that positive selection of TCRα/β⁺ FcεRIγ⁺ IEL is dependent on MHC class I (or perhaps CD1) expression, whereas development of TCRγ/δ⁺ FcεRIγ⁺ IEL is independent of either MHC class I or MHC class II molecules (Simpson et al., 1995).

In this respect it is interesting to note that splenic NKT cells also utilise FcεRIγ in their TCR-CD3 complex (see section 1.7.1). As discussed earlier, these cells also comprise a population that develops extra-thymically. IEL from mice have been shown to include populations which express markers of NK cells that include Ly49A (Roland and Cazenave, 1992; Guy-Grand et al., 1996), CD16 (IgG Fc receptor) and NK1.1 (NKR-P1/CD161) (Guy-Grand et al., 1996). The IEL that express NK-markers belong to the thymus-independent population and exhibit both NK activity and anti-CD3 redirected cytotoxicity (Guy-Grand et al., 1996). Furthermore, IEL from mice express Fas-ligand and are able to kill Fas-bearing targets (Rocha et al., 1995). IEL from humans have also been shown to express NK markers, with many expressing CD56 (Lundqvist et al., 1995). Very few peripheral T cells in humans express CD56 and it has been proposed that they have an extra-thymic origin (Takii et al., 1994). In this respect, it is interesting to note that thymus-independent IEL, NK cells, NKT cells, T cells found in athymic mice (see section 1.4.1), and the murine α/β T cells that express intermediate levels of surface TCR and reside in the liver (see section 1.9.1), all express IL-2Rβ constitutively (Ohtsuka et al., 1994). Furthermore, expression of the β chain of the IL-2R is deemed to be essential for thymus-independent T cell development in the gut microenvironment because thymus-independent IEL fail to develop in IL-2Rβ knockout mice (Suzuki et al., 1997).
A clue which suggests an origin of the thymus-independent IEL is the presence of CD3' lymphocytes in the epithelium of the small intestine. CD3' IEL represent a small percentage of the total IEL in normal adult mice. However, they are more common in very young mice and in athymic mice. Furthermore, they are the only IEL population found in mice with the severe combined immunodeficiency (SCID) mutation and in mice with targeted disruption of the rag-1 gene (reviewed by Rocha et al., 1995). Interestingly, many of these CD3' IEL express CD8αα, suggesting that in this case, expression of this molecule is induced by the gut microenvironment rather than activation via the TCR (Croitoru et al., 1990; Guy-Grand et al., 1991). The identification of RAG-1 expression by isolated IEL has provided the most convincing evidence that the intestinal epithelium is a site at which there is thymus-independent development of T cells. IEL containing RAG-1 mRNA were identified first in mice (Guy-Grand et al., 1991) and later it was shown that it was the CD3' (Guy-Grand et al., 1992) and CD3' CD8αα' (Lin et al., 1994) sub-populations that expressed mRNA transcripts of the rag-1 gene. These studies support the hypothesis that the CD3' sub-population of IEL contains the precursors of thymus-independent IEL. Similarly, expression of the rag-1 and rag-2 genes has been identified in the IEL in adult human intestine, providing strong evidence that extra-thymic T cell differentiation occurs also in the human intestine (Lynch et al., 1995). The cells that express RAG-1 in preparations of IEL from humans are largely TCR' and CD3' (Lundqvist et al., 1995), a phenotype similar to that described in mice.

While there is convincing evidence that some IEL can be generated independently of a thymus in both rodents and humans, there is clear evidence that the thymus can exert an important influence on the expansion of thymus-independent IEL, either directly or indirectly. This statement is supported by numerous observations that have revealed that
the total number of thymus-independent IEL is decreased several fold, while the number of CD3- IEL is increased greatly, in athymic and neonatally thymectomized mice (reviewed by Rocha et al., 1995). Indeed, the grafting of a foetal thymus into either 6 week old nude mice or day 3-thymectomized mice resulted in the generation within 6 weeks of IEL that were numerically and phenotypically indistinguishable from those isolated from euthymic controls (Lin et al., 1993). Genetically marked cells from the thymus grafts gave rise to IEL which were similar phenotypically to thymus-independent T cells (γδTCR+ and αβTCR+ CD8αα+) (Lin et al., 1994). It appears, therefore, that the thymus is (or can be) a source of IEL progenitors which can migrate to the gut. Nevertheless, the presence of IEL that express a TCR in athymic animals indicates that the thymus is not the only source of progenitors for IEL. The presence of FceRI γ chains in thymus-independent IEL but not in thymically-derived T cells (discussed above), argues strongly for local T cell differentiation in the small intestine.

Taken together, various line of evidence suggest that the epithelium of the intestine is a site of extra-thymic T cell differentiation and that IEL constitute a mixture of thymically- and extra-thymically-derived T cells. The ways in which the thymus influences this process are unclear. The thymus may provide some precursors for IEL but it is also possible that the thymus-derived component of IEL regulates production of thymus-independent IEL, perhaps via the production of cytokines. γδ T cells and CD8αα+ αβ T cells could have distinct local functions, some of which may be protective, while others may regulate non-immunological functions such as growth of epithelial cells (Thompson et al., 1996).
1.8.2  Antigen recognition and functions of IEL

The numbers of IEL that express γ/δ TCR are normal in mice that are deficient in either MHC class I or MHC class II molecules and also in mice that express neither class of MHC molecules (Schleussner and Ceredig, 1993). This observation indicates that TCRγ/δ IEL are not dependent on MHC molecules for their development. This could reflect the possibility that γ/δ T cells can recognise protein and non-protein antigens directly, in the manner of antibodies (Schild et al., 1994). Those murine γ/δ T cells which populate the skin and non-intestinal epithelia exhibit canonical TCR repertoires and originate early in thymus ontogeny (reviewed by Rocha et al., 1995). In contrast, the population of thymus-independent γ/δ TCR⁺ IEL that are resident in the intestinal epithelium display considerable TCR diversity (Takagaki et al., 1989).

IEL that express the α/β TCR exhibit important differences. Thymus-independent α/β TCR⁺ IEL develop normally in MHC class II deficient mice but they are essentially absent in β₂-microglobulin knockout mice (Schleussner and Ceredig, 1993). This implicates either MHC class I molecules, or non-classical MHC class I-like molecules, in the selection of IEL. Both groups of molecules are dependent on β₂-microglobulin for their surface expression (discussed above). Of particular significance, intestinal epithelial cells in mice (Bleicher et al., 1990), rats (Kasai et al., 1997; Burke et al., 1994) and humans (Blumberg et al., 1991) have been reported to express the MHC-class I-like molecule, CD1. This raises the possibility that CD1 may play a role in mucosal immunity, either in the presentation of antigen and/or in the selection of some IEL subsets. Indeed, IEL from humans have been shown functionally to recognise members of the CD1 gene family (Balk et al., 1991).
CD1 may prove to be an important molecule for the presentation of antigens to IEL. However, it appears that MHC class I is essential for selection of thymus-independent IEL. Unlike the α/β T cells that develop in the thymus, thymus-independent IEL do not appear to be selected negatively by conventional self-antigens (Rocha et al., 1991) but there does appear to be an absolute requirement for specific antigen for their development ("positive selection"). Using transgenic mice expressing rearranged genes encoding an H-Y-specific TCR in the background of the appropriate MHC class I (H-2D^b), Rocha and colleagues showed that recognition in the context of self-MHC is essential for selection of CD8αα^+ α/β TCR^+ IEL (Rocha et al., 1992). Thymus-independent IEL failed to develop in female transgenic mice. In contrast, thymocytes were selected positively by MHC class I molecules in the absence of H-Y antigen and developed into H-Y specific CD8αβ^+ T cells. However, thymus-independent IEL failed to develop also in male mice in which the H-Y TCR-specific transgenes were backcrossed into an inappropriate H-2D^d background (Rocha et al., 1992). These observations indicate that recognition of the nominal antigen (processed H-Y), in association with MHC class I molecules, is essential for the generation of thymus-independent IEL. Recently, another study has refined this approach by utilising mice in which the transgenes encoding the H-Y-specific TCR were introduced into the rag-2^−/− background (Cruz et al., 1998). These mice could, therefore, express only the H-Y specific TCR, without rearrangement of endogenous TCR α genes. The findings in these studies supported those Rocha and colleagues.

Cruz et al proposed a model in which T cell development is contrasted in the thymus and in the small intestine. This model is based on the finding that CD8β plays an
important role in increasing the avidity of the CD8α/β heterodimer for the MHC class I molecule (Renard et al., 1996). Indeed, CD8α is required for the positive selection of most CD8+ T cells in the thymus (Fung-Leung et al., 1994). The model relates to the expression of CD8αα by IEL, compared with the expression of CD8αβ by double positive and by CD8+ single positive thymocytes. It is suggested that in the thymuses of female mice, which do not express the H-Y antigen, the interaction of the CD8αβ coreceptor with MHC class I results in avidity in the intermediate range, leading to positive selection. However in male mice, which do express the H-Y antigen, the CD8αβ coreceptor interacts with MHC class I molecules with higher avidity and this results in, negative selection. In the epithelial compartment of the gut, where IEL express CD8αα, it is proposed that selection is different. It is proposed that the absence of H-Y antigen in female mice does not allow selection of thymus-independent α/β TCR+ IEL. Positive selection is possible in male mice because antigen is present, while the weaker affinity of the CD8αα coreceptor for MHC class I results in an avidity of interaction that is intermediate and does not induce negative selection (Cruz et al., 1998).

As mentioned already, α/β TCR+ IEL exhibit a distinctive TCR repertoire (reviewed by Rocha et al., 1995; Poussier and Julius, 1994). Study of the TCR β-chain in humans (Van Kerckhove et al., 1992; Pluschke et al., 1994), mice (Regnault et al., 1994; Regnault et al., 1996) and rats (Helgeland et al., 1996) reveals that this repertoire is oligoclonal, consisting of only a few hundred clones. Intriguingly however, these clones are entirely different between individual mice of the same strain and litter (Regnault et al., 1994). In addition, this study showed that although the TCRs of thymus-dependent and thymus-independent IEL were both made up of small numbers of clones, no β-chain
sequence was shared between the subsets. The limited TCR repertoire of IEL has led to the view that these T cells have distinct functions and that they recognise only a limited number of gut-borne antigens. This would explain the limited TCR repertoire of IEL, while the presence of persisting antigens in the gut would account for the observation that IEL have a memory phenotype. However, if IEL respond to a distinct and limited group of antigens, the question arises as to why the clonal composition of IEL isolated from different individuals is distinct. The most likely explanation is that the selecting antigens vary from individual to individual, perhaps related to the composition of the gut flora. Selection could occur through stimulation of mature antigen-specific IEL and expansion of dominant populations in an environment where antigen is present constantly. Alternatively, through the requirement for antigen-driven positive selection, IEL could be generated constantly from immature precursors and express only TCRs that recognise the antigens that prevail in the gut of the individual.

The bacterial microflora has an important effect on the numbers of α/β TCR⁺ IEL found in the gut. Although the numbers of γ/δ TCR⁺ IEL are essentially unchanged in conventional and germ-free mice (Bandeira et al., 1990), the numbers of α/β TCR⁺ IEL are reduced ten-fold in germ-free mice when compared to specific pathogen-free (SPF) mice (Regnault et al., 1996; Bandeira et al., 1990). This observation suggests that IEL respond to bacterial antigens and raises the possibility that the TCR repertoire is influenced by bacterial antigens. This view receives support from studies using rats (Helgeland et al., 1996). Germ-free rats were conventionalised by administration of gut contents from conventional rats by gavage and rectal enemas. As has been demonstrated previously in mice (Guy-Grand et al., 1991; Umesaki et al., 1993), conventionalization increased primarily the number of CD8αβ⁺ IEL. However, the largest effects on the
usage of Vβ genes were found among the CD8αα+ IEL. This finding suggests that the α/β TCR repertoire of IEL in rats is to some extent directed against microbial antigens.

However, this finding is not supported by a study by Regnault and colleagues in mice (Regnault et al., 1996). Although the small intestine of germ-free mice contained only one tenth the number of α/β TCR+ IEL found in conventional mice, the TCRβ repertoire of the sorted CD8αα and CD8αβ sub-populations of the α/β TCR+ IEL showed the same degree of oligoclonality as found in conventional mice. Hence, this finding suggests that the intestinal flora is not responsible for the receptor oligoclonality of α/β TCR+ IEL but rather that the presence of bacteria leads to the expansion of clones which have arisen independently. It remains uncertain therefore, whether bacterial antigens have a role in the positive selection of IEL clones or whether the presence of bacteria simply expands the size of these clones non-specifically. If the latter is the case, the nature of the selecting antigen is unknown and the possibility that it is a component of the normal gut cannot be excluded.

The production of cytokines by IEL subsets highlights the important and distinct roles that these cells may play in maintenance of mucosal tissues and in immunity. As discussed in section 1.5.3, the cytokines produced by T cells can be allocated as either type-1 (classically produced by T_{H1} cells), or type-2 (classically produced by T_{H2} cells). However, the cytokine profiles produced by IEL are quite different to those produced by peripheral T cells. CD8+, γδ TCR+ and CD8+, α/β TCR+ IEL both produce IFN-γ and IL-5 spontaneously, although more of these cytokines are produced by α/β TCR+ IEL (Taguchi et al., 1991). Within the CD8+, α/β TCR+ subset, the Thy-1+ (thymus-dependent) and Thy-1− (thymus-independent) IEL populations contain comparable
numbers of cells that produce IFN-γ and IL-5. Some IEL produce both cytokines and such cells are more prevalent within the γ/δ TCR$^+$ population. Very few T cells in seconding lymphoid organs, with the exception of NKT cells (discussed in section 1.7.3.3), produce both type-1 and type-2 cytokines. This suggests that IEL, like NKT cells, may exhibit unique regulatory functions. Although CD8$^+$ IEL produce type-2 cytokines such as IL-5, a later study showed that only CD4$^+$ and CD4$^+$CD8$^+$ (DP) IEL could provide help for B cell responses (Fujihashi et al., 1993). This finding implies that CD4 expressing IEL (thought to be thymus-dependent) have functions similar to those of peripheral CD4$^+$ T cells, and hence distinct from those of CD8$^+$ IEL.

γ/δ TCR$^+$ IEL may have functions that are distinct from those of α/β TCR$^+$ IEL. It has been observed that γ/δ TCR$^+$ IEL, but not α/β TCR$^+$ IEL or peripheral T cells, produce keratinocyte growth factor (KGF) when cultured in vitro (Boismenu and Havran, 1994). KGF is a growth factor which supports the growth of cultured epithelial cells and presumably it has a similar function in vivo. This finding raises the intriguing possibility that some γ/δ TCR$^+$ IEL may recognise injured (self) epithelial cells. Through the production of KGF (and perhaps other factors), these cells could provide a local regulatory mechanism to maintain the integrity of the epithelium of the small intestine. It has been shown that γ/δ TCR$^+$ IEL produce a range of chemokines (chemotactic cytokines), including large quantities of the lymphocyte attracting lymphotactin (Boismenu et al., 1996). Production of lymphotactin could recruit inflammatory T cells into damaged epithelia. Thus γ/δ TCR$^+$ IEL may recognise damaged epithelia and produce KGF to accelerate repair, while the secretion of chemokines could attract other T cell subsets that have protective functions against infection.
In addition to possible effects on the growth and renewal of intestinal epithelial cells, IEL have been implicated in the induction of epithelial cell death. Administration of IL-12 *in vivo* leads to the activation of IEL and acceleration of epithelial cell damage and renewal (Guy-Grand *et al.*, 1998). By using mutant mice, the authors were able to show that IEL and IFN-γ were essential for this process to occur. Furthermore, administration of IL-12 *in vitro* activates IEL of all subsets (thymus-dependent α/β TCR IEL, thymus-independent α/β TCR IEL and γ/δ TCR IEL) and leads to the production of IFN-γ. Anti-CD3 mAbs induce production of both IFN-γ and TNF-α, by all of the IEL subsets. The results suggest that IEL can be activated either through antigen recognition, or via IL-12 produced by other cells such as macrophages and dendritic cells. It appears, therefore, that IEL could influence turnover of the villus epithelium both through the induction of cell death and through promotion of epithelial cell renewal.

### 1.9 α/β TCR<sub>Intermediate</sub> cells

#### 1.9.1 Surface markers and tissue distribution of α/β TCR<sub>Intermediate</sub> cells

Examination of the expression of the α/β TCR in athymic mice revealed that the TCR was expressed at a level higher than found on immature thymocytes but lower than that expressed by mature α/β T cells in euthymic littermates. As a result, the α/β T cells in athymic mice were termed TCR<sub>Intermediate</sub> cells. Later, a population of α/β T cells with an unusual phenotype were described in the livers of MRL-lpr/lpr mice (Ohteki *et al.*, 1990). Most of these cells, had a DN (CD4<sup>-</sup>CD8<sup>-</sup>) phenotype and their numbers were increased by exposure to bacteria (Abo *et al.*, 1991). Flow cytometric analysis of expression of the α/β TCR by murine liver T cells revealed two sub-populations, one
with the TCR$^{\text{high}}$ phenotype of mature T cells and another which expressed the TCR at a lower level. This pattern was not seen in other lymphoid organs, apart from the thymus (Abo et al., 1991). The latter sub-population of hepatic T cells expresses the TCR at a level which is intermediate between that of immature TCR$^{\text{low}}$ thymocytes and mature TCR$^{\text{high}}$ T cells and they have been termed TCR$^{\text{intermediate}}$ T cells (Watanabe et al., 1992). Comparison of the sizes of TCR$^{\text{intermediate}}$ cells and TCR$^{\text{high}}$ cells from liver revealed that the TCR$^{\text{intermediate}}$ cells were significantly larger (Watanabe et al., 1992; Ohteki et al., 1992).

A number of studies confirm that TCR$^{\text{intermediate}}$ cells include a prominent population which does not express either CD4 or CD8 (Iiai et al., 1992; Watanabe et al., 1992; Ohteki et al., 1992; Ohteki et al., 1990). In the livers of normal mice, most of the TCR$^{\text{intermediate}}$ population expressed CD4, approximately 25-30% expressed the DN phenotype and only a small percentage expressed CD8 (Iiai et al., 1992). When α/β TCR$^{\text{high}}$ cells from peripheral lymphoid organs were examined, most were found to express either CD4 or CD8. Very few expressed the DN phenotype (Iiai et al., 1992).

The TCR$^{\text{intermediate}}$ phenotype was used to gate the population electronically for further analysis. TCR$^{\text{intermediate}}$ cells, like NK cells, were shown to express high levels of LFA-1 (Watanabe et al., 1992; Ohteki et al., 1992) and they expressed IL-2Rβ constitutively (Arai et al., 1995; Watanabe et al., 1993). These features distinguish them from resting TCR$^{\text{high}}$ cells, which express low levels of LFA-1 and express IL-2Rβ only after activation. Further studies have revealed that TCR$^{\text{intermediate}}$ cells express high levels of CD44 (Arai et al., 1995; Ohteki et al., 1992) and that they do not express L-selectin (CD62L) (Arai et al., 1995). This pattern of adhesion molecule expression, constitutive
expression of IL-2Rβ, predominant populations of CD4+ and DN cells, and the TCR\textsuperscript{intermediate} phenotype is shared with NKT cells (discussed in section 1.7.1). Indeed, a considerable population of TCR\textsuperscript{intermediate} cells express NK1.1. The liver contains, therefore, a high frequency of NKT cells (see section 1.7.2). However, unlike orthodox NKT cells, a small yet significant population of TCR\textsuperscript{intermediate} cells express the CD8 co-receptor (Emoto \textit{et al}., 1997; Watanabe \textit{et al}., 1995; Watanabe \textit{et al}., 1992).

Another feature which distinguishes TCR\textsuperscript{intermediate} cells from most TCR\textsuperscript{high} cells is the predominant use of Vβ8 in the rearranged TCR β chain (Emoto \textit{et al}., 1995; Ohteki and MacDonald, 1994; Abo \textit{et al}., 1991) and the existence of potentially self-reactive TCRs (Narita \textit{et al}., 1998; Kawachi \textit{et al}., 1995; Seki \textit{et al}., 1994; Hiromatsu \textit{et al}., 1992; Abo \textit{et al}., 1991). It has been shown that after bacterial infection, the numbers of cells expressing potentially self-reactive TCRs increase in the liver and to a lesser degree, in other organs such as the spleen (Hiromatsu \textit{et al}., 1992; Abo \textit{et al}., 1991). Indeed, proliferative responses in syngeneic MLRs suggest that these T cells are indeed self-reactive (Abo \textit{et al}., 1991) and that the self-reactive T cells are restricted to those expressing intermediate levels of TCR and IL-2Rβ (Kawachi \textit{et al}., 1995). Although the liver contains the highest frequency of TCR\textsuperscript{intermediate} cells, the proportion of TCR\textsuperscript{intermediate} cells in other organs such as the spleen, bone marrow and thymus increases with age (Watanabe \textit{et al}., 1995; Kawachi \textit{et al}., 1995; Arai \textit{et al}., 1995; Seki \textit{et al}., 1994; Watanabe \textit{et al}., 1993; Ohteki \textit{et al}., 1992) and these cells also express potentially self-reactive TCRs (Kawachi \textit{et al}., 1995). In contrast to TCR\textsuperscript{high} cells, TCR\textsuperscript{intermediate} cells isolated from both the liver and the spleen were shown to have potent cytotoxic effects against syngeneic hepatoma cells in the presence of anti-CD3 mAb (Kawachi \textit{et al}., 1995). TCR\textsuperscript{intermediate} cells, irrespective of tissue distribution, share a
similar Vβ repertoire with the DN α/β T cells isolated from the thymus (Seki et al., 1994). NKT cells, which are abundant in the liver, appear to be a subset of this population (see section 1.7.2).

Watanabe and colleagues (Watanabe et al., 1995) examined the relationship between TCRintermediate cells and NKT cells in the tissues of mice. They found that the majority of TCRintermediate cells in the liver and the thymus expressed NK1.1+ (NKT cells), whereas the majority of TCRintermediate cells in the spleen, lymph nodes and bone marrow were NK1.1- . Interestingly, whereas most NK1.1+ cells also expressed CD4+ or had a DN phenotype, the majority of the NK1.1- TCRintermediate cells expressed CD8 (Watanabe et al., 1995).

In conclusion, TCRintermediate cells and NKT cells share a number of characteristics in common (see section 1.7.1). Both cell types exhibit a similar profile of adhesion molecules (CD44hi, LFA-1hi, L-selectin') and express IL-2Rβ constitutively. The TCR β repertoires are similar and the cells have similar morphology (Watanabe et al., 1995). It appears, therefore, that NK1.1- TCRintermediate cells and NK1.1+ TCRintermediate (NKT) cells could be related. Although some NK1.1- TCRintermediate cells express CD8, it is important to note that the CD8α chain is expressed by some T cells after activation (see section of 1.7.1). The CD8α+ phenotype could be superimposed by activation on cells that express a DN phenotype similar to that of many TCRintermediate T cells and some NKT cells. However, it should be recognised also that down regulation of the TCR has been reported following activation's of T cells in vitro (Valitutti et al., 1997) and that in consequence the TCRintermediate cells could contain activated T cells belonging to several distinct subsets.
1.9.2 Origin of α/β TCR\textsuperscript{intermediate} cells

The observation that TCR\textsuperscript{intermediate} cells are essentially the only α/β T cells present in the tissues of congenitally athymic (nu/nu) (Sato et al., 1995; Iiái et al., 1992; Watanabe et al., 1992; Ohteki et al., 1992) and thymectomized mice (Sato et al., 1995; Iiái et al., 1992) suggests that the development of these T cells is thymus-independent. The probable thymus-independence of some NKT cells (see section 1.7.2) and the observation that the majority of TCR\textsuperscript{intermediate} cells present in the livers of mice express NK1.1 antigen supports an extra-thymic origin for that particular population of TCR\textsuperscript{intermediate} cells. The fact that thymic, hepatic and intestinal epithelia are all derived from endoderm provides a speculative link between these organs and sites of T cell development.

The foetal liver is a rich source of haematopoietic progenitor cells and a source of thymocyte progenitors. However, it is interesting to note that haematopoietic progenitor cells residing in the foetal liver of mice are able to differentiate rapidly into TCR\textsuperscript{+} cells in vitro (Kawamoto et al., 1997; Shimamura et al., 1992). During the foetal stage of development in mice, many haematopoietic cells are present within the hepatic parenchyma but lymphocytes are not present within the sinusoids (Iiái et al., 1992). However, after birth and with increased ageing, lymphocytes appear in the hepatic sinusoids and flow cytometric studies reveal that these lymphocytes are largely TCR\textsuperscript{intermediate} cells (Iiái et al., 1992). The numbers of these TCR\textsuperscript{intermediate} cells increase with age, not only in the liver, but also in the periphery (Iiái et al., 1992; Ohteki et al., 1992).

An indication that TCR\textsuperscript{intermediate} cells are generated within the liver is the observation
that some lymphocytes in the hepatic sinusoids of mice synthesise DNA (Ohteki et al., 1992). More recent studies have added strength to the argument that the liver is a major source of these unique cells. As discussed above, rearrangement of the Vα14+ TCR gene takes place within the thymus (Makino et al., 1993) while other Vα14-positive T cells are proposed to develop by an extra-thymic pathway. Because TCR rearrangement is dependent on expression of the rag-1 and rag-2 genes, transcription of these genes provides the most convincing evidence for T cell development. Therefore, the recent detection in mice of rag-1 and rag-2 gene transcripts in hepatic lymphocytes (Narita et al., 1998; Kimura et al., 1995; Sato et al., 1995) suggests strongly that T cells can develop extra-thymically in the livers of mature animals. Furthermore, immature T cells expressing the early T cell markers CD2 and CD7 have been identified in the livers of adult humans (Collins et al., 1996). These cells, but not mature hepatic T cells, were shown to express mRNA for RAG-1, RAG-2 and pTc (Collins et al., 1996), thus providing evidence that T cells can develop extra-thymically in adult humans as well as mice.

1.9.3 Functions of α/β TCR\textsuperscript{intermediate} cells

As discussed in section 1.9.1, bacterial infection leads to an increase in the numbers of TCR\textsuperscript{intermediate} cells in the liver (Abo et al., 1992). This has raised the possibility that these unique T cells might be involved in immune responses against bacteria. For instance, it has been proposed that TCR\textsuperscript{intermediate} cells might play a protective role in the liver during primary murine salmonellosis (Matsumoto et al., 1994). It is thought that those cells could be beneficial during the early stage of Salmonella infection, before a response is mounted by the thymus-derived T cells. Elimination of TCR\textsuperscript{high} T cells with low doses of anti-α/β TCR mAb did not affect bacterial growth in the early stages of
bacterial infection. However, high doses of anti-α/β TCR mAb deleted both the TCR^high cells and TCR^intermediate cells and this treatment was accompanied by an increased rate of bacterial growth. It is postulated that the TCR^intermediate cells, which include potentially autoreactive T cells, may recognise heat shock proteins expressed by infected macrophages. These findings are supported by the work of Ohtsuka and colleagues (Ohtsuka et al., 1995). During infection with *Listeria monocytogenes*, the authors noted a prominent increase in mononuclear cell in the livers and spleens of infected mice. Phenotypic analysis revealed that numbers of NK cells and TCR^intermediate cells increased before there was any rise in the numbers of conventional T cells in these organs, while deletion of NK cells and TCR^intermediate cells by administration of mAbs led to the mice becoming susceptible to sublethal doses of bacteria. Both of these findings indicate, therefore, that TCR^intermediate cells may be important cells in certain bacterial infections. Because of their similarities to NKT cells, the protective effects of the TCR^intermediate cells could be related to their rapid secretion of cytokines such as IFN-γ after stimulation (see section 1.7.3)

The observation that the numbers of potentially autoreactive TCR^intermediate cells increase in the liver following partial hepatectomy has led to the hypothesis that thymus-independent T cells may have a role in regulating regeneration of hepatocytes (Sato *et al.*, 1993). It is also interesting to note that TCR^intermediate cells increase in numbers in the liver and uterus during pregnancy (Kimura *et al.*, 1995) and that the administration of oestrogen enhances the differentiation of TCR^intermediate cells in the liver (Narita *et al.*, 1998; Okuyama *et al.*, 1992). It is interesting to speculate, therefore, that like IEL (see section 1.8.2), TCR^intermediate cells could play a role in regulating growth of non-haematopoietic cells. In a more general sense, thymus-independent T cells (including
NKT cells) could regulate not only growth and regeneration of epithelia, but also immune responses, auto-reactivity and growth of tumours.

1.10 Lymphocyte-filled villi (LFV) and Cryptopatches

1.10.1 Lymphocyte-filled villi (LFV)

Villi are finger-like processes that greatly increase the surface area of the mucosa of the small intestine. Approximately one percent of the villi in the rat small intestine have a modified structure. These villi contain closely packed lymphocytes and dendritic cells, and they are covered by epithelium which contains specialised antigen-absorbing cells (Mayrhofer, unpublished).

Recent work in this laboratory has classified these modified villi as organised lymphoid structures (Mayrhofer and Brooks, 1995). Lymphocyte-filled villi (LFV) are present in specific pathogen free (SPF) rats as well as in conventionally housed rats, suggesting that they are not inflammatory foci. LFV are present also in congenitally athymic (rNu/rNu) rats. These structures do not receive lymphocytes from the recirculating pool, suggesting that they maybe sites of primary lymphopoiesis.

The cellular composition of LFV is distinct from that of Peyer’s patches and isolated lymphoid follicles. Whereas Peyer’s patches and isolated lymphoid follicles contain T cell and B cell rich regions (reviewed by Kagnoff, 1987), LFV do not contain B cells. In fact, the great majority of lymphocytes in LFV do not express markers of either mature B cells or T cells. Only a relatively minor subpopulation of the lymphocytes expresses either the TCR or CD3. Other than MHC class I and CD45, the only markers that have been identified to date on the majority of lymphocytes in LFV are leukosialin.
(CD43) and the IL-2Rα (CD25). The minor subpopulation of lymphocytes in LFV that express markers of classical T cells have mainly the phenotype α/β TCR+ and CD4+.

There is a small sub-population of CD8+ cells but there are no γ/δ TCR+ cells. Autoradiographic studies using tritiated thymidine (3H-TdR) indicate that LFV contain dividing lymphocytes. Kinetic studies have shown that the label is incorporated first by TCR+ lymphocytes and only later enters the population of α/β TCR+ cells.

The cellular composition of LFV in adult nude rats is essentially identical to those in euthymic rats. This finding, together with features found in the thymus (such as the presence of potential antigen presenting cells (DC), a large population of cells that expresses CD25 but lacks TCR, and a smaller population that expresses the α/β TCR), suggests that LFV may be sites of thymus-independent T cell development. Cells expressing the α/β TCR appear in LFV of nude rats at about 9 weeks of age, shortly before α/β T cells are detected in the thoracic duct lymph. As a major contributor of lymphocytes to the thoracic duct, the gut is a candidate for the source of α/β T cells in nude rats. LFV, as organs containing lymphocytes that have the α/β TCR+ CD4+ phenotype, are the most likely sites in the small intestines in which such cells could be generated.

1.10.2 Mouse LFV and Cryptopatches

Recent work in this laboratory (Moghaddami and Mayrhofer, manuscript in preparation) has shown that structures similar to rat LFV can be identified in other rodents (mice and hamsters) and in humans (Moghaddami et al., 1998). LFV in mice share a number of characteristics with those in rats with respect to the maturity of the lymphocytes that
they contain and thymus-independence. However, while structurally similar, LFV in humans contain mature T cells with a memory phenotype (Moghaddami et al., 1998).

The mouse LFV homologue does not contain significant numbers of surface Ig* B cells, although there is a small population of B220* cells. Phenotypic studies (Moghaddami and Mayrhofer, in preparation) reveal that the majority of lymphocytes in this structure express Thy-1 antigen and some surface molecules (e.g. sca-2, TSA-2, HSA, PgP1) that are characteristic of thymocytes but not mature T cells. In addition, significant numbers of the lymphocytes in LFV are labelled by a pulse of tritiated thymidine, indicating that substantial numbers of these cells are dividing. Dendritic cells form an extensive network between the lymphocytes. CD3* and TCR* cells are essentially absent in mouse LFV, although a small population of cells express CD4 and most of the cells express CD25. Work by others has identified similar structures in the mouse, which have been named 'cryptopatches' (Kanamori et al., 1996). LFV appear to be more numerous than the reported incidence of cryptopatches and the relationship between the two structures is unclear. The majority of lymphocytes in cryptopatches have the phenotype of immature thymocytes, characterised by their expression of IL-2R, IL-7R and c-kit (CD117). Significant numbers (10-20%) of lymphocytes in these structures express CD4, although expression of CD3 and TCR was not detected. RAG-1 protein was not detected in the lymphocytes in cryptopatches and this is consistent with the absence of detectable numbers of mature CD3-TCR* cells. If the mouse and rat homologues have similar functions and both are sites of primary T cell differentiation, it appears that developing T cells exit from mouse LFV at a stage before rearrangement of the genes encoding the TCR.
Although T cells do not undergo complete maturation in the mouse LFV, it is possible that they give rise to immature cells that migrate to other sites where they continue their development. The expression of CD4 by lymphocytes in the LFV is consistent with this possibility, because committed T cell progenitors in humans (Bruno et al., 1997) and mice (Wu et al., 1991) express this co-receptor. Indeed, it has been shown recently that cells isolated from cryptopatches and transferred adoptively to SCID recipients give rise to $\alpha/\beta$ TCR$^+$ and $\gamma/\delta$ TCR$^+$ IEL and also to $\alpha/\beta$ T cells which can be recovered from the mesenteric lymph nodes of the recipient. In contrast, cells from Peyer's patches and mesenteric lymph nodes were unable to reconstitute the IEL compartment (Saito et al., 1998). This study provides evidence that sites other than the thymus can initiate the differentiation of T cells. It also indicates that the LFV present in athymic rats could give rise to T cells with the potential to seed the intestinal mucosa and perhaps sites in the periphery.

### 1.11 Project aims and hypotheses

The origins and functions of thymus-independent T cells remain contentious issues. Congenitally athymic laboratory rodents have cells which express T cell markers, and the numbers of these cells increase with age. However, thymus-independent T cells in athymic mice exhibit a number of subtle phenotypic differences when compared with thymus-derived T cells. With the exception of IEL, the thymus-independent T cells in athymic animals are distributed in the same areas occupied by thymus-dependent T cells in euthymic animals. However, there is some evidence that thymus-independent T cells are enriched in certain anatomical sites; namely the bone marrow, the intestinal epithelium and the liver. Such cells have also been reported to increase in number under certain conditions, including bacterial infection and tissue regeneration. Together, these
observations suggest that thymus-independent T cells may be present in normal individuals and have specific (and possibly novel) functions.

1.11.1 Hypotheses

- Thymus-independent development of α/β T cells occurs in LFV and the livers of post-pubertal rats.

- Thymus-independent α/β T cells in athymic rats have a surface antigen phenotype that is distinct from classical α/β T cells.

- Thymus-independent α/β T cells in athymic rats are phenotypically and functionally related to non-classical α/β T cells, including CD161⁺ NKT cells.
Chapter 2

Materials and Methods
2.1 Animals

Female CBHrNu/rNu and CBHrNu/+ rats aged 10-52 weeks were held under specific pathogen free conditions at the Animal Resource Centre (ARC), Perth, until required. They were then transported in isolator cages and housed under barrier conditions at the Medical School Animal House, University of Adelaide. DA rats ranging from 7-14 weeks of age were also obtained from the ARC, Perth.

2.2 Preparation of non-sterile cell suspensions

Phosphate buffered saline (PBS) pH 7.4 was used regularly and comprised of 0.14M NaCl, 3mM KCl, 8mM Na₂HPO₄·12H₂O and 1mM KH₂PO₄ (either BDH AnalAR®, Merck, Kilsyth, Victoria, Australia or 'Univar', Ajax Chemicals, Auburn, NSW, Australia) dissolved in Milli-Q purified water (Millipore Corporation, USA). PBS was prepared as a 20x concentrate: 160g NaCl, 4g KCl, 58g Na₂HPO₄·12H₂O and 4g KH₂PO₄ were dissolved in 900ml of Milli-Q water. The pH was adjusted with 1M HCl (BDH AnalAR®, Merck, Australia) and the volume made up to 1L prior to sterilisation by autoclaving. The solution was diluted 1/20 in Milli-Q water prior to use.

2.2.1 Preparation of cells from lymph nodes and spleens

Lymph nodes (pooled from mesenteric and peripheral sites) and/or spleen were removed from anaesthetised animals and placed in a small volume of PBS containing 1% (v/v) fetal calf serum (FCS) (MultiSer™, Cytosystems, Castle Hill, NSW, Australia) which had been heat inactivated previously by incubating at 56°C for 45 minutes. The tissue was chopped finely with scissors and transferred to a loose fitting glass homogeniser. Following gentle homogenisation, the suspension was filtered through cotton wool and
centrifuged at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). Spleen cells were resuspended in Tris-buffered 0.15M ammonium chloride (1 volume 2.06% (w/v) Tris-HCl pH 7.6 [Boehringer-Mannheim, Germany] to 9 volumes 0.83% (w/v) NH₄Cl ['Univar', Ajax Chemicals, Australia]), incubated for 10 minutes on ice to lyse red blood cells and then centrifuged as described above. The resulting cell pellets were washed twice in PBS containing 1% FCS and 0.01% (w/v) sodium azide (Az) ('Labchem', Ajax Chemicals, Australia) (PBS/FCS/Az) and then resuspended in this solution. Cell density and viability were calculated using a haemocytometer and Trypan blue (ICN Biomedicals, Aurora, Ohio, USA) dye exclusion (0.8% w/v in PBS).

2.2.2 Preparation of cells from Peyer’s patches

Small intestines were removed from anaesthetised animals and the Peyer’s patches excised carefully and placed in a small volume of PBS/FCS/Az. Using two 19 gauge (G) needles, cells were teased gently out of individual Peyer’s patches into the PBS/FCS/Az buffer (suspension 1). Following this process the remnants of the Peyer’s patches were removed and pushed through a sieve with a liberal amount of PBS/FCS/Az to facilitate the isolation of cells remaining in the tissues (suspension 2). The two cell suspensions were then pooled and filtered through cotton wool. After two washes in PBS/FCS/Az, the recovery and viability of the isolated cells was assessed by Trypan blue dye exclusion as described above (see previous section).

2.2.3 Preparation of mononuclear cells from the liver

Livers were removed after exsanguination of the rats. The liver was chopped finely with scissors in a small volume of PBS/FCS/Az, pushed through a sieve and washed with
liberal volumes of the same buffer. The suspension was then filtered through cotton wool and centrifuged at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). After washing twice in PBS/FCS/Az, the pellet was resuspended in this solution and the mononuclear fraction prepared by layering 2 volumes of cell suspension over 1 volume of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The resulting gradient was centrifuged at 500g for 20 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA) and the mononuclear cells were aspirated carefully from the interface. These cells were then washed and counted as described above (see section 2.2.1).

2.2.4 Preparation of cells from bone marrow

Bone marrow cells were isolated from the femur by flushing the shafts with PBS/FCS/Az through a 19G needle. The marrow plugs were aspirated using a 23G needle and syringe to disperse the cells and the resulting cell suspension was filtered through cotton wool. The cells were centrifuged at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA) and washed twice in PBS/FCS/Az. The red blood cells were lysed as described above for spleen preparations, and the resulting cells were washed and counted (see section 2.2.1.).

2.2.5 Isolation of mononuclear cells from peripheral blood

Peripheral blood mononuclear cells were prepared from blood collected into heparanised tubes (Sarstedt, Ingle Farm, S.A., Australia) following cardiac puncture of anaesthetised rats with a 23G needle. An equal volume of PBS/FCS/Az was added to the blood before layering over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifugation at 500g for 20 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). Mononuclear cells, which had formed a band at the interface, were aspirated carefully,
then washed and counted as described above (section 2.2.1). The total number of lymphocytes in the blood of each rat was calculated by weighing each animal and applying the following formula:

Total blood lymphocytes = $1.52 \times 10^8 \times \text{weight (kg)}$ (Trepel, 1974).

### 2.2.6 Thoracic duct lymphocytes obtained by thoracic duct cannulation

Prior to the procedure, cannulae were constructed using 40-50cm lengths of polyethylene tubing (0.5mm internal diameter and 1.0mm outside diameter) (Dural Plastics & Engineering, Dural, N.S.W., Australia, Cat. No. SP 37). The tubing was shaped into a U-bend at one end (approximately 1.5cm in length) by the application of heat. A bevelled edge was then cut at the end of the U-bend to enable ease of insertion into the duct and the cannula bent slightly to enable it to exit the through the dorsal wall of the rat after insertion into the thoracic duct.

The animal was anaesthetised with ether and shaved on its ventral and left hand side. An intravenous drip delivering 1u/ml heparin (Sigma, St Louis, MO, USA) in PBS was then inserted via the tail vein using a 23G needle. A subcostal incision was made on the left hand side from the midline to the mid-axillary line approximately 0.5cm below and parallel to the rib cage. Cotton buds were then employed to displace the left kidney and surrounding fat tissue to expose the aorta. Surrounding tissues were then displaced medially and held in place by cotton gauze soaked in saline, supported by a retractor. The thoracic duct was dissected away from the aorta and a piece of surgical silk placed around the duct and tied loosely. A 12G needle was then passed through the dorsal body wall adjacent to the tie on the thoracic duct and used to pass the cannula through the body wall. The cannula was filled with 1u/ml heparin (Sigma, St Louis, MO, USA) in
PBS, clamped at the distal end and secured loosely with surgical silk to the back wall of the abdomen.

The thoracic duct was engorged by applying pressure below the diaphragm and incised part way through, just anterior to the silk thread. The cannula was then inserted and secured firmly at entry into the duct and at exit from the abdomen by the pre-placed silk ties. The incision was then closed with two layers of continuous silk sutures. The animal was placed in a Bolman cage and provided with food and isotonic saline to drink. The intravenous drip was then removed after a total of 4-5 ml of PBS/heparin was delivered during the course of the procedure.

Thoracic duct lymph (TDL) was collected overnight into 5ml of PBS containing 20u/ml heparin (Sigma, USA) in a sterile 200ml tissue culture flask (Nunc, Denmark). The collection was performed on ice if cells were to be analysed by flow cytometry and at room temperature if cells were to be used for adoptive transfer in CFSE-tracking experiments (section 2.6.6). The cells were resuspended, filtered through cotton wool, washed twice with PBS/FCS/Az and then a viable cell count was performed using a haemocytometer and Trypan blue dye exclusion (see section 2.2.1).

2.3 Tissue culture procedures

All cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air.

2.3.1 Preparation of medium

Roswell Park Memorial Institute (RPMI-1640) culture medium was used in all cell culture
work. RPMI 1640 was prepared by dissolving a sachet of RPMI-1640 powder (Gibco BRL, Gathersburg, MD, USA) and 2g NaHCO₃ ('Univar', Ajax Chemicals, Australia) in Milli-Q purified water (Millipore Corporation, USA). Sterile stock solutions of N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) (Boehringer Mannheim, Germany) pH 7.2, penicillin (Sigma, St Louis, MO, USA) and streptomycin sulphate (Sigma, USA) were added to give final concentrations of 15mM, 67μg/ml and 100μg/ml respectively, and the pH adjusted to 7.4 with 1M HCl. The volume was made up to 1 litre and filter sterilised using a Sterivex GS 0.22μm filter unit with filling bell (Millipore, USA).

Prior to use, medium was supplemented with 2mM glutamine (BDH AnalAr®, Merck, Australia), 0.1mM β-mercaptoethanol (Sigma, Cat. No. M-3148) and 10% v/v heat inactivated FCS (MultiSer™, Cytosystems, Australia) as per section 2.2.1. After 7 days of storage, medium was re-supplemented with glutamine (2mM final concentration).

2.3.2 Preparation of sterile cells for culture

Animals were euthenased and then immersed in 70% ethanol. Lymph nodes (pooled from mesenteric and peripheral sites) were removed aseptically in a laminar flow hood, chopped finely in a Petri dish and transferred to a sterile glass homogeniser, where they were homogenised gently. The cell suspension was filtered through cotton wool with RPMI-1640 culture medium (see section 2.3.1) and then centrifuged at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). Cells were washed twice in RPMI-1640 culture medium and a viable cell count performed using a haemocytometer and Trypan blue dye exclusion (see section 2.2.1). Cells were then cultured as per conditions described in sections 2.6.5 and 2.6.7.
2.4 Antibodies

2.4.1 Primary antibodies

A range of mouse anti-rat monoclonal antibodies (mAbs) were used in flow cytometric and immunohistochemical studies. These monoclonal antibodies are listed in Table 2.1. They were either produced from hybridomas held in the Department of Microbiology and Immunology, or they were obtained as gifts, or purchased from commercial sources (as listed).

The following phycoerythrin (PE)-conjugated mouse anti-rat mAbs were purchased from PharMingen (San Diego, CA, USA) and used in 2-colour flow cytometric and immunofluorescent histological analyses: R73-PE (anti-rat α/β TCR), OX35-PE (anti-rat CD4), OX38-PE (anti-rat CD4), OX8-PE (anti-rat CD8α), 10/78-PE (anti-rat CD161). PE-conjugated mAb 107.3 (anti-trinitrophenol [TNP]) was used as a negative control.

A polyclonal rabbit anti-mouse RAG-1 antibody (cross-reactive with rat RAG-1) was purchased from PharMingen (Cat. No. 13781A) and used to detect RAG-1 protein in rat tissue sections.

With the exception of mAb Mark1 (anti-rat kappa), in all experiments antibodies were used in the presence of 10% heat inactivated normal rat serum (NRS) (as per section 2.2.1) to block non-specific binding through Fc receptors and to absorb any residual cross-reactivity by secondary antibodies for rat Ig.
Table 2.1 Mouse anti-rat monoclonal antibodies used in this study. Target antigens of monoclonal antibodies, their reported distributions on rat leukocytes, the isotypes of the antibodies, their sources and the concentrations at which they were used are listed.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/N</td>
<td>antibody culture supernatant</td>
</tr>
<tr>
<td>mφ</td>
<td>monocytes and macrophages</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>RTE</td>
<td>recent thymic emigrants</td>
</tr>
<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>IEL</td>
<td>intraepithelial lymphocytes</td>
</tr>
</tbody>
</table>

Ascites containing mAb V65 was provided kindly by Dr T. Hünig, Würzburg, Germany.

Culture supernatant containing mAb OX85 was provided kindly by Dr P. Holt, Perth, Australia.
<table>
<thead>
<tr>
<th>Ab Clone</th>
<th>Target Antigen</th>
<th>Distribution on leukocytes</th>
<th>Form</th>
<th>Conc.</th>
<th>Isotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4.18</td>
<td>CD3</td>
<td>T cells, thymocytes</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₃</td>
<td>hybridoma/ Nicolls et al., 1993</td>
</tr>
<tr>
<td>R73</td>
<td>αβ TCR</td>
<td>αβ T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Hüning et al., 1989</td>
</tr>
<tr>
<td>V65</td>
<td>γδ TCR</td>
<td>γδ T cells</td>
<td>Ascites</td>
<td>1/10000</td>
<td>IgG₁</td>
<td>hybridoma/ Dallman et al., 1984</td>
</tr>
<tr>
<td>OX19</td>
<td>CD5</td>
<td>peripheral T cells, thymocytes</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Jefferies et al., 1985</td>
</tr>
<tr>
<td>OX34</td>
<td>CD2 (LFA-2)</td>
<td>peripheral T cells, thymocytes, NK cells, mφ</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Williams et al., 1977</td>
</tr>
<tr>
<td>W3/25</td>
<td>CD4</td>
<td>T cell subset, thymocytes, mφ, DC</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Brideau et al., 1980</td>
</tr>
<tr>
<td>OX8</td>
<td>CD8α-chain</td>
<td>subsets of T cells, thymocytes, NK cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₂a</td>
<td>hybridoma/ Torres-Nagel et al., 1992</td>
</tr>
<tr>
<td>OX33</td>
<td>CD45RA or A/B</td>
<td>B cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Bazin et al., 1984</td>
</tr>
<tr>
<td>MARK-1</td>
<td>rat Ig κ-chain</td>
<td>B cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Kraus et al., 1996</td>
</tr>
<tr>
<td>10/78</td>
<td>CD161 (NKR-P1)</td>
<td>NK cells, subset of T cells, mφ, DC</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Mason and Williams, 1980</td>
</tr>
<tr>
<td>OX7</td>
<td>CD90 (Thy-1)</td>
<td>thymocytes, immature B cells, RTE</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Jefferies et al., 1985</td>
</tr>
<tr>
<td>OX22</td>
<td>CD45RC</td>
<td>CD₈⁺ T cells, NK cells, subset of CD4⁺ T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ McMaster and Williams, 1979</td>
</tr>
<tr>
<td>OX6</td>
<td>MHC class II</td>
<td>B cells, mφ, DC, activated T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Paterson et al., 1987</td>
</tr>
<tr>
<td>OX26</td>
<td>CD71 (Rat transferrin R)</td>
<td>dividing/proliferating cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Paterson et al., 1987</td>
</tr>
<tr>
<td>OX39</td>
<td>CD25 (IL-2 R α chain)</td>
<td>activated T cells, activated B cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₂a</td>
<td>hybridoma/ Paterson et al., 1987</td>
</tr>
<tr>
<td>OX40</td>
<td>CD134 (OX40-antigen)</td>
<td>activated CD4⁺ T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Dr G. Mayrhofer (unpublished)</td>
</tr>
<tr>
<td>UA002</td>
<td>Unknown</td>
<td>B cells, DC, mφ, activated T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₂a</td>
<td>hybridoma/ Tamatani et al., 1991</td>
</tr>
<tr>
<td>WT-1</td>
<td>CD11a (α-subunit of LFA-1)</td>
<td>majority of leukocytes</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₂a</td>
<td>hybridoma/ Tamatani et al., 1993</td>
</tr>
<tr>
<td>WT-5</td>
<td>CD11b (α-subunit of Mac-1)</td>
<td>NK cells, mφ, DC, granulocytes</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Kroese et al., 1985</td>
</tr>
<tr>
<td>W3/13</td>
<td>CD43 (leukosialin)</td>
<td>T cells, plasma cells, NK cells, granulocytes</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₂a</td>
<td>hybridoma/ Paterson et al., 1987</td>
</tr>
<tr>
<td>OX50</td>
<td>CD44 (hyaluronate R)</td>
<td>most leukocytes except for a subset of B cells</td>
<td>Purified</td>
<td>0.5mg/ml</td>
<td>IgG₂a</td>
<td>hybridoma/ D. Mayrhofer (unpublished)</td>
</tr>
<tr>
<td>MRα4-1</td>
<td>CD49d (integrin α₄ subunit)</td>
<td>T cells, B cells, thymocytes, mast cells, mφ</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₂a</td>
<td>hybridoma/ Tamatani and Miyasaka, 1990</td>
</tr>
<tr>
<td>1A29</td>
<td>CD54 (ICAM-1)</td>
<td>B cells, subset of T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Brenan et al., 1992</td>
</tr>
<tr>
<td>OX85</td>
<td>CD62L (L-selectin)</td>
<td>B cells, subset of T cells, neutrophils</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Ramie et al., 1996</td>
</tr>
<tr>
<td>OX82</td>
<td>αE₅- integrin</td>
<td>DC, IEL, dendritic epidermal T cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Dr G. Mayrhofer (unpublished)</td>
</tr>
<tr>
<td>OX81</td>
<td>rat IL-4</td>
<td>produced by T cells</td>
<td>Purified</td>
<td>0.025mg/ml</td>
<td>IgG₁</td>
<td>hybridoma/ Dr G. Mayrhofer (unpublished)</td>
</tr>
<tr>
<td>DB-1</td>
<td>rat IFN-γ</td>
<td>produced by T cells and NK cells</td>
<td>S/N</td>
<td>Neat</td>
<td>IgG₁</td>
<td>hybridoma/ Dr G. Mayrhofer (unpublished)</td>
</tr>
<tr>
<td>1B5</td>
<td>Giardia surface antigen</td>
<td>Giardia (Negative control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.2 Systems for detection of primary antibodies

The secondary antibodies used to detect mouse mAbs all exhibited limited cross-reactivity against rat Ig, which was blocked by use in the presence of 10% NRS. They were as follows:

An affinity purified F(ab')\textsubscript{2} rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Serotec, Oxford, U.K., Cat. No. STAR 41), affinity-purified goat anti-mouse Ig FITC-conjugate (PharMingen, USA, Cat No. 12064D) and goat anti-mouse IgG F(ab')\textsubscript{2} PE-conjugate (Rockland, Gilbertsville, PA, USA, Cat. No. 710-1831) were used for indirect immunofluorescent labelling of cells for flow cytometry and for immunofluorescence staining of frozen tissue sections.

Affinity purified F(ab')\textsubscript{2} sheep-anti-mouse Ig conjugated to horse radish peroxidase (Amersham Life Science, Buckinghamshire, England, Cat No. NA 9310) was used in the indirect immunoperoxidase technique for immunohistochemistry.

Goat anti-mouse IgG, conjugated to biotin (Rockland, Cat No. 610-106-121), was used as the second step reagent to detect mouse anti-rat mAb bound to tissue sections. Positive cells were then identified by the addition of streptavidin-peridinin chlorophyll protein (PerCP) (Becton Dickinson, San Jose, CA, USA, Cat No. 340130).

Biotinylated donkey anti-rabbit Ig F(ab')\textsubscript{2} fragments (Amersham Life Science,) were used in immunohistochemistry to detect rabbit anti-RAG-1 polyclonal antibody bound to tissue sections. RAG-1 positive cells were then detected by labelling with streptavidin-FITC (PharMingen, USA).
2.5 Negative selection of CD4+ cells using immuno-magnetic beads

Thoracic duct lymph (see section 2.2.6) from 3-4 rats was filtered through cotton wool and washed twice in RPMI containing 2% heat inactivated FCS (v/v) (see section 2.3.1) and 0.01% (w/v) sodium azide (RPMI/2%FCS/Az). The cell pellets resulting from centrifugation at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, USA) were pooled and washed once more in RPMI/2%FCS/Az. After centrifugation, the supernatant was removed and the cells resuspended in the first cocktail of depleting mAbs (2ml of each of the following mouse anti-rat mAb supernatants: 341, OX33, MARK-1 [to bind B cells and CD8β+ T cells]) for 30 minutes on ice. After this incubation the cells were pelleted by centrifugation, the supernatant removed and the second cocktail of depleting mAbs added (2ml of each of the following mouse anti-rat mAb : 10/78, V65 and WT-5 [to bind NK cells, γδ T cells and myeloid cells]). The cells were then incubated on ice for a further 30 minutes and washed three times in 40ml of RPMI/2%FCS/Az. After the final wash, the cells were resuspended in 2ml of RPMI/2%FCS/Az. At this stage, M-450 Dynabeads coupled with sheep anti-mouse IgG (Dynal, A.S., Oslo, Norway, Cat. No. 110-02) were resuspended gently at a concentration of 4x10^8/ml and the appropriate volume introduced into 6ml polypropylene tubes (Falcon, Becton Dickinson Labware, New Jersey, USA, Cat. No. 2063) to give a bead to cell ratio of 1 bead per cell. The beads were then washed twice in RPMI/2%FCS/Az, facilitated by the use of a Dynal Magnetic Particle Concentrator (MPC) (Dynal, Norway). Cells were then added to the beads, resuspended gently by the addition of a further 2ml of RPMI/2%FCS/Az and incubated on a rotor at 4°C for 45 minutes. The mixture of cells and beads was then washed twice with 6ml of RPMI/2%FCS/Az using the MPC, and the free cells collected in the washes. The collected
cells were subjected to a second depletion via incubation with 1.5 Dynabeads per cell at 4°C as described above. Following two more washes with 6ml of RPMI/2%FCS/Az using the MPC, the free cells (negatively selected CD4+ T cells) were collected in the supernatant. Sample aliquots of the cells were taken before and after magnetic bead selection and analysed by flow cytometry to assess the success of separation and the purity and yield of the negatively selected CD4+ cells.

2.6 Labelling of cells for flow cytometric analyses

2.6.1 Indirect labelling of cells with monoclonal antibodies (mAbs)

All labelling steps were performed at 4°C. Cells were dispensed in aliquots of 1-1.5x10^6 cells per FACS tube (Falcon, Becton Dickinson Labware, New Jersey, USA, Cat. No. 2008) and centrifuged for 10 minutes at 200g in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). Pellets were resuspended in 50μl aliquots of either culture supernatant, diluted ascites, or purified mAb (prepared as per Table 2.1), each containing 0.01% (w/v) sodium azide ('Labchem', Ajax Chemicals, Australia) and 10% (v/v) heat inactivated NRS (see section 2.2.1). The cells were then incubated on ice for 45-60 minutes. Following this incubation, the cells were washed twice in 2ml aliquots of PBS/FCS/Az and resuspended in 50μl of FITC-conjugated secondary antibody (1/100 dilution of either Serotec rabbit anti-mouse F(ab')2 or PharMingen goat anti-mouse Ig, in PBS/FCS/Az containing 10% (v/v) NRS). After incubation for 45 minutes on ice in the dark, the cells were washed as described above, resuspended in approximately 100μl of PBS and fixed by the addition of 500μl 1% (w/v) paraformaldehyde (PFA) (TAAB, Reading, U.K.) in PBS. Cells were then stored at 4°C in the dark prior to flow cytometric analysis (see section 2.7.1).
2.6.2 Direct labelling of cells with PE-conjugated mAbs

Labelling was carried out at 4°C. Cell suspensions were prepared and cells aliquoted at 1×10^6 cells/6ml polypropylene tube (Falcon, Becton Dickinson, USA). After centrifugation, as described above, the cells were resuspended in 50μl of PBS/FCS/Az containing 0.2-0.3μg/ml of the PE-conjugated mAbs (PharMingen, USA) and incubated for 45-60 minutes on ice in the dark. Following this incubation, the cells were washed twice in 2ml aliquots of PBS/FCS/Az and resuspended in 500μl 1% PFA (TAAB, Reading, U.K.) in PBS. They were then stored at 4°C in the dark prior to flow cytometric analysis (see section 2.7.1).

2.6.3 Dual fluorochrome labelling of cells

This procedure is a combination of the direct and indirect immunofluorescence labelling procedures described above. The first stage was identical to the indirect labelling procedure (section 2.6.1). Cells were then washed twice in PBS/FCS/Az as described, before being resuspended in 5μl of neat heat inactivated normal mouse serum (NMS) (see section 2.2.1) for 10 minutes on ice to block any free valances of the bound anti-mouse immunoglobulin conjugate. Without washing, the cells were labelled directly by addition of PE-conjugated purified mAbs as described in section 2.6.2. Following two final washes in PBS/FCS/Az, the cells were resuspended in approximately 100μl of PBS and fixed by the addition of 500μl 1% PFA in PBS. They were then stored at 4°C in the dark prior to 2 colour flow cytometric analysis (see section 2.7.2).

2.6.4 Labelling DNA with propidium iodide (cell cycle analysis)

To determine the DNA content of α/β TCR^+ and CD4^+ cells, 1-2×10^6 mononuclear cells were labelled with the mAbs R73 and W3/25 respectively by the indirect technique
described above (see section 2.6.1). After washing, the cells were resuspended in 200μl of PBS/FCS/Az and fixed by the addition of 70% ethanol with continuous mixing. The DNA in the cells was then stained using a method described by Ormerod (1990). After fixation for at least 30 minutes, the cells were harvested by centrifugation at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, USA) and resuspended in 800μl of PBS. RNA was digested by the addition of 100μl of RNaseA (Boehringer Mannheim, Germany, Cat. No. 109169) at a concentration of 1mg/ml in PBS containing 0.01% (w/v) sodium azide ('Labchem', Ajax Chemicals, Australia) (PBS/Az). DNA was stained by the further addition of 100μl of propidium iodide (PI) (Sigma, St Louis, MO, USA, Cat. No. P 4170) at 400μg/ml in PBS/Az. The suspension was incubated at 37°C for 30 minutes and the cells were then stored at 4°C in the dark until analysed. The proportions of cells in G0/G1 (2N), S, G2 (4N) phases of cell cycle were determined from histograms generated by flow cytometric analysis (see section 2.7).

2.6.5 Measurement of cell death by Annexin-V binding

Annexin-V is a Ca²⁺-dependent phospholipid-binding protein that binds to phosphatidylserine, which is transferred from the inner to the outer surface of the plasma membrane in cells undergoing apoptosis. However, phosphatidylserine is also exposed in necrotic cells due to loss of membrane integrity, which allows both Annexin-V-FLOUS (Annexin-V-FITC conjugate, Boehringer Mannheim, Germany, Cat. No. 1828681) and propidium iodide (Sigma, St Louis, MO, USA, Cat. No. P 4170) to bind respectively to intracellular phosphatidylserine and DNA of necrotic cells. PI staining can be used, therefore, to distinguish permeable necrotic and late stage apoptotic cells from early apoptotic and apoptotic cells in preparations stained with Annexin-V-FLOUS, and analysed by 2 colour flow cytometry (see section 2.7.2).
CD4+ cells were purified from TDL, as described in section 2.5, and cultured at room temperature in RPMI/5%FCS (see section 2.3.1) for a given period of time. After culturing, cells were pelleted by centrifugation at 200g for 10 minutes and the supernatant aspirated. They were then resuspended in 50μl of 0.5μg/ml Annexin V-FL0US in Hapes buffer (10mM Hapes-NaOH [Boehringer Mannheim, Germany] pH 7.4, 150mM NaCl, 5mM KCl, 1mM MgCl2, 1mM CaCl2 [either BDH Analar®, Merck, Australia or 'Univar', Ajax Chemicals, Australia]), as described previously (Koopman et al., 1994). After incubating at room temperature for 5-10 minutes, the volume was made up to 200μl with Hapes buffer (described above), and PI (Sigma, USA) was then added to a final concentration of 10μg/ml before immediate 2 colour flow cytometric analysis (see section 2.7.2).

2.6.6 Labelling of cells with Carboxyfluorescein diacetate succinimidyl ester (CFSE) and cell tracking in vivo

Thoracic duct lymphocytes from 3-4 animals were collected overnight at room temperature as described above (section 2.2.6), washed twice in RPMI/5%FCS and then pooled in serum-free RPMI 1640 (see section 2.3.1) at a concentration of 5×10⁷ cells/ml. They were then labelled with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) as described by Lyons and Parish (1994). One μl of CFSE stock (2.785mg/ml. in DMSO, obtained from Dr Bruce Lyons, Hobart, Australia) was added to the cell suspension with continuous mixing. The suspension was incubated for 10 minutes at 37°C and then quenched with 5 volumes of cold RPMI/10% FCS or PBS/10% FCS. After repeating this step twice, the cells were resuspended at 5×10⁷ cells/ml in serum free medium and injected intravenously into the tail veins of two groups of syngeneic recipient rats. A sample was withheld and these cells were incubated with PE-conjugated mAbs
(as described in section 2.6.2) and analysed by flow cytometry to determine the numbers of $\alpha/\beta$ TCR$^+$ (indirectly $\alpha/\beta$ TCR), CD4$^+$ and CD8$\alpha$+ lymphocytes that were transferred.

One group of recipient rats was cannulated just prior to intravenous injection, and thoracic duct lymph was collected over 6 successive 12-hour intervals to determine the recirculation kinetics of the CFSE-labelled cells. The second group of rats was not cannulated and these animals were sacrificed after 24 hours to determine the distribution of CFSE-labelled cells to various tissues. Total numbers of CFSE-labelled cells recovered from the TDL and tissues of recipient rats (see sections 2.2.1-2.2.6) were determined by flow cytometric analysis using the PE-conjugated mAbs as described above (section 2.6.2) and the percentage recovery was calculated as follows:

Percentage Recovery = \( \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100 \)

Tissues from recipient rats were also frozen in O.C.T. compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) for the detection of CFSE-labelled cells in frozen sections (see section 2.8).

2.6.7 Detection of intracellular cytokines

Production of IFN-$\gamma$ and IL-4 protein by lymphocytes was determined using a method modified from Assenmacher (1994). Lymphocytes prepared from pooled LN (section 2.3.1) or TDL (section 2.2.6) were cultured in RPMI 1640 (see section 2.3.1) supplemented with 10% FCS (MultiSer$^TM$, Cytosystems), 2mM L-glutamine (BDH) and 0.1mM $\beta$-mercaptoethanol (prepared as in section 2.3.1) for 5 hours at $37^\circ$C in the
presence of 10μg/ml Brefeldin A, 100μM calcium ionophore A23187 and 50ng/ml phorbol myristate acetate (PMA) (all of which were purchased from Sigma, USA). Equal amounts of cells were also cultured in the absence of A23187 and PMA. The cells were then harvested and washed twice in PBS. The resulting cell pellets were then resuspended in 1ml of PBS and fixed by the addition of 1ml of 10% buffered formalin. After fixation for 6 minutes, the cells were washed three times in PBS and counted. Following storage overnight at 4°C, they were aliquoted at 1×10⁶ cells/tube. The cell pellets were then resuspended in the appropriate primary antibody (anti-IL-4, anti-IFN-γ or 1B5, as described in section 2.4.1) containing 10% heat inactivated NRS and 0.1% (w/v) saponin (Sigma, USA) for 45-60 minutes on ice. The cells were then washed twice with PBS/1% FCS/0.1% saponin and then incubated with FITC-conjugated goat anti-mouse antibody (PharMingen, USA – see section 2.4.2) in the presence of 10% NRS and 0.1% saponin for 45 minutes on ice, in the dark. They were then washed twice in PBS/1% FCS/0.1% saponin, followed by one wash in PBS/1%FCS. The cell pellet was resuspended in 5μl of neat heat inactivated NMS and incubated for 10 minutes on ice before the addition of either anti-TNP, anti-CD4 or anti-α/TCR PharMingen PE-conjugated antibodies (described in section 2.4.1). After incubation for 45 minutes, the cells were washed twice in PBS/1% FCS and resuspended in 500μl of PBS for immediate analysis.

2.7 Flow cytometry

Labelled cells were analysed using a FACScan (Becton Dickinson, San Jose, Ca.) equipped with CellQuest software (versions 1.2 and 3.1f). Lymphocyte populations were gated on the basis of their forward (FSC) and side scatter (SSC) characteristics and events were collected from a gate containing all such cells and also separately from
Figure 2.1 Gating of rat lymphocyte populations. Plots of forward light scatter (FSC, proportional to size) and side scatter (SSC) obtained by flow cytometry were used to identify and gate lymphocyte populations. The gate including small cells was placed to include the majority of lymphocytes in the thoracic duct lymph (TDL) from adult rNu/+ rats. Larger cells were included in the large cell gate. A similar gating regime was used in all analysis of lymphocyte populations prepared from tissues from rNu/+ (A-D) and rNu/rNu (E-H) rats. (A and E) TDL cells from 26 week old rats. (B and F) Lymph node cells from 26 week old rats. (C and G) Spleen cells from 26 week old rats. (D and H) Mononuclear cells prepared from the livers of 26 week old rats.
gates limited to small and large lymphocytes (lymphoblasts). The gates used to
distinguish small and large lymphocytes were drawn arbitrarily on the basis of the light
scatter characteristics of TDL, lymph node, spleen and liver mononuclear cell
preparations from normal rats (Figure 2.1). At least 10,000 events were collected from
the total lymphocyte gate and at least 5,000 events were collected from the lymphoblast
gate. In the estimation of DNA content and cytokine production, 10,000 events were
collected by live gating on cells expressing either the α/β TCR or CD4. In the studies
measuring recovery of CFSE-labelled cells, 30,000 events were collected from the
lymphocyte gate.

2.7.1 One-colour flow cytometry

The negative control was used to optimise instrument settings and determine background
fluorescence levels. Gates were positioned to include the desired lymphocyte
populations using the forward scatter (FSC) versus side scatter (SSC) dot plot. The
background fluorescence was defined as that part of the negative peak which contained
98-99% of the recorded events occurring within a gate.

2.7.2 Two-colour flow cytometry

The setting up procedure involved the use of two negative control antibodies that were
labelled with the appropriate fluorochromes (FL-1: FITC signal and FL2: PE signal).
Gates were set as described above and background fluorescence levels for each channel
adjusted with the aid of a FL-1 versus FL2 dot plot. Compensation was made for the
overlap of the FL-1 signal into the FL2 channel using a brightly stained (FITC) positive
control sample and the minimal overlap of the FL2 signal into the FL-1 channel was
compensated by use of a brightly stained (PE) positive control sample.
2.8 Immunohistochemistry

2.8.1 Preparing tissue blocks for sections

Tissue samples from the livers, spleens, lymph nodes and thymii were removed from anaesthetised animals, embedded in O.C.T. compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) and snap frozen in iso-Pentane ('Univar', Ajax Chemical Company, Auburn, NSW, Australia) chilled over liquid nitrogen. Pieces of small intestine (jejunum) approximately 1cm in length were opened longitudinally along the anti-mesenteric border and laid on nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) with the luminal surface exposed. The edges were trimmed and the intestinal samples were then embedded in O.C.T., perpendicular to the base of the tissue block, and snap-frozen in isopentane chilled over liquid nitrogen as described above. All frozen tissue samples were stored at -70°C until required.

2.8.2 Indirect immunoperoxidase

Five μm tissue sections were cut from frozen tissue blocks using a Bright cryostat (Bright Instrument Company, Huntingdon, Cambridgeshire, England) and placed on glass slides. After air-drying, the sections were either used immediately or stored at -70°C until required. The slides were allowed to equilibrate to room temperature (if they had been stored) and a circle was drawn around the section with a PAP PEN (Zymed, San Francisco, CA, USA). They were then fixed in 95% ethanol for 10 minutes, washed 3 times in ice-cold PBS over 6 minutes and then incubated with 50μl of the designated mAbs (see Table 2.1) containing 10% NRS for 1 hour at 4°C in a humid chamber. After washing three times in ice-cold PBS (2 minutes each wash), the sections were incubated with 50μl of the affinity purified F(ab')2 sheep-anti-mouse Ig conjugated to horseradish peroxidase (diluted 1/20) (see section 2.4.2) at 4°C for 1 hour in a humid chamber. After
three more 2 minute washes in ice-cold PBS, the slides were moved to room temperature and incubated with 0.5µg/ml 3-3' diaminobenzidine (DAB) (Sigma, USA) in Tris buffer containing 0.068% H₂O₂ as described previously (Mayrhofer and Schon-Hegrad, 1983). The sections were then counterstained with haematoxylin prior to microscopic examination.

2.8.3 Counterstaining with Haematoxylin

Slides were counterstained by immersion in Gill's haematoxylin for 1 minute and then rinsed briefly in PBS. The slides were then rinsed in acid solution (0.05% HCl in Milli-Q water) followed by a wash in Scott's water (3.5g NaHCO₃, 20g MgSO₄, 0.05% sodium azide [final concentration] in 1 litre of Milli-Q water) for 2 minutes. After a final rinse in water, the sections were dehydrated by passage through an ethanol series (70% ethanol bath for 2 minutes and then transferred to two absolute ethanol baths for periods of 2 minutes in each bath). Lastly, the slides were moved through three Safsolvent ('Labchem', Ajax Chemical Company, Australia) baths, with each step lasting 2 minutes. Sections were mounted using D.P.X neutral mounting medium (Ajax Chemical Company) and viewed under an Olympus BH-2 light microscope (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan). Photomicrographs were shot on Agfacolor Optima 100 colour film (Agfa-Gevaert AG, Germany).

2.8.4 Dual-fluorochrome labelling of tissue sections using indirect and direct immunofluorescent staining

This procedure was identical to that described for the indirect immunoperoxidase technique (see section 2.8.2), until the addition of the secondary antibody. In place of the sheep anti-mouse horseradish peroxidase conjugate, 50µl of the goat anti-mouse
FITC-conjugate (see section 2.4.2) was added at a dilution of 1/50. The sections were then incubated for 45-60 minutes in a humid chamber. This step and all subsequent steps were carried out in the dark to prevent bleaching of the fluorochromes. Following the incubation with the FITC-conjugate, sections were washed three times in ice-cold PBS (2 minutes each wash) and then incubated in 30μl of 50% heat inactivated NMS (see section 2.2.1) for 15 minutes to block any free valances of the bound anti-mouse immunoglobulin conjugate. The slides were then rinsed in three baths of ice-cold PBS, followed by incubation for 45 minutes at 4°C in a humid chamber with 50μl of PE-conjugated mouse anti-rat mAbs (diluted 1/30) (see section 2.4.1) directed against either TNP (negative control), CD4 or the α/β TCR. Sections were washed three times in ice-cold PBS (2 minutes each wash) before mounting in 86% glycerol containing 1% propyl-gallate (Sigma, USA, Cat. No. P-3130). Sections were then analysed and photographed with a fluorescence microscope using single and double-exposure photography (see sections 2.8.7 and 2.8.8).

2.8.5 Indirect immunofluorescence staining to detect RAG-1 protein in tissue sections

Initial staining with the rabbit anti-mouse RAG-1 polyclonal antibody (see section 2.4.1) produced considerable background on rat intestinal sections. This background was reduced using the following absorption protocol. Ten μm tissue sections of rat small intestine (LFV-free) were cut (see section 2.8.2) and collected into cold reaction tubes (Sarstedt, Australia). This tissue was fixed with 95% ethanol (which had been stored at -20°C) for 10 minutes on ice. After washing in PBS to remove O.C.T. (Tissue-Tek, Miles Inc, USA), 50μl of 50% FCS/PBS was incubated with the tissue for 5 minutes. After removing the supernatant, an approximately equal volume of antibody was
incubated with the tissue preparation for 2 hours with regular resuspension. The tissue absorbed antibody was stored at 4°C and diluted 1/2 in PBS containing 10% NRS and 0.5% Tween-20® for use.

The staining protocol described here was adapted from a method described by Fuschiotti et al. (1997). All procedures were carried out at 4°C. Freshly cut sections were fixed in 10% formalin for 5mins, followed by methanol at -20°C for 30mins. After washing three times (2 minutes each wash) in PBS containing 0.1% Triton X-100 (Labchem, Ajax Chemicals, Sydney, Australia), the previously described diluted rabbit anti-RAG-1 antibody was added. After incubating for 1-2 hours in a humid chamber, the sections were washed in PBS (three 2 minute washes) and incubated with biotinylated donkey anti-rabbit Ig (see section 2.4.2) diluted 1/1000 in PBS containing 10% NRS and 0.5% Tween-20® for 1 hour in a humid chamber. The slides were then washed three times in PBS (2 minute washes) and incubated for 30 minutes in the dark with streptavidin-FITC (see section 2.4.2) diluted 1/100 in PBS containing 10% NRS and 0.5% Tween-20® to detect bound biotinylated donkey anti-rabbit Ig polyclonal antibody. After three final 2 minute washes in PBS (performed in the dark), the sections were mounted in 86% glycerol containing 1% propyl-gallate (Sigma, USA) and analysed by both fluorescence and confocal microscopy (see section 2.8.7 and 2.8.8).

2.8.6 Detection of CFSE-labelled cells in tissue sections

Two methods were employed to examine the distribution and phenotype of CFSE-labelled cells in the tissues of recipient rats. Recipient rats, which had been injected with CFSE-labelled cells, were sacrificed after 24 hours and tissues samples were taken from these animals and frozen in O.C.T compound (Tissue-Tek, Miles Inc., USA) as
described in section 2.8.1. Five µm cryostat sections were cut, fixed in 95% ethanol for 10 minutes and washed in ice-cold PBS as described above (see section 2.8.2).

In the first method, fixed sections were stained directly with 50 µl of PE-conjugated mAbs against either the α/β TCR or CD4 (see section 2.4.1) (diluted 1/30 in PBS containing 10% NRS) to enable phenotypic analysis of the CFSE-labelled (green) cells in the sections. After a 45 minute incubation at 4°C in a humid chamber, the slides were washed three times in ice-cold PBS and mounted in 86% glycerol containing 1% propyl-gallate as described in section 2.8.4. The slides were then analysed by fluorescence microscopy (see section 2.8.7).

The second method involved a three-step labelling procedure. After washing, the fixed sections were incubated for 1 hour at 4°C with mouse mAb against either the α/β TCR (R73) or CD4 (W3/25) (see section 2.4.1) containing 10% NRS. The sections were then washed in three baths of ice-cold PBS over 6 minutes and incubated with biotinylated goat anti-mouse IgG (see section 2.4.2) diluted 1/50 in PBS and containing 10% NRS. After three more washes in ice-cold PBS, bound biotinylated antibody was detected by incubating the sections for 30 minutes with streptavidin-PerCP (see section 2.4.2) diluted 1/8 in PBS containing 10% NRS. The slides were then washed three more times in ice-cold PBS, mounted in 86% glycerol containing 1% propyl-gallate (see section 2.8.4) and analysed by confocal microscopy (see section 2.8.8).

2.8.7 Fluorescence microscopy

An Olympus BH-2 microscope associated with an epi-illumination fluorescence attachment (model BH-RFL-W, Olympus, Tokyo, Japan) was used to examine and
photograph tissue sections which had been stained for immunofluorescence. Appropriate negative controls were included to confirm the specificity of the staining. Double exposure and single exposure photographs were taken on ‘Provia’ Fujichrome 400 (Fuji, Tokyo, Japan) colour reversal film. To identify double-labelled cells, an exposure was taken firstly for 32 seconds using the filter set-up to detect PE-labelled cells (590 barrier filter and green dichroic mirror) and then for a further 32 seconds after adjusting the filter set-up to detect FITC/CFSE-labelled cells (530 barrier filter and blue dichroic mirror). A separate exposure using the latter filter was taken for 1 minute to photograph just the FITC/CFSE-labelled cells in the field. After returning to the 590 barrier filter and green dichroic mirror set-up, the PE-labelled cells were photographed using a 1-2 minute exposure. For interpretation of the merged images, where the staining was not superimposed, the colours were either orange-red or green, representing PE or FITC/CFSE staining, respectively. In regions where the staining was superimposed, the colour was yellow, indicating that the two antigens under investigation were co-localised.

2.8.8 Confocal microscopy

Confocal microscopy was performed using a MRC 600 confocal microscope (Bio-Rad Microscience, Cambridge, MA, USA). Monoclonal antibody 1B5 (see section 2.4.1), labelled indirectly with PerCP, was used as a negative control in the experiments examining CFSE-labelled cells (see section 2.8.6) and normal rabbit serum was used as the negative control in studies to detect RAG-1 protein (see section 2.8.5). For the detection of RAG-1 staining (single colour, FITC), the sample was excited at 488nm and viewed through a 500nm long pass filter. For detection of CFSE- and PerCP-labelled cells (2-colour labelling), the sample was excited at 514nm, and then viewed firstly
through 527nm band pass filter (FITC/CFSE, FL-2) and subsequently through a 600nm long pass filter (PerCP, FL-1). Single fluorochrome (CFSE, or PerCP) labelled controls were included to ensure that no overlap of signal occurred between the channels. Images collected during these experiments were analysed using Confocal Assistant™ (Version 4.02, ©1994-1996, Todd Clark Brelge) software.

2.9 Molecular Biology Techniques

2.9.1 Bacterial Growth Media and Buffers

Luria Bertani (LB) broth: 10g Bacto-tryptone (Difco, USA), 10g NaCl (BDH Analar®, Merck, Australia) and 5g Bacto-yeast (Difco, USA) were dissolved in Milli-Q purified water. The pH was adjusted to 7 with NaOH (BDH Analar®, Merck, Australia) and the volume made up to 1 litre prior to autoclaving.

LB Agar Plates: Agar plates were prepared by supplementing the above media with 1.5% (w/v) Bacto-agar (Difco, USA). Following autoclaving, the agar was allowed to cool to approximately 56°C prior to the addition of 50µg/ml ampicillin (Sigma, USA). Agar was then poured into Petri dishes (Techno-Plas, Australia), allowed to set, dried and stored at 4°C.

TE: 10mM Tris-HCl (Sigma, USA) pH 8.0 and 0.1mM ethylenediaminetetra-acetic acid (EDTA) (Sigma, USA) pH 8.0 in Milli-Q water.

TAE: 40mM Tris-acetate (Sigma, USA) and 1mM EDTA (Sigma, USA) in Milli-Q water. Prepared as a 50x stock consisting of 242g Tris base, 57.1ml glacial acetic acid
(BDH, Merck, Australia), and 100ml 0.5M EDTA pH 8, which was autoclaved and diluted in Milli-Q water prior to use.

10x DNA loading buffer: 15% (w/v) Ficoll 400 (Pharmacia Biotech, Sweden), 0.3% (w/v) bromophenol blue (Progen, Australia), and 0.1 mg/ml RNaseA (Boehringer-Mannheim, Germany). Stored at 4°C and diluted 1/10 for use

2.9.2 Preparation of genomic DNA

Genomic DNA was prepared from rat spleens essentially as outlined by Strauss (1990). The spleen was removed and frozen immediately in liquid nitrogen. The frozen tissue was then crushed into a fine powder using a pre-chilled mortar and pestle. The powder was then suspended in 1.2 ml of digestion buffer (100mM NaCl; 10mM Tris-HCl, pH 8; 25mM EDTA, pH 8; 0.5% SDS; 0.1 mg/ml proteinase K [all from Sigma, USA.]) per 100mg of tissue. This suspension was incubated with shaking at 50°C for 12 hours. After this time, the nucleic acids were extracted from the suspension by addition of an equal volume of phenol/chloroform/iso-amyl (25:24:1), followed by centrifugation for 10 minutes at 1700g in a Beckman J2-21M centrifuge (Beckman, Palo Alto, CA, USA). The aqueous layer was transferred to a new tube and the DNA precipitated by the addition of ¼ volume of 7.5M ammonium acetate (BDH, AnalaR®, Merck, Australia) and 2 volumes of ice-cold absolute ethanol (BDH, AnalaR®, Merck, Australia). Following centrifugation at 1700g for 2 minutes, as described above, the pellet was rinsed with 70% ethanol. The ethanol was poured off carefully and the DNA pellet allowed to air dry. The pellet was then resuspended in 500µl of TE buffer (see section 2.9.1) and stored at 4°C.
Mouse genomic DNA (produced by the same protocol) and human genomic DNA were provided kindly by Mr Brett Aplin (Department of Microbiology and Immunology, University of Adelaide, Australia).

2.9.3 Oligonucleotide primers

Degenerate primers were designed to regions with high interspecies homology and low levels of redundancy, based on the homology between mouse and human rag-I cDNA and amino acid sequences obtained from GenBank® database at the National Centre for Biotechnological Information (NCBI). Primer Designer software (version 2.0; Scientific and Educational Software) was utilised to check potential primers for complementarity, secondary structure and to determine the melting temperatures (Tm°C). Sequences homologies were assessed using DNASIS (version 7.0; Hitachi) and PROSIS (version 7.0; Pharmacia Biotechnology, Uppsala, Sweden). Multiple alignments were generated using CLUSTAL W (Thompson et al., 1994).

The following degenerate primers were designed:

5′ rag-I primer (2034): GCT CTA GAT GGA CAT GGA A6GA A6GA C6AT
3′ rag-I primer (2035): ACC ATG CAT CTT CCA T6T C6T A6C A6T T

These primers were designed to amplify a fragment in excess of 1200bp at the 3′ end of the rag-I coding sequence. The XbaI and NsiI restriction sites incorporated in the 5′ and 3′ rag-I primers respectively are underlined (see above).

The oligonucleotide primers were synthesised on an Applied Biosystems 381A DNA synthesiser in the Department of Microbiology and Immunology.
de-protected oligonucleotides was performed by butanol extraction (Sawadogo and Van Dyke, 1991). Concentration of oligonucleotides was determined by spectrophotometry 

\[ A_{260} \, l = 33 \mu g/ml \] and the molarity determined via the following formula:

\[
\text{Concentration of oligo(M)} = \frac{x \, \mu g \times 10^6}{[\text{MW of oligonucleotide} \times 10^3]}
\]

(Molecular weight (mwt) of dNTPs: A=347.2, T=332.2, G=363.2, C=323.2)

### 2.9.4 Polymerase Chain Reaction (PCR)

Fifty µl reactions were set up in 0.5ml microcentrifuge tubes (Sarstedt, SA, Australia). Each reaction contained approximately 100ng of template DNA, 0.5µM of each primer (5' and 3'), 0.2mM of each of dATP, dTTP, dCTP, dGTP (Pharmacia Biotech, Sweden), 5µl of 9mM MgCl₂, 5µl of 10x Tth PCR buffer (100mM Tris-HCl pH 8.9, 1M KCl, 15mM MgCl₂, 500µg/ml bovine serum albumin (BSA), 0.5%(v/v) Tween 20° - Boehringer-Mannheim, Germany), 1 unit of Tth DNA polymerase (Boehringer-Mannheim, Germany) and sterile Milli-Q water to a final volume of 50µl. Each reaction mix was overlayed with approximately 100µl of mineral oil (Sigma, USA).

DNA was amplified using a Corbett FTS-320 thermal cycler (Corbett Research, Sydney, Australia) by denaturing the DNA initially at 95°C for 2 minutes prior to 30 cycles consisting of: 95°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute. These cycles were followed by a final extension at 72°C for 10 minutes. Tubes were then held at 4°C and 10µl of each reaction was analysed by agarose gel electrophoresis (see section 2.9.5).
2.9.5 Separation of DNA fragments by agarose gel electrophoresis

Following restriction endonuclease digestion or PCR amplification, a portion of the reaction was mixed with a 1/10 dilution of 10x loading buffer (see section 2.9.1). Electrophoresis of digested DNA was carried out on 1% (w/v) agarose (Progen, Australia) gels prepared and run in TAE (see section 2.9.1) at 100V in a horizontal gel apparatus. The gels were stained in ethidium bromide (2μg/ml in distilled water - Sigma, USA) for 5 minutes and then destained in H2O for 10 minutes whilst being rocked gently. DNA fragments were visualised by 254nm short wave UV light and photographed using Polaroid 667 film or Tracktel GDS-2 gel documentation system (Vision Systems, The Levels, SA, Australia).

2.9.6 Determination of DNA fragment size

The sizes of restriction fragments were calculated by comparing their relative mobilities in agarose with those of DNA molecules of known sizes (ie DNA molecular weight markers). The molecular weight markers used were EcoRI digested Bacillus subtilis phage SPP1 DNA (Bresatec, Adelaide, Australia, Cat. No. DMW-S1). The estimated sizes of these molecular weight markers in kilobases (kb) are as follows:

8.51; 7.35; 6.11; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.48; 0.36

2.9.7 DNA purification by phenol/chloroform extraction

Between DNA manipulations, DNA was purified by phenol/chloroform extraction. The volume of the DNA solution was made up to 400μl with TE (see section 2.9.1). An equal volume of phenol/chloroform/isoamyl (25:24:1) was added, vortexed throughly and spun for 3 minutes at 13000rpm in a microcentrifuge (Eppendorf 5417R, Hamburg, Germany). The aqueous layer was collected and to it was added a 1/10 volume of 3M
sodium acetate (NaAc) (BDH, AnalaR®, Merck, Australia) pH 4.6 and 2 volumes of 95% ethanol (BDH, AnalaR®, Merck, Australia). The DNA was precipitated at -20°C for at least 20-30 minutes and then collected by microcentrifugation at 13000rpm for 15 minutes at 4°C. The DNA pellet was then washed in 500μl of 70% ethanol (BDH, AnalaR®, Merck, Australia), dried and dissolved in an appropriate volume of TE (see section 2.9.1).

2.9.8 Restriction endonuclease digestion

PCR products or vector DNA were digested in the presence of the appropriate restriction endonuclease (3-5 units/μg DNA) according to the method recommended by Boehringer-Mannheim (Germany). Digests were incubated for a minimum of 2 hours at the temperature recommended by the manufacturer. The reaction was then terminated by heating at 65°C for 30 minutes. Double digests were performed simultaneously when the recommended buffer was identical.

2.9.9 Purification of DNA from agarose using Geneclean®

This method was carried out essentially as recommended by the manufacturer (BIO 101, Vista, CA, USA) and was used to purify DNA fragments of between 0.5 and 3kb from agarose gels. The excised agarose gel containing the desired DNA fragment was placed into a 1.5ml reaction tube (Eppendorf, Germany) and the mass determined. To this were added 3 volumes of sodium iodide (NaI) stock solution (supplied by the manufacturer) and the mixture was heated at 55°C in a heat block until the agarose melted. Five μl of thoroughly resuspended Glassmilk (provided by the manufacturer) was added to the melted agarose, the solution was mixed gently and then incubated at room temperature for 15 minutes, inverting occasionally. DNA bound to the silica matrix was pelleted in a
microcentrifuge (Eppendorf 5417R, Germany) for 1 minute at 13000rpm and the supernatant was aspirated. The pellet was resuspended and washed 3 times with 700μl of ice-cold "New Wash" solution (supplied by the manufacturer). Residual wash solution was removed after centrifugation and the silica matrix pellet was dried in a heat block for 5 minutes at 55°C. The DNA was then eluted from the silica matrix with 25μl of TE or Milli-Q water by heating the tube at 55°C for 10 minutes. The silica matrix was then pelleted and the supernatant containing the DNA was removed.

2.9.10 Quantitation of DNA

The concentration of DNA in solution was determined either by measuring the absorption at 260nm in a spectrophotometer (Ultraspec® Plus, Pharmacia Biotech, Sweden), assuming that an A_{260} 1 = 50μg/ml of DNA, or by electrophoresis of DNA in agarose gels (see section 2.9.5) and comparing the intensities of ethidium bromide-stained bands with the intensities of bands containing known amounts of DNA.

2.9.11 Ligation of restriction digested fragments to vector

A 10μl ligation reaction contained 1-2 Weiss units of T4 DNA ligase (Pharmacia Biotech, Sweden), 20-50ng of digested vector DNA and a 2-3 fold molar excess of the restriction fragment insert DNA in 1x ligation buffer (20mM Tris-HCl (Sigma, USA) pH 7.6, 5mM MgCl₂ (BDH, Analar®, Merck, Australia), 5mM dithiothreitol (DTT) (Sigma, USA), 0.5mM ATP (Pharmacia Biotech, Sweden). The ligation reaction was incubated overnight at 16°C in an ice water bath. Control reactions containing vector but no insert were set up and included in subsequent transformations (see section 2.9.13) to determine the levels of background uncut or recircularised vector. pGEM®-7Zf(+) vector (Promega, WI, USA) was used in all ligation reactions.
2.9.12 Preparation of chemically competent cells for transformation

*E. coli* DH5α strain (*E. coli* Genetic Stock Centre, Yale University, New Haven, USA) were made competent for transformation by a procedure similar to that described by Sambrook *et al.*, (1989). Cells were streaked from -70°C glycerol stocks on to LB agar plates (see section 2.9.1) and grown overnight at 37°C. A single colony was used to inoculate 5ml of Ψ broth (20g Bacto-tryptone [Difco, USA], 5g Bacto-yeast extract [Difco, USA], 5g MgSO₄ [BDH, AnalaR®, Merck, Australia], pH adjusted to 7.6 with KOH [BDH, AnalaR®, Merck, Australia] and made up to 1L with Milli-Q water before autoclaving), which was incubated overnight with shaking at 37°C. The overnight culture was then subcultured 1:20 into 100ml of Ψ broth (prewarmed at 37°C) and grown for approximately 2 hours until A₆₀₀ = 0.5-0.6. The culture was chilled on ice for 15 minutes with occasional mixing and then centrifuged in a Beckman J2-21M induction drive centrifuge at 5500g using a JA20 rotor for 5 minutes at 4°C. The pelleted cells were resuspended in 40 ml of ice-cold Tfb I Buffer (30mM KOAc, 100mM KCl, 10mM CaCl₂·2H₂O, 50mM MnCl₂·4H₂O and 15% (v/v) glycerol [all from BDH, AnalaR®, Merck, Australia] in Milli-Q water, pH adjusted to 5.8 with 0.2M HAc [BDH, AnalaR®, Merck, Australia] and sterilised through 0.2μM filter [Satorius, Germany]) and incubated on ice for 5 minutes prior to pelleting by centrifugation as described above. The pellet was resuspended in 4ml of ice-cold Tfb II Buffer (10mM MOPS, 75mM CaCl₂·2H₂O, 10mM KCl, 15% (v/v) glycerol [all from BDH, AnalaR®, Merck, Australia] in Milli-Q water, pH adjusted to 6.5 with 0.5M KOH [BDH, AnalaR®, Merck, Australia] and sterilised through 0.2μM filter [Satorius, Germany]). Following incubation on ice for 15 minutes, 200μl volumes of cells were aliquoted into cold 1.5ml
reaction tubes (Sarstedt, SA, Australia) on dry ice. The cells were snap frozen in the tubes and stored at -70°C.

2.9.13 Transformation of chemically competent E.coli.

Competent cells, prepared as described in section 2.9.12, were thawed on ice and 50-100μl aliquots were transferred to fresh 1.5ml reaction tubes (Sarastedt, Australia), to which 5-10μl of ligation reaction (see section 2.9.11) was added. This mixture was incubated on ice for 20 minutes, heat shocked at 45°C for 90 seconds and then placed immediately on ice for a further 10 minutes. Cells were then left at room temperature for 5 minutes before the addition of 500μl of LB broth (section 2.9.1) and further incubation for 45 minutes at 37°C with shaking. Following this incubation, cells were pelleted in a microcentrifuge (Eppendorf 5417R, Germany) for 30 seconds at 13000rpm. The cells were resuspended in 160μl of PBS and from this 20μl and 140μl aliquots were spread onto two ampicillin LB agar plates (see section 2.9.1). Bacterial cells transformed with pGEM®-Zf(+) plasmid (Progen, USA) were selected using LB agar plates (see section 2.9.1) containing 50μg/ml ampicillin (Sigma, USA), 25μg/ml X-gal (Sigma, USA) and 24μg/ml IPTG (Sigma, USA). Plates were incubated overnight at 37°C.

2.9.14 Small ‘Miniprep’ plasmid DNA preparations

Isolated white colonies transformed with pGEM®-Zf(+) plasmid (Progen, USA) were selected from ampicillin/X-gal/IPTG LB agar plates (see section 2.9.13) and cultured overnight at 37°C with shaking in 2ml of LB broth (see section 2.9.1) containing 50μg/ml ampicillin (Sigma, USA). Cells from 1.5ml of overnight culture were pelleted in a microcentrifuge (Eppendorf 5417R, Germany) at 13000rpm for 30 seconds. The
supernatant was discarded, the pellet was resuspended in 100\(\mu l\) of Solution 1 (50mM glucose [BDH, Analar®, Merck, Australia], 25mM Tris-HCl [Sigma, USA] pH 8.0, 10mM EDTA [Sigma, USA] pH 8.0) and the tubes were then incubated for 5 minutes at room temperature. Two hundred microlitres of freshly prepared Solution 2 (0.2 M NaOH [BDH, Analar®, Merck, Australia], 1% sodium dodecyl sulphate (SDS) [Sigma, USA]) was added and mixed by gentle inversion of the tubes several times prior to incubation on ice for 5 minutes. Chromosomal DNA was precipitated by adding 150\(\mu l\) of ice-cold Solution 3 (3M KAc, pH adjusted to 4.5 with glacial acetic acid [both from BDH, Analar®, Merck, Australia]) and incubated on ice for 5 minutes. The mixture was microcentrifuged at 13000rpm for 10 minutes at 4°C and the supernatant containing the plasmid DNA was removed carefully and transferred to a fresh 1.5ml reaction tube. The plasmid DNA was phenol/chloroform extracted and precipitated as described in section 2.9.7. The resulting plasmid DNA pellet was then dissolved in 20\(\mu l\) of Milli-Q water and analysed subsequently by restriction endonuclease digestion (see section 2.9.8) and agarose gel electrophoresis (see section 2.9.5) to detect the presence of an insert of the correct size. Clones exhibiting fragments of the appropriate size were selected for large scale plasmid DNA purification (see following section).

2.9.15 Large ‘Miniprep’ plasmid DNA preparations

Single white colonies transformed with pGEM®-7Zf(+) plasmid (Progen, USA) were selected from ampicillin/X-gal/IPTG LB agar plates (see section 2.9.13) and cultured overnight at 37°C with shaking in 10ml of LB broth (see section 2.9.1) containing 50\(\mu g/ml\) ampicillin (Sigma, USA). Cells from the overnight culture were pelleted in a Beckman J2-M centrifuge (Beckman, USA) using a JA20 rotor for 10 minutes at 2500g. The supernatant was discarded and the pellet was resuspended in 1ml of Solution 1 (see
section 2.9.14) then incubated for 5 minutes at room temperature. Two millilitres of freshly prepared Solution 2 (see section 2.9.14) was added and mixed by gentle inversion of the tubes several times prior to incubation on ice for 5 minutes. Chromosomal DNA was precipitated by adding 1.5ml of ice-cold Solution 3 (see section 2.9.14) and incubated on ice for 5 minutes. The mixture was centrifuged at 17,700g in a Beckman J2-21M centrifuge using a JA20 rotor for 10 minutes at 4°C. The supernatant containing the plasmid DNA was removed carefully to a fresh tube and 2 volumes of cold 95% ethanol (BDH, AnalaR®, Merck, Australia) were added. The plasmid DNA was precipitated on ice for 10 minutes and then collected by centrifugation at 17,700g for 10 minutes at 4°C as described above. The supernatant was discarded, the pellet was resuspended in 0.4ml of TE (see section 2.9.1) and it was then transferred into a 1.5ml reaction tube (Sarstedt, Australia). The plasmid DNA was phenol/chloroform extracted and precipitated as described in section 2.9.7. The resulting pellet of plasmid DNA was then dissolved in 100μl of TE (see section 2.9.1).

2.9.16 DNA sequence analysis

Plasmid DNA, prepared by the large plasmid miniprep method (see above section), was further purified using the Geneclean® method (see section 2.9.9) and quantitated as described in section 2.9.10. Sequence analysis of recombinant plasmid DNA constructs was performed using the PRISM™ Ready Reaction Dye Primer Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturers instructions. The concentration of plasmid DNA was adjusted to 1-1.5μg/μl and the following added to separate reaction tubes: 4μl A mix and 1μl DNA, 4μl C mix and 1μl DNA, 8μl T mix and 2μl DNA, 8μl of G mix and 2μl DNA. Separate A, C, T and G mixes were utilised for forward and reverse sequencing with the rag-1 primers described
in section 2.9.3. The reaction mixes were overlayed with 1-2 drops of mineral oil (Sigma, USA) and placed in a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT, USA) pre-heated to 95°C. The amplification cycle consisted of the following: 95°C for 30 seconds, 55°C for 30 seconds and 70°C for 1 minute (15 cycles in total), followed by 95°C for 30 seconds and 70°C for 1 minute (15 cycles in total). The single stranded fluorescent DNA products were concentrated by pooling each set of A, C, T and G reaction mixes together with 80μl of 95% ethanol (BDH, Analar®, Merck, Australia) and 1.5μl of 3M NaAc (BDH, Analar®, Merck, Australia) pH 5.2. The tubes were mixed and incubated on ice for 10-15 minutes. DNA was pelleted by microcentrifugation (Eppendorf 5417R, Germany) at 13000rpm for 15 minutes at 4°C, washed in 70% ethanol (BDH, Analar®, Merck, Australia), dried and stored at -20°C. Sequence analysis was carried out on an Applied Biosystems 373A Automated DNA Sequencer (Applied Biosystems, CA, USA) in the Department of Microbiology and Immunology, University of Adelaide.

2.10 Preparation and analysis of mRNA

2.10.1 RNase-free conditions and buffer preparations

General laboratory glassware was filled with a solution of 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma, USA), a potent inhibitor of RNases. Vessels were allowed to stand at 37°C for 2 hours, rinsed several times with sterile water and autoclaved for 30 minutes. The autoclave treatment removes all traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation.

All solutions were prepared in RNase-free glassware, using Milli-Q water containing 0.1% (v/v) DEPC, treated and autoclaved as above. Alternatively, where possible,
solutions were prepared and treated with 0.1% (v/v) DEPC for a minimum of 12 hours at 37°C, prior to autoclaving as above.

Sterile disposable plasticware was considered to be RNase-free and was used for the precipitation and storage of RNA without pre-treatment.

2.10.2 Isolation of total RNA

Total mRNA was extracted from both tissues and isolated mononuclear cells using the RNAzol™ B method (Biotecx Laboratories, Houston, TX, USA). For isolation of RNA from tissues (i.e. thymi and lymph nodes of rats), the organs were first rinsed in PBS and then homogenised in RNAzol™ B (2ml per 100mg of tissue) with 30 strokes of a DEPC-treated glass homogeniser. RNA was also isolated from mononuclear cell suspensions prepared from the livers, lymph nodes, blood and spleens of rats (see section 2.2 for cell isolation procedures). For this purpose, 5×10⁶-1×10⁷ cells were resuspended in 1ml of PBS and microcentrifuged (Eppendorf 5417R, Germany) at 4°C for 5 minutes at 13,000 rpm. The supernatant was removed and the cells were resuspended gently in 1-2ml of RNAzol™ B (0.2ml of RNAzol™ B per 10⁶ cells). The RNA was extracted by the addition of 1/10 volume of chloroform and vortexing for 1 minute. The samples were then placed on ice for 5 minutes. After centrifugation at 13,000 rpm for 15 minutes at 4°C as described above, the upper aqueous layer was removed carefully and transferred to a fresh 1.5ml reaction tube. The total RNA was precipitated by the addition of an equal volume of isopropanol, followed by a quick vortex and incubated on ice for 15 minutes. The RNA was pelleted by centrifugation at 13,000 rpm for 15 minutes at 4°C as described above and the supernatant was tipped off carefully in one movement. The pellet was then washed in 70% ethanol and allowed to air dry for approximately 30
minutes. After drying, the pellet was dissolved in 20µl of DEPC-treated water. The RNA concentration and purity was then measured by reading absorbance at both 260nm and 280nm with a spectrophotometer (Ultraspec® Plus, Pharmacia Biotech, Sweden). The A_{260}/A_{280} ratios of the RNA samples were typically in the range of 1.6-2.0, indicating good purity. The concentration of RNA was calculated using the formula:

RNA concentration (µg/µl) = (A_{260} \times \text{dilution factor} \times 40)/1000

(assuming an A_{260} value of 1 = 40µg/ml RNA)

RNA samples were stored at -70°C.

2.10.3 Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Five µg of RNA was resuspended in 45µl of DEPC-treated water and heated at 65°C for 5-10 minutes. The RNA suspension was allowed to cool to 4°C and the following cocktail was added: 16µl of RT buffer (Gibco Life Technologies, Australia), 8µl 0.1M DTT (Sigma, USA), 0.5µl of random hexamers at 100nmol/µl, 8µl of 10mM dNTPs (Pharmacia Biotech, Sweden), 1.5µl of RNAGuard (Pharmacia Biotech, Sweden) and 0.5µl of Superscript II reverse transcriptase at 200units/µl (Gibco BRL, Gaithersburg, MD, USA, Cat No. 18064/104). After incubating at 37°C for 90 minutes, the reverse transcriptase was inactivated by heating for 5-10 minutes at 95°C and then cooled to 4°C. The resultant complementary (c)DNA was stored at -20°C until required.

A standard PCR reaction contained 3-5µl of cDNA. The cDNA was amplified using a Corbett FTS-320 Thermal Cycler (Corbett Research, Sydney, Australia) by denaturing the cDNA initially at 95°C for 2 minutes prior to 40 cycles consisting of: 95°C for
30 seconds, 58°C for 1 minute and 72°C for 1 minute. These cycles were followed by a final extension at 72°C for 10 minutes. RT-PCR products were analysed by agarose gel electrophoresis as described previously (see section 2.9.5).
Chapter 3

Extra-thymic T cell development in the rat:
Investigations on the liver and lymphocyte-filled villi (LFV)
3.1 Introduction

Lymphocytes expressing the $\alpha/\beta$ TCR exist in the lymphoid tissues of athymic rodents (Kennedy et al., 1992; Sarawar et al., 1991; Vaessen et al., 1986) and the question arises, therefore, as to where these T cells develop. Recent research has indicated that some T cells can develop extra-thymically. Studies into IEL have revealed that subpopulations of these lymphocytes (largely those that express the CD8$\alpha\alpha$ homodimer) are present in athymic rodents and it is likely, therefore, that these IEL develop in a thymus-independent manner. Support for local extra-thymic development of some IEL comes from the presence of Thy-1+, CD8$\alpha^+$ precursors in mouse intestinal epithelium (Croitoru et al., 1990) and the detection of rag-1 gene activity in immature mouse (Lin et al., 1994; Guy-Grand et al., 1991) and human (Lynch et al., 1995; Lundqvist et al., 1995) IEL. Most of the IEL in mice (reviewed by Rocha et al., 1995; Poussier and Julius 1994), humans (Latthe et al., 1994; Jarry et al., 1990) and rats (Kearsy and Stadnyk, 1996; Lyscomb and Brueton, 1982) express CD8, whereas the majority of $\alpha/\beta$ TCR$^+$ cells in the spleens and lymph nodes of nude rats express CD4 (Sarawar et al., 1991). It is unlikely, therefore, that these CD4$^+$ $\alpha/\beta$ TCR$^+$ cells arise from the IEL compartment in the intestine.

It has been shown that RNA extracted from mononuclear cells isolated from the livers of mice (Narita et al., 1998; Kimura et al., 1995, Sato et al., 1995) and humans (Collins et al., 1996) contains mRNA transcripts of the rag-1 gene. In addition, putative lymphocyte precursors have been described in the livers of adult mice (Narita et al., 1998) and humans (Collins et al., 1996). However, the evidence for hepatic extra-thymic T cell differentiation is not definitive. The presence of mRNA encoding the RAG-1 protein could be interpreted as evidence of migration to the liver by recent
emigrants from other sites of primary T cell development. Furthermore, the expression of B220 antigen by some hepatic T cells has been interpreted to indicate that the liver is a site of accumulation for pre-apoptotic T cells and that the unusual phenotype of the cells is related to their targeting for cell death (Huang et al., 1994).

In addition to these proposed avenues of thymus-independent T cell development, other research suggests that specific anatomical structures in the mucosa of the small intestine may support extra-thymic differentiation of T cells. Recent work in this laboratory has characterised the lymphocyte-filled villi (LFV) in the rat small intestine (Mayrhofer and Brooks, 1995). Others (Saito et al., 1998; Kanamori et al., 1996) have identified similar structures in the mouse small intestine and further work on these structures is in progress (Moghaddami and Mayrhofer, manuscript in preparation). Importantly, with respect to the α/β T cells present in athymic rats, LFV are potential sources of the CD4+ subset that dominates in most of the lymphoid tissues in these animals.

Approximately 1% of villi in the rat small intestine have the structure of LFV. These villi are characterised by a lamina propria that is filled with closely packed lymphocytes and dendritic cells. LFV have a covering epithelium which contains specialised antigen-absorbing cells and they exist in conventional, SPF and athymic (rNu/rNu) rats. They are distinguished from other gut lymphoid tissues, namely Peyer’s patches and isolated lymphoid follicles, by not being compartmentalised into T cell- and B cell-rich regions. Most of the lymphocytes in LFV do not express markers of either T cells or B cell cells. Only a relatively minor subpopulation expresses CD3. Apart from MHC class I and CD45, the only markers that have been identified on the major LFV population to date are leukosialin (CD43) and the α-chain of the IL-2R (CD25). These are both markers
associated with immature thymocytes (reviewed by Godfrey and Zlotnik, 1993), while CD43 is also expressed by mature T cells. Minor sub-populations in LFV express markers of classical T cells. Approximately equal numbers of cells express CD3, the \( \alpha/\beta \) TCR (cells expressing the \( \gamma/\delta \) TCR are not present), CD4, CD5 and CD2. However, it is not certain that these markers are expressed simultaneously by a single subset. Very few express CD8\( \alpha \). In support of LFV being sites of primary lymphopoiesis, autoradiographic studies using tritiated thymidine (\( ^3 \)H-TdR) have shown that considerable numbers of dividing lymphocytes are present, while kinetic studies have shown that label can be chased into the \( \alpha/\beta \) TCR\(^+ \) population. Furthermore, LFV do not receive lymphocytes from the recirculating pool. There is substantial evidence, therefore, to suggest that LFV may be sites of primary lymphopoiesis.

Expression of the \textit{rag-1} gene by lymphocyte populations provides strong indication of ongoing or recent receptor rearrangement. To investigate whether in the mononuclear cells in the rat liver contain transcripts that encode RAG-1, a reverse transcription (RT)-PCR assay was established. However, this could not be applied to RNA extracted from the mucosa of the small intestine because it is not feasible to isolate LFV and it has been shown that some IEL in the mouse and human small intestine express mRNA for RAG-1. A preliminary attempt to detect transcripts of the \textit{rag-1} gene by \textit{in situ} hybridisation was unsuccessful for technical reasons. A cross-reactive polyclonal antibody against a fragment of mouse RAG-1 was utilised to overcome this problem. Further characterisation of the surface antigens expressed by the major lymphocyte population in LFV was carried out to explore the relationship between these cells and thymic T cell precursors.
3.2 Results

3.2.1 Expression of RAG-1 mRNA by mononuclear cells in the rat liver

To investigate the possibility that the liver in rats is a site of thymus-independent T cell development, RT-PCR was employed to detect mRNA transcripts encoding RAG-1 among isolated liver mononuclear cells. Oligonucleotide primers were designed which would amplify a PCR product in excess of 1200 bp from rat genomic DNA. The primers made use of the high degree of similarity (approximately 86%) that exists between mouse and human rag-1 sequences (Figure 3.2.1), allowing prediction of degenerate oligonucleotides with complementarity for the homogeneous sequences in the rat gene. Using these primers, forward/5' (2034) and reverse/3' (2035) bands of expected size were amplified from total genomic DNA derived from mouse, human and DA rat lymphocytes (Figure 3.2.2). The amplified product from the rat gene was cloned and sequenced. Sequence alignment revealed a high degree of similarity (85.6%) with the corresponding fragment amplified from mouse DNA (Figure 3.2.3). In addition, most restriction sites were conserved across the two species (data not shown). Having confirmed the specificity of these primers, total RNA was prepared from mononuclear cells isolated from the liver of a 7 week old DA rat and reverse transcribed. The primers were then used to explore whether the rag-1 fragment could be amplified from liver mononuclear cell cDNA. Bands of expected size were produced from these cells and from cDNA prepared from thymi of DA rats (Figure 3.2.4). The liver contains, therefore, cells in which the rag-1 gene is (or has been recently) transcriptionally active. This suggests that it is a potential site of thymus-independent T cell development in the rat, as described in mice and humans.
Figure 3.2.1  Sequence similarity between the coding regions of human and mouse RAG-1. The complete coding sequences of human and mouse RAG-1 genes were obtained from Genbank and similarity between the sequences was compared using the DNASIS software. The regions to which degenerate primers were designed to amplify a portion of the rat RAG-1 gene are indicated in bold and the direction of the forward (2034) and reverse 92035) primers are indicated with arrows. Overall sequence similarity over the coding region was approximately 86%.

--- Gaps inserted during alignment
|| Identical nucleotides
85.8% identity

**HUMAN.SEQ**

| 1 | ATGGCAGCTCTTTCCACCCACCTTGAGATCGCTTCGCCCCAGATGAAATTGAGCAACCC |
| 63 |

**MOUSE.SEQ**

| 64 | ATGGCTGCCCTTTCCGCCTCTACCTGTAGATTCGCTTCGCCCCAGATGAAATTGAGCAACCC |

| 127 | CAAATCCAAATTTCCGAGTGGAAATTTAACGCTGTTTCGGGTGAGATGCTTTG |
| 189 |

**HUMAN.SEQ**

| 190 | GAAAGACTGCAAAGGAGGAGAAAGCTCCTTTAGGAGCATCTAAAAGGACCCCAAC |
| 252 |

**MOUSE.SEQ**

| 253 | GTAGTCCAGAAGAGGTGGTGGTCTGACAGAACTCTCAGAAGAGACTGTCAGTCCC |

| 315 | CCAAATTTTCAAGAAGATTCCAGATGCTGAGGAGGAAGCAGAGCGCATCACAGG |
| 378 |

**HUMAN.SEQ**

| 316 | AACCCCTGACATCTGCGACCCACTCTGTGAGATGCTTTAGGAGCATCTAAAAGGACCCCAAC |
| 379 |

**MOUSE.SEQ**

| 379 | TATCCAGCTGCGTTGCTGAGATGCTTTTAGGAGCATCTAAAAGGACCCCAAC |

| 441 | TACCTTTCTGCGACCCACTCTGTGAGATGCTTTTAGGAGCATCTAAAAGGACCCCAAC |
| 504 |

**HUMAN.SEQ**

| 442 | GCCCTTACATCTGCAACACTGCCCTGGGAGCATGTAAGGAGCTTACGACACTGAGG |
| 505 |

**MOUSE.SEQ**

| 505 | TCCATCCACGCCAGTAATGTGATGCTGTGGAGATGCAAGAGTGGAGTTCGGAGG |

| 567 | TGGTCAGCAGGAGTGGAGTTCGGAGGAGATGCAAGAGTGGAGTTCGGAGG |
| 630 |

**HUMAN.SEQ**

| 568 | GCCCATGTGAGTTTTACCTCCTCCGAGGAGTAGGAGTAGTGGAGTCCAGACACCACCAAC |
| 693 |

**MOUSE.SEQ**

| 631 | TGGTGCAGGAGTGGAGTTCGGAGGAGATGCAAGAGTGGAGTTCGGAGG |

| 694 | TGGTGCAGGAGTGGAGTTCGGAGGAGATGCAAGAGTGGAGTTCGGAGG |

| 756 | TGGTGCAGGAGTGGAGTTCGGAGGAGATGCAAGAGTGGAGTTCGGAGG |
Figure 3.2.2  Amplification of segments of the rag-1 gene from rat, mouse and human genomic DNA. Degenerate primers 2034 (forward) and 2035 (reverse) were used to amplify a portion of the rag-1 gene (in excess of 1200bp) from genomic DNA. Amplification was performed as detailed in Materials and Methods (section 2.9.4), after which the PCR products were separated on a 1% agarose gel and visualised by ethidium bromide staining.

<table>
<thead>
<tr>
<th>M</th>
<th>SPP1 markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>no DNA control</td>
</tr>
<tr>
<td>Lane 2</td>
<td>rat genomic DNA</td>
</tr>
<tr>
<td>Lane 3</td>
<td>mouse genomic DNA</td>
</tr>
<tr>
<td>Lane 4</td>
<td>human genomic DNA</td>
</tr>
</tbody>
</table>
RAG-1
(1268 bp)
Figure 3.2.3  Sequence similarity between a cloned portion of the rat rag-1 gene and the corresponding region of the mouse rag-1 sequence.

The complete coding sequence of the mouse rag-1 gene (Schatz et al., 1989) obtained from Genbank was aligned with the sequence obtained from the cloned portion of the rat rag-1 gene, using the DNASIS software. Sequence similarity over a 521 bp overlap of the two sequences was approximately 90%.

---  Gaps inserted during alignment
||  Identical nucleotides
90.2% identity in 521 bp overlap

**MOUSE.SEQ**
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**RATp.SEQ**
```
ATGGACATGGAGGACATCTTGGACGGCATGAGATCCCAA
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**MOUSE.SEQ**
```
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```

**RATp.SEQ**
```
AATGGTCCCTCAATGTTGGTGGATAGGATTTGCGATGAGATCCCAA
```

**MOUSE.SEQ**
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```

**RATp.SEQ**
```
AGCTCTGGGAGTGGCCCGCACTTCAGAGCACTCTTTCGTTTCCTTTTCACAGCATG
```

**MOUSE.SEQ**
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**RATp.SEQ**
```
AGAATTAGTATAGCAGTTGCAAGAACGCTGATTGCCAAAAGAGAAAAGAAAAGAA
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**MOUSE.SEQ**
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**RATp.SEQ**
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**MOUSE.SEQ**
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**RATp.SEQ**
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**MOUSE.SEQ**
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**RATp.SEQ**
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**MOUSE.SEQ**
```
GATGAAAAACCTCTGCGGGAAGTA-GAGGCTTTGGAGCTTGGCTCACTGATCCTGTG
```

**RATp.SEQ**
```
GATGAAAAACCTCTGCGGGAAGTA-GAGGCTTTGGAGCTTGGCTCACTGATCCTGTG
```

**MOUSE.SEQ**
```
TACACCTGTCAACAACCGCGTGTGGAAGCTCTCAAGAAATC
```

**RATp.SEQ**
```
TACACCTGTCAACAACCGCGTGTGGAAGCTCTCAAGAAATC
```
Figure 3.2.4 Detection of mRNA encoding RAG-1 among thymocytes and mononuclear cells isolated from the liver of a rat. RNA extracted from thymocytes and liver mononuclear cells (MNCs) was reverse transcribed and the *rag-1* specific primers 2034 (forward) and 2035 (reverse) used to amplify *rag-1* mRNA as detailed in *Materials and Methods* (section 2.10.3). Primers against β-actin were included as internal controls. Amplification products were separated and visualised by ethidium bromide staining of a 1% agarose gel.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
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<tr>
<td>M</td>
<td>SPP1 markers</td>
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<tr>
<td>Lane 1</td>
<td>cDNA from thymocytes amplified with β-Actin primers</td>
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<tr>
<td>Lane 2</td>
<td>cDNA from thymocytes amplified with RAG-1 primers</td>
</tr>
<tr>
<td>Lane 3</td>
<td>cDNA from liver MNCs amplified with β-Actin primers</td>
</tr>
<tr>
<td>Lane 4</td>
<td>cDNA from liver MNCs amplified with RAG-1 primers</td>
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RAG-1 (1268 bp)
β-actin (607 bp)
3.2.2 Attempted detection of transcripts encoding RAG-1 in lymphocytes in LFV

In an attempt to determine whether lymphocytes in LFV contained mRNA encoding RAG-1, *in situ* hybridisation was employed. Digoxigenin (DIG)-labelled riboprobes, in both sense and anti-sense orientations, were generated using the portion of the cloned rat *rag-1* gene described in 3.2.1 as a template. These probes were then hybridised with sections of intestine containing LFV and with thymus (positive control) and lymph node (negative control) sections. Unfortunately, hybridisation with both the sense and anti-sense probes produced non-specific background staining in the medulla of the thymus and among considerable numbers of lymphocytes in lymph nodes. All attempts to block non-specific binding by increasing the stringency of the hybridisation, along with varying the concentrations of non-specific DNA (salmon sperm DNA), transfer (t) RNA and BSA in the probe mix proved unsuccessful. This specificity problem is most likely due to the probes themselves because the *in situ* hybridisation protocol yielded expected results when sense and anti-sense riboprobes specific for class I MHC (provided by Dr Rosemary Periera, Medical Virology, IMVS, Adelaide, South Australia) were used. The large size of the probes (in excess of 1000bp) could be responsible for the non-specific binding observed. In the future, smaller probes could be generated using known restriction sites within the cloned portion of the rat *rag-1* gene (Murphy, unpublished results).

3.2.3 Phenotypic characterisation of cells present in LFV

Lymphocytes in LFV were examined for the expression of a number of markers associated with immature thymocytes. The data presented were obtained from LFV in the small intestines of DA rats. However, similar results were observed using tissue
prepared from CBH rNu/+ (euthymic) and rNu/rNu (athymic) rats. LFV were identified in random cryostat sections and further serial sections were prepared. After air-drying and fixation in 95% ethanol, the sections were labelled with mAb using an indirect immunoperoxidase technique. Sections from two LFV, stained with the negative control mAb (1B5), are shown in Figure 3.2.5.

CD44 expression by early thymocytes is important during thymic development in the mouse (Patel et al., 1995). Monoclonal antibody OX50 (anti-CD44) stained essentially all of the lymphocytes in LFV (Figure 3.2.6 [C]).

During T cell development in the mouse thymus, expression of NKR-P1 (CD161) appears to coincide with commitment to the T and NK lineages and loss of ability to give rise to DC and B cells (Carlyle et al., 1997). A subpopulation of immature human thymocytes also expresses CD161 (Poggi et al., 1996). These reports prompted an investigation into the expression of CD161 by lymphocytes in LFV. Figure 3.2.6 (D) shows that most lymphocytes in LFV also stain with the mAb 10/78 (anti-CD161). As described previously (Mayrhofer and Brooks, 1995), the majority of lymphocytes in LFV were stained by mAbs OX39 (anti-CD25) and W3/13 (anti-CD43) (Figure 3.2.6 [A] and [B]). Therefore, the phenotype of the majority of the lymphocytes in LFV is CD25⁺ CD43⁺ CD44⁺ CD161⁺.

As described earlier, minor populations of lymphocytes in LFV express markers of mature T cells. As shown in Figure 3.2.7 (A), there is a small population of α/β TCR⁺ cells which occupy mainly a peripheral distribution in the subepithelial region of the LFV. Lymphocytes that express CD4 have a similar distribution and number but in
Figure 3.2.5  Identification of cell surface antigens in lymphocyte-filled villi (LFV) (negative control). Frozen sections from rat small intestine were stained by the indirect immunoperoxidase technique. (A) and (B) represent sectioned LFV, showing lack of staining by the negative control mAb 1B5. Original magnification ×82.5.
Figure 3.2.6  Antibodies that stain major lymphocyte populations in lymphocyte-filled villi (LFV). Frozen sections from rat small intestine were stained by the indirect immunoperoxidase technique. (A) a section stained with mAb OX39 (anti-CD25), (B) a section stained with mAb W3/13 (anti-CD43), (C) a section stained with mAb OX50 (anti-CD44) and (D) a section stained with mAb 10/78 (anti-CD161). Original magnification ×82.5.
addition some large CD4+ cells are present that appear to be dendritic cells. The latter are found in a subepithelial region and in the base of the LFV (Figure 3.2.7 [B]). Very few lymphocytes were found to stain with the mAb OX8 (anti-CD8α), although CD8α+ cells were common in the overlaying epithelium of both LFV and classical villi (Figure 3.2.7 [C]). Because gut associated/derived CD8+ T cells express mainly CD8αα homodimers rather than the CD8αβ heterodimer (reviewed by Rocha et al., 1995; Poussier and Julius 1994), LFV were also stained with mAb 341 (anti-CD8β). Staining of serial sections with anti-CD8α (Figure 3.2.7 [C]) and anti-CD8β (Figure 3.2.7 [D]) revealed that most CD8+ cells in LFV expressed CD8αα homodimers. Cells that expressed the CD8αβ heterodimer were essentially absent within the LFV and were infrequent in the overlaying epithelium. The observation that relatively few lymphocytes in LFV express CD8α indicates also that the majority of the CD161+ lymphocytes in LFV are unlikely to be NK cells (Torres-Nagel et al., 1992). Moreover, most of the lymphocytes in LFV do not express CD11b (data not shown). Taken together, the absence of CD11b and CD8α expression by most of the lymphocytes in LFV makes it unlikely that most of the CD161+ cells are NK cells.

Dual-immunofluorescence was performed to examine whether the lymphocytes that express the α/β TCR are a subset of the majority CD43+, CD25+, CD44+, CD161+ population in LFV. As shown in Figure 3.2.8, α/β TCR+ cells did not express CD161. Furthermore, preliminary observations also indicate that most α/β TCR+ cells were also negative for CD25 (data not shown). If the CD43+, CD25+, CD44+, CD161+ cells are indeed a precursor population, it appears that expression of CD25 and CD161 ceases by the time a TCR is expressed on the cell surface.
Figure 3.2.7  Antibodies that stain minor lymphocyte populations in lymphocyte-filled villi (LFV). Frozen sections from rat small intestine were stained by the indirect immunoperoxidase technique. (A) a section stained with mAb R73 (anti-α/β TCR), (B) a section stained with mAb W3/25 (anti-CD4), (C) a section stained with mAb OX8 (anti-CD8α) and (D) a section stained with mAb 341 (anti-CD8β). Original magnification ×82.5.
The mAb OX62 (anti-αE2-integrin) labels DC (Brenan and Puklavec, 1992; Griffin and Mayrhofer, unpublished results), IEL and some lamina propria T cells (Brenan and Rees, 1997). Figure 3.2.9 (A) shows that IEL and some cells within the LFV are stained by mAb OX62. However, many of the cells stained by this mAb within LFV did not appear to be lymphocytes. The staining pattern produced by mAb OX6 (anti-MHC class II) on an adjacent section in the series revealed clusters of large branching cells (Figure 3.2.9 [B]), similar to those stained with mAb OX62. It appears that the large cells stained by these mAbs are DC. Simultaneous expression of αE2 or MHC-class II by α/β TCR+ cells was examined by dual-fluochrome staining. Some α/β TCR+ cells appeared to express each of these molecules. However, the ramification of processes from the putative DC (Figure 3.2.9 [A] and [B]) between the lymphocytes made this observation inconclusive (data not shown).

A number of reports have highlighted the importance of VLA-4 (α4β1) expression in the differentiation of progenitor cells (Lévesque et al., 1995; Miyake et al., 1991). Monoclonal antibody MRα4-1 (anti-CD49d) was used to investigate the expression by LFV lymphocytes of integrins expressing the α4 chain. Figure 3.2.9 (C) shows that CD49d is expressed in LFV. However, because rat DC express CD49d (Griffin and Mayrhofer, unpublished results), it is difficult to interpret this result (for the reasons outlined above). Nevertheless, the profiles of some stained cells were consistent with those of lymphocytes. In the future, confocal microscopy may be useful in accurately determining co-expression of surface markers such as αE2-integrin and CD49d by lymphocytes in LFV.
Figure 3.2.8  Co-expression of surface antigens by lymphocytes in lymphocyte filled villi (LFV). Co-expression of surface antigens by lymphocytes within LFV was addressed by dual fluochrome staining of rat intestinal sections. A section stained indirectly with mAb 10/78 (anti-CD161; FITC) followed by direct staining with mAb R73 (anti-α/β TCR) conjugated to PE. Original magnification x82.5.
Figure 3.2.9  Expression of MHC class II, transferrin receptor, CD49d and αE2-integrin by lymphocytes in lymphocyte-filled villi (LFV). Frozen sections from rat small intestine were stained by the indirect immunoperoxidase technique. (A) a section stained with mAb OX62 (anti-αE2), (B) a section stained with mAb OX6 (anti-MHC class II), (C) a section stained with mAb MRα4-1 (anti-CD49d) and (D) a section stained with mAb OX26 (anti-CD71). Original magnification ×82.5.
Earlier work has shown that some lymphocytes in LFV incorporated \(^3\)H-TdR (Mayrhofer and Brooks, 1995). Dividing cells also express the transferrin receptor (CD71) (Trowbridge and Omary, 1981). Expression of CD71 by LFV lymphocytes was examined using mAb OX26 (anti-CD71). Figure 3.2.9(D) demonstrates the presence of CD71 expressing cells in LFV and also in the epithelium at the bases of villi and in the crypts. Lymphocytes expressing CD71 were most frequent at the base of the LFV.

### 3.2.4 Detection of RAG-1 protein in LFV

The presence of dividing lymphocytes and of a putative T cell precursor population in LFV suggests strongly that the \(\alpha/\beta\) TCR\(^+\) cells develop locally within these structures. To examine whether there is evidence of TCR gene-re-arrangement within lymphocytes in LFV, sections were stained, using an indirect immunofluorescence technique, with an affinity purified polyclonal antibody raised against a fragment of mouse RAG-1 protein (amino acids 56-123) (Leu and Schatz, 1995). As a positive control, Figure 3.2.10 (A) illustrates that RAG-1 protein could be detected by this technique in lymphocytes in the thymic cortex. Conversely, there was no staining of lymphocytes in the T-dependent regions of Peyer’s patches (Figure 3.2.10 [C]). These observations and others made on lymph node sections (data not shown), indicate that the antibody was specific for RAG-1. Lymphocytes expressing RAG-1 protein were detected in LFV and were a minor population (<10%) and were found largely in the centre of these structures (Figure 3.2.11 [A] and [C]). In contrast, lymphocytes in the lamina propria of adjacent villi did not express RAG-1 protein (Figure 3.2.11 [C]).
Figure 3.2.10  Confocal microscopic images of tissue sections stained with anti-RAG-1 antibody. Detection of RAG-1 in frozen tissue sections by immunofluorescent staining. (A) Section of rat thymus labelled indirectly with anti-mouse RAG-1 polyclonal antibody as described in Materials and Methods. (B) Phase contrast image of the field shown in (A). (C) Peyer’s patch section labelled indirectly with anti-mouse RAG-1 polyclonal antibody. (D) Phase contrast image of the field shown in (C). The horizontal bar represents 40μm.
Figure 3.2.11 Confocal microscopic images of lymphocyte-filled villi (LFV) stained with anti-RAG-1 antibody. Detection of RAG-1 in frozen tissue sections by immunofluorescent staining. (A) Section of a lymphocyte-filled villus labelled indirectly with anti-mouse RAG-1 polyclonal antibody as described in Materials and Methods. (B) Phase contrast image of the field shown in (A), where the horizontal bar represents 40μm. (C) Magnification at a lower power of the same lymphocyte filled villus (boxed) shown in (A). (D) Phase contrast image of the field shown in (C), where the horizontal bar represents 80μm.
3.3 Discussion

3.3.1 Expression of RAG-1 by rat liver mononuclear cells: A Summary

Recent studies have implicated the liver as a site of thymus-independent T cell development. DNA synthesis by lymphocytes in hepatic sinusoids (Ohteki et al., 1992), together with the detection of mRNA transcripts encoding RAG-1 (Narita et al., 1998; Kimura et al., 1995; Sato et al., 1995) or RAG-2 (Narita et al., 1998) in isolated hepatic mononuclear cells, suggests strongly that T cells can develop extra-thymically in the livers of mice. Furthermore, cells sharing phenotypic characteristics with T cell precursors in the thymus have been identified in the livers of adult mice (Narita et al., 1998). In humans, hepatic lymphocytes that express CD2 and CD7, in the absence of surface TCR, have been shown to contain mRNA transcripts encoding RAG-1, RAG-2 and the pre-T cell α-chain (pTα) (Collins et al., 1996). The present study extend these observations to the rat.

Approximately one third of the coding sequence of the rat rag-1 gene was cloned. This portion shared a high degree of similarity with the corresponding portion of the mouse rag-1 gene. The primers used in cloning the rat rag-1 gene segment were also used in an RT-PCR protocol. This protocol detected mRNA transcripts encoding RAG-1 in thymocytes and also in mononuclear cells isolated from adult rat liver (Figure 3.2.4). The identification of mRNA transcripts encoding RAG-1 among isolated liver mononuclear cells suggests that thymus-independent T cell development could be occurring in the livers of adult rats. Future work will be required to determine the phenotype of those cells that express transcripts of the rag-1 gene. This is important, not only to examine whether such cells have an immature surface antigen phenotype but also to exclude the possibility that some could be precursors of B cells.
3.3.2 Thymus-independent T cell development in LFV: A Summary

The work described in this chapter adds important information to the initial characterisation of LFV in the rat small intestine. Previous work has shown that LFV constitute approximately 1% of the villi in the small intestine. They have a modified structure and they are filled with closely packed lymphocytes. These structures are present in SPF and conventional rats and they are also present in athymic rats. Most of the lymphocytes in LFV were shown to express CD43 and CD25. CD43 is expressed by both immature and mature T cells in rats, while CD25 is expressed by a subpopulation of immature thymocytes. Small numbers of lymphocytes in LFV were shown to express CD4 and other markers of mature T cells, including CD3, CD5 and the α/β TCR. Lymphocytes in LFV were also shown to be excluded from the recirculating pool and to contain a population of dividing cells. These observations, together with the finding that incorporation of $^3$H-TdR by lymphocytes in LFV occurred earlier in the CD3/TCR$^-$ population than in the CD3/TCR$^+$ population, supported the hypothesis that LFV are sites of extra-thymic T cell differentiation. The present studies strengthen this hypothesis.

Studies in mice have revealed that during T cell development in the thymus, commitment to both the T and NK cell lineages is marked by the expression of CD161 (Carlyle et al., 1997). Human thymocytes have also been shown to express this molecule (Poggi et al., 1996). Another marker expressed by early thymocytes is CD44. Expression of CD44 by thymocytes is thought to mediate essential interactions during thymic development (Patel et al., 1995). Expression of a number of integrins, including integrins VLA-4 and VLA-5, are also thought to be essential for the differentiation of progenitor cells, including lymphoid progenitors. Finally, CD25 is expressed by
thymocytes in mice at the triple negative (TN) stage. Detectable levels of CD44 and CD25 are no longer expressed by thymocytes that express low levels of the CD3-TCR complex (Godfrey et al., 1993).

Therefore, just prior to rearrangement of the TCR genes, thymocytes in the mouse thymus have the surface antigen phenotype CD44⁺CD25⁺CD161⁺ (Carlyle et al., 1997). The results in this chapter show that the majority of lymphocytes in LFV share a similar phenotype, namely CD43⁺CD44⁺CD25⁺CD161⁺ (Figure 3.2.6). This can be inferred from the fact that each marker is expressed by a large proportion of the lymphocytes in LFV. However, formal proof that they are expressed simultaneously and investigation of subsets that do not express all of the markers, will require future multi-colour immunofluorescence or immunohistochemistry. In accordance with the observations made on mouse thymocytes (Carlyle et al., 1997), a preliminary study suggests that at about the time of acquisition of the α/β TCR, lymphocytes in LFV cease to express CD161 (NKR-P1) (Figure 3.2.8) or CD25 (preliminary observation, data not shown).

Previous autoradiographic studies have shown that labelled cells can be detected in LFV after intravenous infusion of ³H-TdR into rats. The labelled cells were more frequent at the bases of LFV. It is of interest, therefore, that some lymphocytes in LFV express CD71 and that these also were found at the basal region (Figure 3.2.9 [D]). These findings, together with the observation on the phenotype of lymphocytes in LFV, suggest that the base of LFV is a region of lymphopoiesis. The cells produced in LFV appear to acquire the CD3-α/β TCR complex and to migrate towards the tip and subepithelial regions as they mature. The detection of RAG-1 protein in lymphocytes
within LFV provides important corroborative evidence that rearrangement of TCRs occurs within LFV.

LFV contained a population of CD4+ lymphocytes which had a size and location similar to α/β TCR+ cells. Of the few CD8+ cells present, it appeared that most expressed the CD8αα homodimer as CD8β+ were rare. Expression of the CD8αα homodimer, rather than the CD8αβ heterodimer, is a feature of the subpopulation of IEL that is believed to be thymus-independent (reviewed by Rocha et al., 1995 and Latthe et al., 1994; Torres-Nagel et al., 1992) and to develop locally in the gut (Rocha et al., 1994; Guy-Grand et al., 1991). It is, therefore, of considerable interest that putative thymus-independent CD8+ cells in LFV also appear to express CD8αα+. NK cells in rats express CD8α in the absence of CD8β chains (Torres-Nagel et al., 1992) but for reasons discussed earlier, it is unlikely that the cells in LFV are NK cells. Nevertheless, expression of the CD8αα homodimer may be a characteristic of cells of the NK cell-T cell lineage that pre-date the evolution of the thymic maturation pathway.

Preliminary dual-fluorescence studies indicated that the number of CD4+ cells in LFV exceeded the number of α/β TCR+ cells (data not shown). It is worth noting that during thymocyte development, the earliest T cell precursors express CD4 (Wu et al., 1991). Precursor cells in LFV could, therefore, also express CD4, prior to expression of CD3-TCR. It is worth noting that studies in this laboratory (Moghaddami and Mayrhofer, in preparation) and by others (Kanamori et al., 1996) on a potential homologue of LFV in mice show that while some CD4+ cells are present, cells expressing the TCR are absent or rare. Expression of RAG-1 protein was not detected in cryptopatches. A technique has been developed recently to isolate cells from cryptopatches (Saito et al., 1998).
Despite the absence of TCR<sup>+</sup> cells in these structures, adoptive transfer of precursor cells (TCR<sup>-</sup> cells) into SCID mice resulted in the appearance of lymphocytes that expressed either the α/β or the γ/δ TCR in the IEL population and in the mesenteric lymph nodes. Given this observation, it is possible that immature cells in LFV/cryptopatches express CD4 and that, in the case of mice, progenitor cells leave the LFV and differentiate further at other sites, including the epithelium of the small intestine. However, the expression of RAG-1 and the presence of cells expressing the α/β TCR in LFV in rats indicates that it is highly likely that T cells can develop in a thymus-independent manner within these structures in this species.

A number of questions arise from these studies. (1) Are the DC present in LFV integral to lymphocyte development and TCR selection? Perhaps they have a role in antigen presentation. In this respect, non-classical MHC molecules such as CD1 have been shown to present antigens to NKT cells (Bendelac et al., 1995) and to IEL (Balk et al., 1991). It will be interesting to investigate whether CD1 has a role in the selection of T cells in LFV. Local antigen may have a role in T cell selection because work in this laboratory has shown that horseradish peroxidase is taken up from the gut lumen by the epithelium of LFV in a manner similar to transport through the follicle-associated epithelium of Peyer’s patches (Mayrhofer, unpublished results). It is possible that development of T cells in LFV is different from thymic development with respect to the role of exogenous antigens. Perhaps T cell development in LFV is antigen-driven, leading to the production of cells that are already activated within the site of primary generation. A system such as this could be geared to deliver a continuous stream of effector cells and pre-date the evolution of immunological memory. (2) In such a system, exogenous antigen, presented in association with perhaps novel MHC
molecules, could be responsible for positive selection of cells that would have an immediate utility. It would be of interest to see whether any cells in LFV undergo apoptosis. This could reflect negative selection and apoptosis should be detected by the TUNEL technique. In regard to this, it is interesting to note that using this method, apoptotic lymphocytes were not detected in cryptopatches (Kanamori et al., 1996).

(3) It is pertinent to enquire as to the function of cells generated (positively selected) in LFV. While they might have protective effector functions against pathogens, it is also possible that their function is regulatory. A role for LFV in the development of regulatory and/or protective roles is feasible and it would be of use to develop a technique to examine these possibilities. (4) Expression of CD49d has been shown to be important during progenitor cell differentiation (Lévesque et al., 1995; Miyake et al., 1991). Immuno-electron microscopic analysis could be utilised to resolve whether it is the lymphocytes and/or DC in LFV that express this antigen. Given the importance of CD49d ($\alpha_4\beta_7$ and VLA-4) (Hamann et al., 1994; Briskin et al., 1993) and $\alpha_E$-integrin (Brenan and Rees, 1997; Cerf-Bensussan et al., 1987) as homing receptors among mucosal T cells, this technique may provide insights into the possible destinations of the cells that are generated in LFV.

In conclusion, the data presented here indicate that LFV are sites of thymus-independent T cell development. The majority of lymphocytes present in these structures share with thymic T cell precursors the phenotype CD44$^+$CD25$^+$CD161$^+$ (and possibly CD4$^+$). A smaller population consists of cells that express the $\alpha/\beta$ TCR, CD4, CD49d and the $\alpha_E$-integrin, while a few cells express CD8$\alpha$ but not CD8$\beta$. The evidence provides a strong case that T cell precursors differentiate into $\alpha/\beta$ TCR$^+$ T cells using a process that involves RAG-1 expression and related TCR rearrangement. It is possible that some
precursor cells may exit LFV and undergo maturation at other sites such as the epithelium of the small intestine. However, unlike cryptopatches in mice, it is likely that mature \( \alpha/\beta \) T cells which express mainly the CD4 co-receptor leave LFV and migrate to other sites—perhaps via the thoracic duct.
Chapter 4

Characterisation of T cells in athymic (rNu/rNu) rats
4.1 Introduction

Thymus-deficiency, shown later to result from a mutation of the \textit{whn} gene (Nehls \textit{et al}., 1994), was reported first in mice (Pantelouris, 1968) and later in rats (Festing \textit{et al}., 1978). Paradoxically, lymphocytes that express an antigen specific T cell receptor (TCR) are present in these athymic (nude) animals. In both nude mice (MacDonald \textit{et al}., 1981; Lawetzky and Hünig, 1988; Kennedy \textit{et al}., 1992) and nude rats (Vos \textit{et al}., 1980; Vaessen \textit{et al}., 1986), there is an age-dependent increase in the numbers of cells expressing the $\alpha/\beta$ TCR in the spleens and lymph nodes of the animals. Studies on the thoracic duct lymph (TDL) of nude rats show that cells expressing the $\alpha/\beta$ TCR ($\alpha/\beta$ T cells) are also present in efferent lymph (Sarawar \textit{et al}., 1991). Analysis of the $\alpha/\beta$ T cells present in the lymph nodes of athymic rats reveals that the proportions of CD4$^+$ and CD8$^+$ cells are similar to those observed in euthymic littermates (Sarawar \textit{et al}., 1991), although the form of the CD8 dimer that is expressed is distinct between athymic and euthymic rats. A high proportion of the CD8$^+$, $\alpha/\beta$ T cells present in the lymph nodes and spleens of athymic rats expresses the CD8$\alpha\alpha$ homodimer, whereas in euthymic rats essentially all of the CD8$^+$, $\alpha/\beta$ T cells express the CD8$\alpha\beta$ heterodimer (Torres-Nagel \textit{et al}., 1992). Differences also exist among CD4$^+$ cells from euthymic and athymic rats. The majority of CD4$^+$ T cells from normal rats express the CD45RC isoform, whereas in athymic rats most are CD45RC' (Sarawar \textit{et al}., 1991). With respect to function, the $\alpha/\beta$ TCR$^+$ cells that are present in nude rats are responsive to T cell mitogens (Vaessen \textit{et al}., 1986; Sarawar \textit{et al}., 1991) and respond to alloantigens \textit{in vitro}. However, they do not mediate rejection of allogeneic skin grafts (Sarawar \textit{et al}., 1991). These observations suggest that the $\alpha/\beta$ TCR$^+$ T cells in athymic rats are not functional equivalents of the classical $\alpha/\beta$ T cells found in euthymic animals.
Studies since 1990 on α/β T cells isolated from the livers of mice have identified a population of α/β TCR<sup>+</sup> cells that is distinct phenotypically. These cells express reduced levels of the TCR on the cell surface and they have, therefore, hence been termed TCR<sub>intermediate</sub> cells (Watanabe et al., 1992). Utilising this characteristic, flow cytometric analysis reveals distinct α/β TCR<sup>+</sup> cell populations in the livers of normal mice (Watanabe et al., 1992). Further analysis of the TCR<sub>intermediate</sub> cells has revealed a prominent subpopulation (25-30%) with a CD4<sup>-</sup>CD8<sup>-</sup> (DN) phenotype (Iiai et al., 1992; Ohteki et al., 1992; Watanabe et al., 1992; Ohteki et al., 1990), while the majority were CD4<sup>+</sup>. Only a small proportion of TCR<sub>intermediate</sub> cells express the CD8 co-receptor (Iiai et al., 1992). Further characterisation has revealed that TCR<sub>intermediate</sub> cells express high levels of LFA-1 (CD11a) (Watanabe et al., 1992; Ohteki et al., 1992) and the cells express the β-chain of the IL-2 receptor (CD122) (Arai et al., 1995; Watanabe et al., 1993) constitutively. In addition, it has been shown that TCR<sub>intermediate</sub> cells express high levels of CD44 (Arai et al., 1995; Ohteki et al., 1992) but low levels of L-selectin (CD62L<sup>lo</sup>) (Arai et al., 1995. Many of these TCR<sub>intermediate</sub> cells have been shown also to express NKR-P1/NK1.1 (CD161).

Although the liver is the richest source of TCR<sub>intermediate</sub> cells; these cells are also present in the spleens, lymph nodes, and the bone marrow of normal mice. However, whereas TCR<sub>intermediate</sub> cells in the liver express CD161 and most express the CD4<sup>+</sup> or DN phenotypes, the majority of TCR<sub>intermediate</sub> cells present in these other tissues express the CD8 co-receptor (Watanabe et al., 1995).

It is interesting to note that TCR<sub>intermediate</sub> cells are essentially the only α/β TCR<sup>+</sup> cells present in the tissues of both nude mice (Sato et al., 1995; Iiai et al., 1992, Watanabe et
Ohteki et al., 1992; Lawetzky and Hünig, 1988) and thymectomized, bone
marrow reconstituted mice (Sato et al., 1995; Ii et al., 1992). These observations
suggest that cells with a TCR<sub>intermediate</sub> phenotype have developed extra-thymically.

LFV are a potential source of CD4<sup>+</sup> α/β TCR<sup>+</sup> cells. Because this phenotype is
prominent in the lymphoid tissues of aged athymic rats (Vaessen et al., 1986; Sarawar et
al., 1991), an investigation was undertaken into the accumulation of α/β TCR<sup>+</sup> cells in
athymic rats. This chapter details phenotypic differences between the α/β T cells
present in athymic rats and age-matched euthymic controls. More specifically, it shows
that α/β TCR<sup>+</sup> cells in nude (rNu/rNu) rats appear in the TDL before their appearance in
secondary lymphoid organs, and that these cells are larger in size than the corresponding
cells present in control (rNu/+ ) rats. In addition, α/β T cells from rNu/rNu rats are
shown to express a different pattern of adhesion molecules compared with rNu/+ rats.
This pattern is consistent with activation rather than with a resting phenotype.
4.2 Results

4.2.1 Appearance of $\alpha/\beta$ T cells in rNu/rNu rats

Work in this laboratory (Lee, 1993) showed that small numbers of cells expressing the $\alpha/\beta$ TCR could be detected immunohistochemically in the spleens and lymph nodes of rNu/rNu rats at approximately 16 weeks of age. Cells expressing the $\alpha/\beta$ TCR were detected in the TDL at a slightly earlier age than they could be detected in the peripheral lymphoid organs. In addition, flow cytometric analysis revealed that the $\alpha/\beta$ TCR$^+$ cells in TDL were present only in the large lymphocyte (blast) population. Because lymphocyte drainage from the gut accounts for approximately 90% of the cells in the TDL of normal rats (Mann and Higgins, 1950; Mayrhofer, unpublished observations and Spargo et al., 1996), it was reasoned that the gut was the most likely source of these cells. It was possible, therefore, that the $\alpha/\beta$ TCR$^+$ cells appearing in the TDL of young rNu/rNu rats were newly formed cells and that they could arise from the LFV. Lee (1993) had shown that $\alpha/\beta$ TCR$^+$ cells were present in LFV at approximately 11 weeks of age, before they could be detected elsewhere in lymphoid tissues in nude rats.

The thoracic ducts of four rNu/rNu and four rNu/+ rats, aged 9-10 weeks of age (w/o), were cannulated. TDL was collected over six subsequent 12 hour intervals and the lymphocytes were labelled by the direct technique with mAb R73 (anti-$\alpha/\beta$ TCR). The proportion of $\alpha/\beta$ TCR$^+$ cells in each collection was then determined by flow cytometry. In all cases, $\alpha/\beta$ TCR$^+$ cells contributed no more than 1.5% of the total lymphocyte population (data not shown). With the prior knowledge that $\alpha/\beta$ TCR$^+$ cells present in the TDL of 15 w/o rNu/rNu rat are found only in the blast population (Lee, 1993), small and large lymphocytes were electronically gated and analysed for forward- and side-
light scatter (Figure 4.2.1 [A] and [B]). The proportion of lymphoblasts collected from rNu/+ and rNu/rNu rats increased steadily over the period of thoracic duct drainage, reflecting the removal of small lymphocytes from the recirculating pool (Figure 4.2.1). Analysis of the small lymphocytes (R1, Figure 4.2.1) from 9-10 w/o rNu/rNu rats highlights the essential absence of α/β TCR+ cells (illustrated in Figure 4.2.2 [A]). However, a small population of labelled cells does become evident over the 72 hours of thoracic duct drainage (collection 1-6), although considerable variability was seen in the lymph collected from the four rats. In comparison, small α/β TCR+ cells were a major population in the TDL of age-matched euthymic rats (Figure 4.2.2 [B]). The proportion of these cells, relative to the α/β TCR- cells, declined in successive collections over the time course.

Examination of the lymphoblast population, which comprised 5-10% of total lymphocytes during the first 12 hours of drainage (R2, Figure 4.2.1), revealed that a significant proportion of these cells expressed the α/β TCR in all of the rNu/rNu rats examined (Figure 4.2.3 [A]). Furthermore, the proportion of α/β TCR+ cells in this lymphoblast population increased over the first 36-48 hours of collection (from approximately 29% to approximately 50%, collections 1-4 in the animal illustrated), although the proportion of T cells varied considerably between rats. As expected, examination of the lymphoblast populations of 9-10 w/o rNu/+ rats identified a high proportion of α/β T cells (Figure 4.2.3 [B]), although in contrast to rNu/rNu rats, there was a general decline in the proportion of α/β TCR+ cells relative to the α/β TCR- (B cells (data not shown) over the period of the collections (collection 1-6).
A

9-10w/o rNu/rNu

Collection 1

Collection 2

Collection 3

Collection 4

Collection 5

Collection 6
B

9-10w/o rNu/+
Figure 4.2.2  Proportions of $\alpha/\beta$ T cells among small lymphocytes in thoracic duct lymph (R1). Small lymphocytes were gated as shown in Figure 4.2.1. Lymphocytes falling within this region in the six sequential collections of thoracic duct lymph shown in Figure 4.2.1 were examined for the expression of the $\alpha/\beta$ TCR by labelling directly with phycoerythrin-conjugated mAb R73 (R73-PE). The dashed line represents the autofluorescence of lymphocytes incubated with the negative control antibody 1B5. The staining profile of cells incubated with R73-PE is superimposed. The proportion of labelled R73$^+$ cells is indicated above the marker in each panel.

A. Thoracic duct lymph collections from a rNu/rNu rat aged 9-10 weeks

B. Thoracic duct lymph collections from a rNu/+ rat aged 9-10 weeks
A 9-10w/o rNu/rNu - α/β T cells in R1

Collection 1

Collection 2

Collection 3

Collection 4

Collection 5

Collection 6
B

9-10w/o rNu/+ - α/β T cells in R1

Collection 1

Collection 2

Collection 3

Collection 4

Collection 5

Collection 6
**Figure 4.2.3** Proportions of α/β T cells among large lymphocytes in thoracic duct lymph (R2). Large lymphocytes were gated as shown in Figure 4.2.1. Lymphocytes falling within this region in the six sequential collections of thoracic duct lymph shown in Figure 4.2.1 were examined for the expression of the α/β TCR by labelling directly with phycoerythrin-conjugated mAb R73 (R73-PE). The dashed line represents the autofluorescence of lymphocytes incubated with the negative control antibody 1B5. The staining profile of cells incubated with R73-PE is superimposed. The proportion of labelled R73* cells is indicated above the marker in each panel.

A. Thoracic duct lymph collections from a rNu/rNu rat aged 9-10 weeks

B. Thoracic duct lymph collections from a rNu/+ rat aged 9-10 weeks
9-10w/o rNu/rNu - α/β T cells in R2

Collection 1

Collection 2

29.1%

32.4%

Collection 3

Collection 4

42.3%

50.2%

Collection 5

Collection 6

48.0%

49.7%
9-10w/o rNu/+ - \( \alpha/\beta \) T cells in R2

Collection 1

Collection 2

Collection 3

Collection 4

Collection 5

Collection 6
From the proportions of small and large (blast) lymphocytes determined by flow cytometry (Figure 4.2.1 [A] and [B]) and the count of total viable lymphocytes in TDL, the total numbers of small and large lymphocytes were estimated for each 12 hour collection. Furthermore, the output of various subsets could be calculated from the flow cytometric determination of the proportions of cells expressing the α/β TCR (mAb R73), CD4 (mAbs OX35/OX38) and CD8α (mAb OX8) in small and large cell gates. Previous studies have shown that during thoracic duct drainage, the number of small lymphocytes collected in the lymph declines over time (Mann and Higgins, 1950; Gowans and Knight, 1964; Westermann et al., 1994), whereas the number of large lymphocytes remains relatively constant (Mann and Higgins, 1950; Gowans and Knight, 1964). Figure 4.2.4(C) shows the decline in the numbers of small lymphocytes in euthymic rats during thoracic duct drainage. Small α/β T cells and small B cells (α/β TCR− cells) both decline, although this decline is more pronounced in recirculating α/β T cells. Figures 4.2.4 (A) and (B) depict the effect prolonged drainage of the thoracic duct on the output of small lymphocytes from two 9-10 w/o rNu/rNu rats. The essential absence of cells expressing the α/β TCR is clearly evident in both Figures. Although low, the numbers of these cells remained relatively constant, or increased slightly in some cases (Figure 4.2.4 [B]). The numbers of α/β TCR− cells in the TDL from rNu/rNu rats declined in a manner similar to that seen in rNu/+ animals (Figure 4.2.4 [A], [B] and [C]).

As expected, examination of the output of large lymphocytes in the TDL from rNu/+ rats (Figure 4.2.5 [C]) revealed little change over 72 hours and this observation extended to both the large α/β TCR+ cells and large α/β TCR− cells. Similarly, the output of large α/β TCR− cells in 9-10 w/o rNu/rNu rats remained relatively unchanged during thoracic
duct drainage (Figure 4.2.5 [A] and [B]). However, there was an increase (1.5-2.5 fold) in the output of cells expressing the α/β TCR over the time course and this was reflected in an increase in the output of total lymphocytes (Figure 4.2.5 [A] and [B]). In the two examples shown, the output of large α/β TCR<sup>+</sup> cells rose to be equivalent to, or greater than, the output of large α/β TCR<sup>-</sup> lymphocytes in the final period of collection (50-72 hours of drainage, collection 5-6). When examined further, it was concluded that essentially all of the α/β TCR<sup>+</sup> cells present in the TDL from rNu/rNu rats co-expressed the CD4 co-receptor (in excess of 95%). Of the CD8α<sup>+</sup> cells present, it was concluded that the majority expressed the γ/δ TCR (data not shown). In contrast, after a comparable period of thoracic duct drainage, the TCR<sup>+</sup> cells from rNu/+ rats consisted of both CD4<sup>+</sup> (approximately 69%) and CD8<sup>+</sup> (approximately 35%) populations (data not shown).

4.2.2 Extended phenotype of α/β T cells in rNu/rNu rats

When the total numbers of B cells were calculated by the use of total cell counts and the proportion of cells labelled with the mAbs MARK-1 and OX33, it was found that similar numbers of B cells were present in the spleens and lymph nodes of rNu/rNu rats and rNu/+ age-matched controls (data not shown). This observation applied also to the output of B cells in TDL, as illustrated in 9-10 week old rats by the output of α/β TCR<sup>-</sup> cells in the first 12 hour collections (Figures 4.2.4 and 4.2.5). Therefore, the numbers of B cells in each tissue could be used as a common reference point with which to exclude factors such as differences in organ sizes when comparing the numbers of α/β T cells in the tissues of rNu/rNu and control rats. The ratio of α/β T cells to B cells in various organs of rats at ages from 13 weeks to 52 weeks are shown in Table 4.2.1. As described in Table 4.2.1, small numbers of cells that express the α/β TCR were present
Figure 4.2.4  Numbers of small lymphocytes recovered during drainage of the thoracic duct. The total numbers of small lymphocytes collected from the thoracic duct lymph (TDL) of individual rats were determined by multiplying the total lymphocyte count for each collection by the proportion of cells classified as small (R1) by the gating criteria shown in Figure 4.2.1. The total numbers of small $\alpha/\beta$ TCR$^+$ cells (and $\alpha/\beta$ TCR$^-$ cells) were then calculated by multiplying this number by the proportions of small lymphocytes which were $\alpha/\beta$ TCR$^+$ and $\alpha/\beta$ TCR$^-$ respectively (illustrated in Figure 4.2.2). Total numbers of small cells recovered in the TDL over the six 12 hour collections (as shown in Figure 4.2.1) are plotted, together with the numbers of $\alpha/\beta$ TCR$^+$ and $\alpha/\beta$ TCR$^-$ (B) cells.

A. Total cell numbers from a rNu/rNu rat aged 9-10 weeks (shown in Figure 4.2.1)

B. Total cell numbers from a second r Nu/rNu rat aged 9-10 weeks

C. Mean total cell numbers from four rNu/rNu rat aged 9-10 weeks (+/- S.E.M.)
Small Lymphocyte Numbers during TDL Drainage - 9-10w/o rNu/rNu rat (1)

Small Lymphocyte Numbers during TDL Drainage - 9-10w/o rNu/rNu rat (2)

Small Lymphocyte Numbers during TDL Drainage - 9-10 w/o rNu/+ rats (n=4)
**Figure 4.2.5** Numbers of large lymphocytes recovered during drainage of the thoracic duct. The total numbers of large lymphocytes collected from the thoracic duct lymph (TDL) of individual rats were determined by multiplying the total lymphocyte count for each collection by the proportion of cells classified as large (R2) by the gating criteria shown in Figure 4.2.1. The total numbers of large α/β TCR<sup>+</sup> cells (and α/β TCR<sup>-</sup> cells) were then calculated by multiplying this number by the proportions of large lymphocytes which were α/β TCR<sup>+</sup> and α/β TCR<sup>-</sup> respectively (illustrated in Figure 4.2.3). Total numbers of large cells recovered in the TDL over the six 12 hour collections (as shown in Figure 4.2.1) are plotted, together with the numbers of α/β TCR<sup>+</sup> and α/β TCR<sup>-</sup> (B) cells.

A. Total cell numbers from a rNu/rNu rat aged 9-10 weeks (shown in Figure 4.2.1)

B. Total cell numbers from a second rNu/rNu rat aged 9-10 weeks

D. Mean total cell numbers from four rNu/rNu rat aged 9-10 weeks (+/- S.E.M.)
Large Lymphocyte Numbers during TDL Drainage - 9-10 w/o rNu/rNu rat (1)

Large Lymphocyte Numbers during TDL Drainage - 9-10 w/o rNu/rNu rat (2)

Large Lymphocyte Numbers during TDL Drainage - 9-10 w/o rNu/+ rats (n=4)
Table 4.2.1  Ratios of $\alpha/\beta$ T cells to B cells in lymphocyte populations from rNu/+ and rNu/rNu rats. Flow cytometric analysis was used to determine the proportion of $\alpha/\beta$ TCR$^+$ cells (stained with mAb R73) and B cells (stained with mAbs OX33 and MARK-1) in thoracic duct lymph (TDL), lymph nodes (LN) and spleens from rNu/+ and rNu/rNu rats aged 13, 26, 39 and 52 weeks of age (2-4 animals per data set).

Ratio: Percentage of $\alpha/\beta$ TCR$^+$ : Percentage of B cells (± SD)
<table>
<thead>
<tr>
<th></th>
<th>13 W/O RATS</th>
<th>26 W/O RATS</th>
<th>39 W/O RATS</th>
<th>52 W/O RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TDL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rNu/+ (+/- SD)</td>
<td>1.00 (+/- 0.11)</td>
<td>1.09 (+/- 0.16)</td>
<td>1.26 (+/- 0.21)</td>
<td>0.83 (+/- 0.23)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>0.04 (+/- 0.03)</td>
<td>0.25 (+/- 0.03)</td>
<td>0.50 (+/- 0.18)</td>
<td>0.42 (+/- 0.11)</td>
</tr>
<tr>
<td><strong>LN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rNu/+ (+/- SD)</td>
<td>1.44 (+/- 0.32)</td>
<td>1.26 (+/- 0.12)</td>
<td>1.20 (+/- 0.11)</td>
<td>1.13 (+/- 0.15)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>0.08 (+/- 0.05)</td>
<td>0.29 (+/- 0.06)</td>
<td>0.36 (+/- 0.02)</td>
<td>0.52 (+/- 0.17)</td>
</tr>
<tr>
<td><strong>SPLEEN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rNu/+ (+/- SD)</td>
<td>0.61 (+/- 0.15)</td>
<td>0.45 (+/- 0.16)</td>
<td>0.44 (+/- 0.05)</td>
<td>0.57 (+/- 0.04)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>0.07 (+/- 0.04)</td>
<td>0.06 (+/- 0.01)</td>
<td>0.17 (+/- 0.01)</td>
<td>0.19 (+/- 0.04)</td>
</tr>
</tbody>
</table>
in the TDL from rNu/rNu rats aged approximately 10 weeks. At this age, α/β TCR⁺ cells were virtually undetectable in the lymph nodes or spleen but by 13 weeks, they constituted approximately 2-5% of the lymphocytes (approximately 1 α/β T cell per 20 B cells) in these organs (Table 4.2.1). It was concluded that at 13 weeks of age, enough cells expressing the α/β TCR were present in the TDL and lymphoid tissues of rNu/rNu rats to commence a Multi-parameter flow cytometric analysis of a range of surface antigens expressed by α/β T cells. Rats aged 13, 26, 39 and 52 weeks were examined.

The ratios of α/β T cells to B cells were similar in the TDL, lymph nodes and spleens of rNu/rNu rats at age 13 weeks (Table 4.2.1) and they were also evident in the liver (see section 4.2.7). There was a progressive rise in the proportion of α/β T cells in the TDL and lymph nodes in rNu/rNu rats, reaching approximately 30% of the total lymphocytes (1:2 ratio of α/β T cells to B cells) in animals aged between 39 and 52 weeks (Table 4.2.1). A rise in the proportion of α/β T cells in the spleen occurred later and even at 52 weeks of age, α/β T cells rarely exceeded 10-15% of total lymphocytes. In rNu/+ rats, although the ratios of α/β T cells to B cells in TDL, lymph nodes (LN) and spleen were different (LN>TDL>spleen), the proportions of α/β T cells in each remained relatively unchanged with age (Table 4.2.1).

Rat intraepithelial lymphocytes (IEL) expressing the α/β TCR can be distinguished from peripheral α/β T cells because most are CD2⁻ and a large proportion are either CD5⁻ or CD5lo (Helgeland et al., 1996; Fangmann et al., 1991). Dual fluochrome flow cytometry revealed that the α/β T cells in TDL, lymph nodes and spleens from rNu/+ and rNu/rNu rats of all ages were largely CD5⁺ (mAb OX19) and CD2⁺ (mAb OX34).
Representative findings in the TDL from 26 week old rNu/rNu and rNu/+ rats are shown in Figure 4.2.6. CD5- α/β T cells were essentially undetectable in the TDL from rNu/+ rats and comprised only a small proportion (<5% of α/β TCR+ cells) of α/β T cells in rNu/rNu rats (Figure 4.2.6 [A] and [B]). Similar findings were made in preparations of lymph node and spleen cells. Only a small population of CD2- α/β TCR+ cells (approximately 1% of α/β TCR+ cells) was detected in rNu/+ rats (Figure 4.2.6 [C]). In rNu/rNu rats, this population accounted for less than 7% of α/β T cells (Figure 4.2.6 [D]). Similar observations were made on lymph node and spleen cell preparations. The α/β TCR- CD2+ and α/β TCR- CD5+ populations which are evident in Figure 4.2.6 are probably γ/δ T cells.

γ/δ T cells were detected in the TDL (Figure 4.2.7) and secondary lymphoid organs (data not shown) of both rNu/rNu and rNu/+ rats by the use of mAb V65 (anti-γ/δ TCR). The proportions of γ/δ T cells rats did not greatly vary at any of these sites in rNu/rNu rats aged 13-52 weeks, where they accounted for 1-2% of total lymphocytes (illustrated in Figure 4.2.7 [B] and [D]). In rNu/+ rats, γ/δ T cells in TDL and lymphoid tissues constituted approximately 0.5-1.0% of total lymphocytes (Figure 4.2.7 [A] and [C]). In both rNu/rNu and control rats, most of the γ/δ T cells expressed CD8α but there was also a variable proportion of CD8- cells that are probably part of a double negative (DN) population.

Sarawar et al. (1991) had examined the ratio of CD4+ to CD8+ cells among α/β TCR+ lymphocytes in the lymph nodes of aged athymic rats and they concluded that the ratio of CD4+ to CD8+ cells was similar to that seen in heterozygous littermates. They did not
Figure 4.2.6   Expression of CD5 and CD2 by α/β T cells in thoracic duct lymph. Thoracic duct lymphocytes from (A and C) a 26 week old rNu/+ rat and (B and D) a 26 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with either OX19 (anti-CD5; FITC) or OX34 (anti-CD2; FITC), followed by direct labelling with R73 (anti-α/β TCR; PE). The percentage of events in each quadrant is indicated.
Figure 4.2.7 Detection of CD8α⁺ γδ T cells in thoracic duct lymph.

Thoracic duct lymphocytes from (A) a 13 week old rNu/+ rat, (B) a 13 week old rNu/rNu rat, (C) a 52 week old rNu/+ rat and (D) a 52 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with diluted ascites containing mAb V65 (anti-γδ TCR; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). The percentage of events in each quadrant is indicated.
report significant numbers of CD4+CD8−, DN α/β T cells. In the study discussed herein, most (>70%) α/β TCR+ cells in rNu/rNu rats and age-matched littermates (13-52 weeks of age) were found to express the CD4 co-receptor in all tissues studied. However, the ratio of α/β T cells expressing CD4 and CD8 varied depending on the tissue analysed (Table 4.2.2). The ratio of CD4+ α/β T cells to CD8+ α/β T cells for both rNu/+ and rNu/rNu rats was similar in cells prepared from lymph nodes and spleens for all ages studied. However, with increasing age, the ratio of CD4+ α/β T cells to CD8+ α/β T cells in TDL of rNu/rNu rats increased. This was especially evident in rats aged 39 and 52 weeks of age (Table 4.2.2).

Expression of the CD8αα homodimer and the CD8αβ heterodimer among CD8+ cells in rNu/rNu and age-matched rNu/+ rats was examined using mAbs against CD8α (OX8) and CD8β (341). T cells were defined as cells that expressed high levels of CD8α, allowing exclusion of the CD8αdim population, which included NK cells (see Figure 4.2.9). T cells expressing only CD8α were present in rNu/rNu rats but essentially all CD8α+ T cells in rNu/+ rats expressed CD8β (Figure 4.2.8). This was observed in both young (13 weeks) aged rats (52 weeks) (Figure 4.2.8). Dual fluochrome flow cytometry, utilising the labelling of CD8α in combination with anti CD8β (Figure 4.2.9 [A]), anti-CD161 (to define the population of CD161bright CD8αdim NK cells, Figure 4.2.9 [B]), anti-γ/δ TCR (Figure 4.2.9 [C]) or anti-α/β TCR (Figure 4.2.9 [D]) were used to estimate the proportions of α/β TCR+ and γ/δ TCR+ T cells which expressed the CD8α+β+ and CD8α−β− phenotypes of the CD8 co-receptor. T lymphocyte populations which expressed CD8α chains in the absence of CD8β chains (Figures 4.2.8 [B], [D] and 4.2.9 [A]) expressed higher mean levels of CD8α when compared directly to T cells
Table 4.2.2 Ratios of CD4⁺ α/β T cells to CD8α⁺ α/β T cells in lymphocyte populations from rNu/+ and rNu/rNu rats. Dual fluochrome flow cytometric analysis was used to determine the proportions of CD4⁺ α/β T cells (stained indirectly with mAb W3/25 [anti-CD4; FITC] followed by direct labelling with mAb R73 [anti-α/β TCR; PE]) and CD8⁺ α/β T cells (stained with indirectly with mAb OX8 [anti-CD8α; FITC] followed by direct labelling with mAb R73 [anti-α/β TCR; PE]) in thoracic duct lymph (TDL), lymph nodes (LN) and spleens from rNu/+ and rNu/rNu rats aged 13, 26, 39 and 52 weeks of age (2-4 animals per data set).

Ratio: Percentage of α/β TCR⁺ / Percentage of B cells (± SD)
<table>
<thead>
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<tr>
<td><strong>TDL</strong></td>
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<td></td>
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<tr>
<td>rNu/+ (+/- SD)</td>
<td>4.38 (+/- 0.49)</td>
<td>5.60 (+/- 0.49)</td>
<td>5.88 (+/- 0.18)</td>
<td>5.72 (+/- 0.45)</td>
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<td>rNu/rNu (+/- SD)</td>
<td>3.37 (+/- 2.12)</td>
<td>6.85 (+/- 2.88)</td>
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<td>20.18 (+/- 0.15)</td>
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</tr>
<tr>
<td>rNu/+ (+/- SD)</td>
<td>5.25 (+/- 0.80)</td>
<td>5.44 (+/- 0.63)</td>
<td>6.42 (+/- 1.39)</td>
<td>6.59 (+/- 0.08)</td>
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<td>rNu/rNu (+/- SD)</td>
<td>4.60 (+/- 1.00)</td>
<td>4.10 (+/- 1.06)</td>
<td>2.97 (+/- 0.37)</td>
<td>7.15 (+/- 2.47)</td>
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</tr>
<tr>
<td>rNu/+ (+/- SD)</td>
<td>3.70 (+/- 0.59)</td>
<td>2.93 (+/- 0.06)</td>
<td>2.56 (+/- 0.06)</td>
<td>4.62 (+/- 1.23)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>3.26 (+/- 0.32)</td>
<td>4.73 (+/- 1.03)</td>
<td>2.76 (+/- 0.49)</td>
<td>5.54 (+/- 2.35)</td>
</tr>
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</table>
Figure 4.2.8  Detection of CD8α⁺ CD8β⁻ T cells in thoracic duct lymph. Thoracic duct lymphocytes from (A) a 13 week old rNu/+ rat, (B) a 13 week old rNu/rNu rat, (C) a 52 week old rNu/+ rat and (D) a 52 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with mAb 341 (anti-CD8β; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). The percentage of events in each quadrant is indicated. In addition, the CD8αᵇright CD8β⁻ cells have been gated separately and the percentage of these cells in the preparation indicated.
Figure 4.2.9  Characterisation of CD8α^{bright} CD8β^{-} cells in thoracic duct lymph. Thoracic duct lymphocytes from a 26 week old rNu/rNu rat were stained with a panel of mAbs in association with anti-CD8α to characterise the CD8α^{bright} CD8β^{-} population. Lymphocytes were labelled indirectly with one of the following mAbs: (A) 341 (anti-CD8β; FITC), (B) 10/78 (anti-CD161; FITC), (C) V65 (anti-γ/δ TCR; FITC) or (D) R73 (anti-α/β TCR) before direct labelling with mAb OX8 (anti-CD8α; PE). Region 1 in (A) highlights the CD8α^{bright} CD8β^{-} cells (note that the level of CD8α expression by these cells is higher than that expressed by many CD8α^{+}β^{+} cells). Region 2 in (B) isolates the CD8α^{bright} population, some of which express lower levels of CD161 than the NK cells gated in region 4. The CD161^{bright} NK cells (region 4), along with a population of CD161^{-} cells (region 3) express lower levels of CD8α than the CD8α^{+} γ/δ TCR^{+} (C) and CD8α^{+} α/β TCR^{+} (D) T cells. Regions 6 and 7 indicate that the γ/δ TCR^{+} population, as a whole, expresses higher levels of CD8α than the CD8α^{+} α/β TCR^{+} (regions 5 and 8) population in this example.
Figure 4.2.10 Detection of $\alpha/\beta$ TCR$^+$ CD4$^-$ CD8$^-$ (DN) T cells.

Lymphocytes from (A) thoracic duct lymph (TDL) from a 52 week old rNu/+ rat, (B) TDL from a 52 week old rNu/rNu rat, (C) lymph nodes (LN) from a 52 week old rNu/+ rat and (D) LN from a 52 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with mAb R73 (anti-$\alpha/\beta$ TCR; FITC), followed by direct labelling with mAbs OX8 and OX38 in combination (anti-CD8$\alpha$ + anti-CD4; PE). The percentage of events in each quadrant is indicated.
Table 4.2.3: Proportions of α/β T cells with the CD4⁻ CD8α⁻ (DN) phenotype among lymphocytes isolated from rNu/rNu and rNu/+ rats. Dual fluochrome flow cytometric analysis was used to determine the proportions of DN T cells in thoracic duct lymph (TDL), lymph nodes (LN) and spleens from rNu/+ and rNu/rNu rats aged 13, 26, 39 and 52 weeks of age (2-4 animals per data set). Cells were stained indirectly with mAb R73 (anti-α/β TCR; FITC) followed by direct labelling with mAbs OX8 and OX38 (CD8α + CD4; PE).

Proportion: Percentage of DN α/β TCR⁺ + Percentage of α/β TCR⁺ cells (± SD)
<table>
<thead>
<tr>
<th></th>
<th>13 W/O RATS</th>
<th>26 W/O RATS</th>
<th>39 W/O RATS</th>
<th>52 W/O RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TDL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rNu/+ (+/ SD)</td>
<td>0.63 (+/- 0.15)</td>
<td>0.28 (+/- 0.36)</td>
<td>0.33 (+/- 3.09)</td>
<td>0.77 (+/- 0.60)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>9.06 (+/- 4.73)</td>
<td>8.98 (+/- 2.38)</td>
<td>5.94 (+/- 3.92)</td>
<td>3.99 (+/- 0.71)</td>
</tr>
<tr>
<td><strong>LN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rNu/+ (+/ SD)</td>
<td>0.33 (+/- 0.28)</td>
<td>0.52 (+/- 0.59)</td>
<td>N.D.</td>
<td>0.44 (+/- 0.18)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>13.35 (+/- 1.48)</td>
<td>10.40 (+/- 4.43)</td>
<td>N.D.</td>
<td>4.89 (+/- 1.17)</td>
</tr>
<tr>
<td><strong>SPLEEN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rNu/+ (+/ SD)</td>
<td>0.53 (+/- 0.13)</td>
<td>1.99 (+/- 0.04)</td>
<td>N.D.</td>
<td>0.98 (+/- 0.42)</td>
</tr>
<tr>
<td>rNu/rNu (+/- SD)</td>
<td>9.64 (+/- 1.36)</td>
<td>17.38 (+/- 8.06)</td>
<td>N.D.</td>
<td>6.90 (+/- 3.34)</td>
</tr>
</tbody>
</table>

Proportion of α/β TCR⁺ cells with the DN phenotype
expressing CD8α chains in association with CD8β chains. In most cases (illustrated in Figure 4.2.9 [C] and [D]) 50% or greater of the T cells which expressed the CD8α<sup>hi</sup>β<sup>+</sup> phenotype expressed the γ/δ TCR. These results extend a previous observation regarding CD8<sup>+</sup> T cells isolated from the spleens and lymph nodes of athymic rats (Torres-Nagel et al., 1992). It appears, therefore, that CD8<sup>+</sup> T cells in rNu/rNu rats are composed of α/β T cells expressing CD8α/β, a variable population of α/β T cells that presumably expresses CD8α/α (β<sup>+</sup>) and a population of γ/δ T cells, many of which express CD8αα.

It had been reported previously that DN T cells were not a significant population of the α/β T cells in athymic rats (Sarawar et al., 1991). As shown in Figure 4.2.10 (B) and (D), DN cells comprise a distinct population of α/β TCR<sup>+</sup> cells in CBH rNu/rNu rats but they are barely detectable in rNu/+ rats (Figure 4.2.10 [A] and [C]). DN α/β T cells were present at all ages examined, and in all tissues studied (Table 4.2.3). They comprised 5-15% of α/β T cells in rNu/rNu rats and typically less than 1% of α/β T cells in rNu/+ rats (Table 4.2.3). Interestingly, DN T cells make up a similar proportion (up to 16%) of the total α/β T population in athymic mice (Lawetzky and Hünig, 1988).

**4.2.3 α/β T cells in rNu/rNu rats are larger, express reduced levels of surface TCR but most exhibit 2N levels of DNA**

**4.2.3.1 Cell size**

As shown in Figure 4.2.11 [B] and [C], α/β T cells in TDL from a 26 week old rNu/rNu rat are larger than the corresponding cells in TDL from an age-matched rNu/+ rat (indicated by greater forward light scatter [FSC]) and also display greater cytoplasmic
complexity (indicated by greater side scatter of light [SSC]). This was a consistent finding in rNu/rNu rats at all ages (9 weeks to 52 weeks). However, the difference in size of α/β T cells was greatest in young animals. Examination of TDL from rats aged 9-10 weeks (Figures 4.2.1 [A], 4.2.2 [A] and 4.2.3 [A]) revealed that essentially all of the α/β T cells were contained within the large cell gate. Thus the differences in inferred cell size and cytoplasmic complexity among α/β T cells were greater in the youngest rats examined. It is interesting to note that with advancing age, increased numbers of α/β T cells in TDL from rNu/rNu rats appeared in the small lymphocyte gate. Figure 4.2.12 compares the flow cytometric dot-plots (FSC Vs SSC) obtained from analysis of thoracic duct lymphocytes from rNu/rNu and rNu/+ rats. In this analysis, gates were drawn arbitrarily to include the populations of small and large lymphocytes, based on the FSC Vs SSC plots of lymphocytes in TDL from rNu/+ rats (Figure 4.2.12 [E]). The majority (>95%) of the α/β T cells in TDL from the 26 week old rNu/+ rat fall in the small cell gate when they are back-gated to the plot of FSC Vs SSC. However, when cells in TDL from 13 week old rNu/rNu rats are examined (Figure 4.2.12), back-gating shows that the majority of α/β T cells are large or intermediate in size (Figure 4.2.12 [B]), despite the fact that the majority (86%) of the lymphocytes are small in size (Figure 4.2.12 [A]). At 26 weeks of age, the majority (approximately 70%) of the α/β T cells in TDL from rNu/rNu rats lie in the small cell gate (Figure 4.2.12 [D]), although most appear larger than the α/β T cells in TDL from rNu/+ rats (Figure 4.2.12 [F]).

Similar results were obtained for lymph node and spleen cell preparations from rNu/rNu rats at all ages. However, at 13 weeks, the distinction between small and large cell populations was greater (data not shown).
Figure 4.2.11  Size of α/β T cells in thoracic duct lymph from rNu/rNu rats and surface expression of the TCR.  (A) Lymphocytes from the thoracic duct lymph of 26 week old rats were stained by the indirect immunofluorescence technique with mAb R73 and analysed by flow cytometry. The fluorescence profiles of cells from a rNu/+ rat (teal line) and a rNu/rNu rat (purple line) are superimposed and the mean fluorescence intensity for each α/β TCR⁺ population is shown. (B) Forward light scatter (FSC) of those cells gated as ‘TCR positive’ in ‘A’. The FSC profiles of the gated cells from a rNu/+ rat (teal line) and rNu/rNu rat (purple line) are superimposed and the mean FSC of each peak is indicated.
Figure 4.2.12  Sizes of α/β T cells in the thoracic duct lymph (TDL) of rNu/rNu and rNu/+ rats. Forward light scatter (FSC, proportional to size) and side scatter (SSC, an indicator of cellular complexity) were measured by flow cytometry on thoracic duct lymphocytes. All larger cells were included in the large cell gate. The FSC Vs SSC dotplots are displayed for (A) a 13 week old rNu/rNu rat, (C) a 26 week old and (E) a 26 week old rNu/+ rat. The gate including small cells was placed to include the majority of lymphocytes in the TDL from the rNu/+ rat. The distribution of directly-labelled α/β TCR⁺ TDL cells within these gates is shown for cells from (B) a 13 week old rNu/rNu rat, (D) a 26 week old rNu/rNu rat and (F) a 26 week old rNu/+ rat. The proportion of the total cells included in each gate is indicated as a percentage.
4.2.3.2 Surface TCR

The levels of TCR expressed on the surfaces of α/β T cells was compared between lymphocytes isolated from the TDL and the lymphoid tissues of age-matched rNu/rNu and rNu/+ rats. The mean fluorescence intensity (MFI) of the α/β TCR⁺ populations was measured on thoracic duct lymphocytes from 26 w/o rats after labelling with mAb R73. The MFI of α/β TCR⁺ cells from rNu/rNu rats was 85.0, a value that was approximately 65% of that observed for the corresponding population from rNu/+ rats (MFI=131.6) (Figure 4.2.11 [A]), despite the larger mean size of these cells. This was a consistent finding in animals of all ages (data not shown). Examination of surface TCR expression by α/β TCR⁺ cells in the secondary lymphoid organs and the liver also followed a similar pattern (data not shown).

4.2.3.3 DNA content

Because T cells in rNu/rNu rats have relatively large mean size, DNA content was measured to determine the proportions of cells that were in cell cycle. Lymphocytes were isolated from the TDL, lymph nodes, spleens and livers of rats from each of the age groups. Lymphocytes were labelled with mAbs R73 and W3/25 to identify α/β TCR⁺ cells and CD4⁺ cells respectively, made permeable by fixation with 70% ethanol, and the DNA stained with propidium iodide. As depicted in Figure 4.2.13, binding of propidium iodide indicated that the proportions of α/β T cells containing 2N and 4N multiples of the haploid DNA content were not grossly different in the TDL from 26 week old rNu/rNu and rNu/+. Similar observations were made on thoracic duct lymphocytes from rats of all ages and also in cells isolated from lymph nodes, spleens and livers. It was often noted, however, that the proportion of α/β T cells from rNu/rNu
Figure 4.2.13  DNA content of α/β T cells in thoracic duct lymph from rNu/rNu and rNu/+ rats, measured by binding of propidium iodide. Thoracic duct lymphocytes from 26 week old rats were labelled indirectly with mAb R73 (FITC), fixed in 70% ethanol and stained with propidium iodide (PI). The fluorescence from PI (proportional to DNA content) was measured on 10,000 gated α/β TCR⁺ cells from (A) a rNu/+ rat and (B) a rNu/rNu rat and recorded on a linear scale. The proportion of cells within each of the populations indicated by the horizontal bars is indicated as a percentage of all α/β T cells.
A

![Histogram with different DNA content proportions]

Counts

Propidium Iodide

B

![Histogram with different DNA content proportions]

Counts

Propidium Iodide
rats that were in S-phase was greater than in the corresponding cells from rNu/+ rats (Figure 4.2.13. rNu/rNu, 2.1%; rNu/+, 0.6%). Therefore, a greater proportion of α/β TCR⁺ cells from rNu/rNu rats have DNA contents greater than 2N. However, it is unlikely that the larger mean size of α/β TCR⁺ T cells from rNu/rNu rats is explained by this small difference in the proportion of cycling cells (Figure 4.2.13. rNu/rNu, 4.8%; rNu/+ 3.4%). Of interest was a small population (1.5%) of α/β T cells from rNu/rNu rats that exhibited a DNA content less than 2N (Figure 4.2.13 [B]). These cells, which were not present in the TDL from rNu/+ rats (Figure 4.2.13 [A]), may be apoptotic.

4.2.4 Comparison of activation marker expression by α/β TCR⁺ cells in rNu/rNu and rNu/+ rats

4.2.4.1 Activation markers

To address the possibility that the greater size of α/β TCR⁺ T cells in rNu/rNu rats could be due to either immaturity (recent release from site of production) or to activation and/or recent cell division, dual-fluorochrome flow cytometry was undertaken using a panel of monoclonal antibodies that detect surface antigens whose expression on T cells is associated with either the naive phenotype or with activation. Gating on the total lymphocyte population, it was found that the majority of α/β T cells in the TDL from both rNu/+ and rNu/rNu rats did not express either CD25 (IL-2 receptor α chain) or CD134 (MRC OX40-antigen), indicating that most of these cells have not been activated recently. However, the percentage of α/β T cells from rNu/rNu rats that expressed these molecules was typically in the range of 20-40% for all ages studied (illustrated in Figure 4.2.14 [B] and [D] for cells from a 26 week old rat), compared with 10% for thoracic duct T cells from age-matched rNu/+ rats (Figure 4.2.14 [A] and [C]). The CD71 molecule (transferrin receptor), expressed by cycling cells, was detected on
approximately 10-20% of α/β T cells in the TDL from rNu/rNu rats and on 5-10% of α/β T cells in the TDL from rNu/+ rats (Figure 4.2.14 [E] and [F]). The observation that few α/β T cells from either rNu/rNu and rNu/+ rats express CD71 supports the studies on DNA content (Figure 4.2.13) and the conclusion that most of these cells in rNu/rNu rats are not in cell cycle.

Although α/β T cells were essentially all either large or intermediate sized cells in 13 week old (Figure 4.2.12 [B]) and 26 week old (Figure 4.2.12 [D]) rNu/rNu rats, most of the α/β T cells in the TDL from rNu/+ rats aged 26 weeks were smaller in size (Figure 4.2.12 [F]). However, a population of large cells, similar to that seen in rNu/rNu rats, is evident. These large α/β T cells in the TDL from rNu/+ rats contained a greater proportion cells expressing activation markers (Figures 4.2.14 [A, C, E] versus Figure 4.2.15 [A, C, E]) than was observed among the small α/β T cells. Up to 50% of the α/β T cells in the large cell gate were CD71+ (44% in Figure 4.2.15 [E]) and up to 40% expressed CD25 or CD134 (26% and 40% respectively in Figures 4.2.15 [A] and [C]). While, the proportions of large α/β T cells (Figure 4.2.15 [B], [D] and [F]) from the TDL of rNu/rNu rats that expressed these activation markers was similar to that observed in the large cells from rNu/+ rats, similar proportions of positive cells were seen in TDL from rNu/rNu rats irrespective of whether analysis was performed on the whole α/β T cell population (Figure 4.2.14 [B], [D] and [F]) or only those cells which fell within the large cell gate.

CD4+ T cells in rats have been shown to express CD8α chains upon activation (Torres-Nagel et al., 1992; Ramirez et al., 1992). Dual-fluorochrome flow cytometry was employed to examine CD8α expression by CD4+ populations in rNu/+ and rNu/rNu rats.
Figure 4.2.14 Activation markers expressed by $\alpha/\beta$ T cells in thoracic duct lymph. Thoracic duct lymphocytes from (A, C and E) a 26 week old rNu/+ rat and (B, D and F) a 26 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with either mAb OX39 (anti-CD25; FITC), mAb OX40 (anti-CD134; FITC) or mAb OX26 (anti-CD71; FITC), followed by direct labelling with mAb R73 (anti-$\alpha/\beta$ TCR; PE). The proportion of gated events in each quadrant is presented as a percentage.
Figure 4.2.15  Activation markers expressed by large αβ T cells in thoracic duct lymph. Thoracic duct lymphocytes falling in the large cell gate from (A, C and E) a 26 week old rNu/+ rat and (B, D and F) a 26 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with either mAb OX39 (anti-CD25; FITC), mAb OX40 (anti-CD134; FITC) or mAb OX26 (anti-CD71; FITC), followed by direct labelling with mAb R73 (anti-αβ TCR; PE). The proportion of gated events in each quadrant is presented as a percentage. Data was obtained from the analysis presented in Figure 4.2.14.
Only a small proportion (approximately 5%) of CD4+ cells from either rNu/rNu and rNu/+ rats were shown to express CD8α chains (data not shown). These observations support the conclusion that most of the α/β T cells have not been activated recently.

Analysis of the α/β T cells from lymph nodes and spleens revealed a similar pattern. Approximately 10% of the total α/β T cell populations from these tissues of rNu/+ rats expressed the activation markers CD25, CD134 and CD71, whereas 20-30% of the corresponding populations in rNu/rNu rats expressed these markers (data not shown).

4.2.4.2 Expression of MHC class II molecules

Activated T cells in rats have been shown to express MHC class II molecules, both in vitro and in vivo (Seddon et al., 1996; Broeren et al., 1995; Reizis et al., 1994). Most of the α/β T cells in the TDL from rNu/rNu rats expressed this molecule (Figure 4.2.16 [B], [D] and [F]). However, with increasing age the proportion of MHC class II-positive T cells declined (Figure 4.2.17 [A]). At 13 weeks of age, up to 90% of the α/β T cells in the TDL expressed MHC class II (Figure 4.2.16 [B] and Figure 4.2.17 [A]). By 26 weeks, less than 70% of the α/β T cells in TDL expressed MHC class II molecules (Figure 4.2.16 [D] and Figure 4.2.17 [A]) and by the age of 52 weeks, only 40-60% were positive (Figure 4.2.17 [F] and Figure 4.2.18 [A]). In contrast, most α/β T cells in TDL from rNu/+ rats did not express MHC class II molecules (Figure 4.2.16 [A], [C] and [E]) and the proportion of MHC class II-positive cells (5-10% of total α/β T cells) did not change with age (Figure 4.2.17 [A]). A similar pattern also emerged upon analysis of the CD4+ cell populations (Figure 4.2.18 [A] and [B]).
Figure 4.2.16  Expression of MHC class II molecules by α/β T cells in thoracic duct lymph from rNu/rNu and rNu/+ rats. Thoracic duct lymphocytes from (A) a 13 week old rNu/+ rat, (B) a 13 week old rNu/rNu rat, (C) a 26 week old rNu/+ rat, (D) a 26 week old rNu/rNu rat, (E) a 52 week old rNu/+ rat and (F) a 52 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with mAb OX6 (anti-MHC class II; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). The proportion of gated events in each quadrant is presented as a percentage.
Figure 4.2.17  Expression of MHC class II molecules by α/β TCR⁺ cells in thoracic duct lymph and lymph nodes from rNu/rNu and rNu/+ rats as a function of age. The proportion of α/β T cells expressing MHC class II in thoracic duct lymph from rNu/+ and rNu/rNu rats was determined by flow cytometric analysis (Figure 4.2.16) and calculated as a percentage of total α/β T cells. A similar study was done on lymphocytes prepared from pooled lymph nodes from animals of the same ages.

A. MHC class II expression by α/β T cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.

B. MHC class II expression by α/β T cells from lymph nodes (LN) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.
A

MHC class II expression by α/β T cells (TDL)

Percentage of α/β T cells

Age of rats

B

MHC class II expression by α/β T cells (LN)

Percentage of α/β T cells

Age of rats
Figure 4.2.18  Expression of MHC class II molecules by CD4+ lymphocytes in thoracic duct lymph and lymph nodes from rNu/rNu and rNu/+ rats as a function of age. The proportion of α/β T cells in the thoracic duct lymph expressing MHC class II from rNu/+ and rNu/rNu rats was determined by flow cytometric analysis and calculated as a percentage of total α/β T cells. A similar study was done on lymphocytes prepared from pooled lymph nodes from animals of the same ages.

A. MHC class II expression by CD4+ cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.

B. MHC class II expression by CD4+ cells from lymph nodes (LN) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.
A

MHC class II expression by CD4* cells (TDL)

<table>
<thead>
<tr>
<th>Percentage of αβ T cells</th>
<th>13 w/o</th>
<th>26 w/o</th>
<th>39 w/o</th>
<th>52 w/o</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNu/rNu</td>
<td>90</td>
<td>90</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>rNu/+</td>
<td>80</td>
<td>80</td>
<td>75</td>
<td>65</td>
</tr>
</tbody>
</table>

Age of rats

B

MHC class II expression by CD4* cells (LN)

<table>
<thead>
<tr>
<th>Percentage of αβ T cells</th>
<th>13 w/o</th>
<th>26 w/o</th>
<th>39 w/o</th>
<th>52 w/o</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNu/rNu</td>
<td>90</td>
<td>90</td>
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<td>60</td>
</tr>
<tr>
<td>rNu/+</td>
<td>80</td>
<td>80</td>
<td>75</td>
<td>65</td>
</tr>
</tbody>
</table>

Age of rats
Figure 4.2.19  Expression of MHC class II molecules by large α/β T cells in the thoracic duct lymph from rNu/rNu and rNu/+ rats. Thoracic duct lymphocytes falling within the large cell gate from (A) a 26 week old rNu/+ rat and (B) a 26 week old rNu/rNu rat, were labelled first by the indirect immunofluorescence technique with mAb OX6 (anti-MHC class II; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). The proportion of gated events in each quadrant is presented as a percentage.
Expression of MHC class II molecules by α/β T cells from the TDL of rNu/+ rats was restricted largely to the large (lymphoblast) cell population. Approximately 40-60% of the large α/β T cells in TDL from rNu/+ rats were shown to express MHC class II molecules (Figure 4.2.19 [A]). In contrast, only approximately 1-5% of α/β T cells in the small cell gate expressed MHC class II molecules (data not shown). Analysis of the α/β T cells from the TDL of 26 week old rNu/rNu rats revealed that 80-90% expressed MHC class II molecules (Figure 4.2.19 [B]). In contrast to other activation markers, expression of MHC class II molecules was greater, therefore, by large α/β T cells from rNu/rNu rats than by the whole α/β TCR+ population (Figure 4.2.19 [B] versus Figure 4.2.16 D). MHC class II expression was also examined among α/β T cells from the spleens and lymph nodes of age-matched animals. As shown in Figure 4.2.17 (B), the proportion of α/β T cells that express MHC class II molecules declined with age in the lymph nodes of rNu/rNu rats, albeit at a slower rate than was observed in the TDL (Figure 4.2.17 [A]).

4.2.4.3 Expression of CD45 isoforms

It has been shown previously that the majority of α/β T cells in the lymph nodes of rNu/rNu rats do not express the CD45RC+ isoform of CD45 (Sarawar et al., 1991). In this study, expression of CD45RC by α/β T cells was assessed in the TDL, as well as in the spleens and lymph nodes of rNu/rNu rats and age-matched rNu/+ rats. The results of dual-fluochrome analysis, utilising mAb OX22 (anti-CD45RC) in combination with mAb R73, are presented in figure 4.2.20. Fewer than 20% of the α/β T cells in the TDL from 13 week old rNu/rNu rats expressed CD45RC, compared with approximately 70% of the α/β T cells from rNu/+ TDL (Figure 4.2.20 [A]). Interestingly, with advancing
Figure 4.2.20  Expression of CD45RC by \( \alpha/\beta \) TCR\(^{+} \) cells from rNu/rNu and rNu/+ rats as a function of age. The proportion of \( \alpha/\beta \) T cells from rNu/+ and rNu/rNu rats expressing CD45RC was determined by dual fluochrome flow cytometric analysis and calculated as a percentage of total \( \alpha/\beta \) T cells.

A.  CD45RC expression by \( \alpha/\beta \) T cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.

B.  CD45RC expression by \( \alpha/\beta \) T cells from lymph nodes (LN) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.
A

CD45RC expression by α/β T cells (TDL)

- rNu/rNu
- rNu/+ 

Percentage of α/β T cells

Age of Rats

13 w/o 26 w/o 39 w/o 52 w/o

B

CD45RC expression by α/β T cells (LN)

- rNu/rNu
- rNu/+ 

Percentage of α/β T cells

Age of rats

13 w/o 26 w/o 39 w/o 52 w/o
Figure 4.2.21  Expression of CD45RC by CD4\(^+\) lymphocytes from rNu/rNu and rNu/+ rats as a function of age. The proportion of CD4\(^+\) T cells from rNu/+ and rNu/rNu rats expressing CD45RC was determined by dual fluochrome flow cytometric analysis and calculated as a percentage of total CD4\(^+\) T cells.

A.  CD45RC expression by CD4\(^+\) cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.

B.  CD45RC expression by CD4\(^+\) cells from lymph nodes (LN) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.
A

CD45RC expression by CD4\(^{+}\) cells

(TDL)

- rNu/rNu
- rNu/+ (rDL)

<table>
<thead>
<tr>
<th>Age of Rats</th>
<th>Percentage of α/β T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 w/o</td>
<td>52</td>
</tr>
<tr>
<td>26 w/o</td>
<td>39</td>
</tr>
<tr>
<td>39 w/o</td>
<td>26</td>
</tr>
<tr>
<td>52 w/o</td>
<td>13</td>
</tr>
</tbody>
</table>

B

CD45RC expression by CD4\(^{+}\) cells

(LN)

- rNu/rNu
- rNu/+ (lrNu/rNu/ IrNu/+)

<table>
<thead>
<tr>
<th>Age of Rats</th>
<th>Percentage of α/β T cells</th>
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<tbody>
<tr>
<td>13 w/o</td>
<td>52</td>
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<tr>
<td>26 w/o</td>
<td>39</td>
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<tr>
<td>39 w/o</td>
<td>26</td>
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<td>52 w/o</td>
<td>13</td>
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age there was an overall trend towards an increasing proportion of the $\alpha/\beta$ T cells from the TDL of rNu/rNu rats that expressed CD45RC (Figure 4.2.20 [A]) but this was not significant statistically. However, the proportion of $\alpha/\beta$ T cells expressing CD45RC did not exceed 30%, even at 52 weeks of age. Contrary to expectations, with increasing age there was an increase in the proportion of $\alpha/\beta$ T cells in TDL from rNu/+ rats that expressed CD45RC (Figure 4.2.20 [A]).

Examination of CD45RC expression by $\alpha/\beta$ T cells from the lymph nodes of rNu/rNu rats revealed a similar trend to that observed for thoracic duct lymphocytes (Figure 4.2.20 [B]). However, in rNu/+ rats, the proportion of $\alpha/\beta$ T cells that expressed CD45RC remained relatively constant up to 39 weeks of age, before declining at 52 weeks of age (Figure 4.2.20 [B]). Similar results were obtained when expression of CD45RC was examined in the CD4$^+$ populations of lymphocytes (Figure 4.2.21 [A] and [B]).

4.2.5 Expression of adhesion molecules by $\alpha/\beta$ T cells in rNu/rNu and rNu/+ rats

Naive T cells express high levels of CD62L (L-selectin) (reviewed by Sprent, 1997; Bradley et al., 1993) and a high molecular weight isoform of CD45 (CD45RC in the rat [Spickett et al., 1983]). Surface adhesion molecules such as CD54 (ICAM-1), CD11a (LFA-1) and CD49d ($\alpha$-chain of VLA-4 and $\alpha4\beta7$) are expressed at relatively low levels prior to activation (reviewed by Sprent, 1997; Bradley et al., 1993). Because most of the $\alpha/\beta$ T cells in the TDL and lymphoid tissues from rNu/rNu rats did not express CD45RC, it was anticipated that this population would exhibit a pattern of adhesion molecule consistent with that expressed by CD4$^+$ cells with memory or effector function.
(memory/effecter) in normal rats. To examine whether this was the case, expression of adhesion molecules by α/β T cells from the TDL and peripheral lymphoid organs of rNu/rNu and age-matched rNu/+ rats was analysed by dual-fluochrome flow cytometry, utilising a panel of mAbs. As illustrated in Figure 4.2.22 (A-D), the majority of the α/β T cells present in the TDL from rNu/+ rats were CD62L+, expressed low levels of CD54 and CD49d and in comparison with cells from rNu/rNu rats (Figure 4.2.22 [E]), expressed lower levels of CD11a. In comparison essentially all of the α/β T cells in TDL from rNu/rNu rats expressed high levels of CD11a, CD54 and CD49d (Figure 4.2.22 [E-G]), while most were CD62L- (Figure 4.2.22 [H]). Phenotypic analysis of the α/β T cell populations in lymph nodes and spleens produced similar findings (data not shown). These differences in the expression of adhesion molecules by α/β T cells from rNu/+ and rNu/Nu rats are summarised in Table 4.2.4.

Because adhesion molecules are upregulated upon activation, the large cells in TDL from rNu/+ rats were examined separately. Analysis of the α/β T cells included in the large cell gate revealed that expression of CD11a (Figure 4.2.23 [A] Vs Figure 4.2.22 [A]) appeared to be upregulated. Furthermore, in comparison with the total population of α/β T cells (Figure 4.2.22 [A-D]), the proportion of large cells (Figure 4.2.23) that expressed CD54 (range: 75-79% compared to 14-15%, n=2) and CD49d (range: 70-71% compared to 24-30%, n=2) was greater, while more than half of the large cells were CD62L- (range: 33-46% compared to 56-66%, n=2). The large α/β T cells in TDL from rNu/+ rats did not express adhesion molecules in the relatively homogeneous pattern that was characteristic of the entire α/β TCR+ population from rNu/rNu rats, especially in the cases of CD54, CD49d and CD62L (illustrated in Figure 4.2.22 [E-H]).
Figure 4.2.22  Comparison of adhesion molecules expressed by α/β T cells in thoracic duct lymph from rNu/+ and rNu/rNu rats. Thoracic duct lymphocytes from (A-D) a 26 week old rNu/+ and (E-H) a 26 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique using one of the following mAbs: WT-1 (anti-CD11a; FITC), 1A29 (anti-CD54; FITC), MRα4-1 (anti-CD49d; FITC) and OX85 (anti-CD62L; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). The proportion of cells in each quadrant is presented as a percentage of gated events.
Table 4.2.4  Expression of adhesion molecules by $\alpha/\beta$ T cells in thoracic duct lymph. The table summarises the expression of adhesion molecules by $\alpha/\beta$ T cells from rNu/+ and rNu/rNu rats.

+ , ++, etc.  Indicator of the level of antigen expression

-  Indicates that there is no expression of the particular antigen

+/-  Indicates that most cells are positive

+/-  Indicates that most cells are negative
<table>
<thead>
<tr>
<th>Adhesion Molecule</th>
<th>rNu/+</th>
<th>rNu/rNu</th>
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<tbody>
<tr>
<td>CD11a</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>CD54</td>
<td>-/+</td>
<td>++</td>
</tr>
<tr>
<td>CD49d</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>CD62L</td>
<td>+/-</td>
<td>-/+</td>
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Figure 4.2.23 Expression of adhesion molecules by large α/β T cells in thoracic duct lymph from rNu/+ rats. Thoracic duct lymphocytes from a 26 week old rNu/+ rat falling within the large cell gate were labelled first by the indirect immunofluorescence technique with one of the following mAbs: (A) WT-1 (anti-CD11a; FITC), (B) 1A29 (anti-CD54; FITC), (C) MRα4-1 (anti-CD49d; FITC) and (D) OX85 (anti-CD62L; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). The proportion of cells in each quadrant is presented as a percentage of gated events.
4.2.6 Other unique differences in surface molecule expression by $\alpha/\beta$ TCR$^+$ cells in rNu/rNu and rNu/+ rats

4.2.6.1 Expression of $\alpha_{E2}$-integrin

The rat homologue of the $\alpha_E$ subunit (CD103) of the human $\alpha_E\beta_7$ integrin ($\alpha_{E2}$) has been described recently (Brenan and Rees, 1997). In mice, rats and humans this integrin is expressed by IEL and by a large proportion of the CD$^+$ cells in the lamina propria of the gut. However, it is expressed by very few peripheral $\alpha/\beta$ T cells (Brenan and Rees, 1997; Cerf-Bensussan et al., 1987). Because at least some $\alpha/\beta$ T cells found in rNu/rNu rats could originate from the gut (LFV), expression of the $\alpha_{E2}$-integrin was examined in lymphocytes obtained from the TDL and secondary lymphoid organs from rNu/rNu rats and age-matched heterozygous littermates. Using dual-fluochrome flow cytometry, a population of cells in the TDL from rNu/rNu rats was found to express both the $\alpha_{E2}$-integrin and CD3 (Figure 4.2.24 [B]). This population accounted for only approximately 0.5% of lymphocytes in age-matched rNu/+ rats (Figure 4.2.24 [A]). The data in Figure 4.2.24 (C) and (D) illustrate the co-expression of $\alpha_{E2}$ by $\alpha/\beta$ T cells in TDL from rNu/+ and rNu/rNu rats aged 26 weeks. Approximately 15% of the $\alpha/\beta$ T cells from rNu/rNu rats co-expressed the $\alpha_{E2}$ integrin (illustrated in Figure 4.2.24 [D] and Figure 4.2.25 [A]), while only approximately 1% of $\alpha/\beta$ T cells from rNu/+ rats expressed this molecule (illustrated in Figure 4.2.24 [C] and Figure 4.2.25 [A]). Although there was a small increase in the proportion of $\alpha_{E2}$-integrin$^+$ $\alpha/\beta$ T cells in TDL from older rNu/+ rats, the percentage of positive cells reached only 2% by 52 weeks of age (Figure 4.2.25 [A]).

The clear difference between rNu/+ and rNu/rNu rats in the expression of $\alpha_{E2}$-integrin
Figure 4.2.24  Expression of the $\alpha_{E2}$-integrin by $\alpha/\beta$ T cells in thoracic duct lymph from rNu/+ and rNu/rNu rats. Thoracic duct lymphocytes from (A, C and E) a 26 week old rNu/+, and (B, D and F) a 26 week old rNu/rNu rat were labelled by the indirect immunofluorescence technique with the mAb OX62 (anti-$\alpha_{E2}$; FITC), followed by direct labelling with either mAb G4.18 (anti-CD3), mAb R73 (anti-$\alpha/\beta$ TCR; PE) or mAb OX38 (anti-CD4; PE). The proportion of gated cells in each quadrant is presented as a percentage.
Figure 4.2.25  Expression of the $\alpha_{E2}$-integrin by $\alpha/\beta$ T cells from rNu/+ and rNu/rNu rats. The proportion of $\alpha/\beta$ T cells from rNu/+ and rNu/rNu rats expressing the $\alpha_{E2}$-integrin was determined by flow cytometric analysis and calculated as a percentage of total $\alpha/\beta$ T cells.

A. Expression of the $\alpha_{E2}$-integrin by $\alpha/\beta$ T cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 26 and 52 weeks of age. Each data set represents the mean of 2 rats ± SD.

B. Expression of $\alpha_{E2}$-integrin by $\alpha/\beta$ T cells from the lymph nodes (LN) and spleens of rNu/+ and rNu/rNu rats. Each data set represents the mean of 2 rats ± SD.
A

Expression of $\alpha_{E2}$ by TDL $\alpha/\beta$ T cells

- rNu/rNu
- rNu/+  

Percentage of $\alpha/\beta$ T cells

Rats

26 w/o  52 w/o

---

B

Expression of $\alpha_{E2}$ by $\alpha/\beta$ T cells

- rNu/rNu
- rNu/+  

Percentage of $\alpha/\beta$ T cells

Lymphoid Tissue

LN  Spleen
was also evident among peripheral CD4\(^+\) T cells (Figure 4.2.24 [E] and Figure 4.2.24 [F] respectively). Similar observations were made on the \(\alpha/\beta\) T cells and CD4\(^+\) cells in lymphocytes prepared from the spleens and lymph nodes (Figure 4.2.25 [B]) of 52 week old rNu/rNu and rNu/+ rats although the proportion of \(\alpha_{E2}^+\alpha/\beta\) T cells in the spleens of rNu/rNu rats was reduced slightly compared to those found in TDL and lymph nodes (Figure 4.2.25 [A] and [B]). The proportions of \(\alpha_{E2}\)-expressing cells among \(\alpha/\beta\) T cells from rNu/+ rats aged 52 weeks did not vary greatly between lymphocytes from TDL and those from spleen and lymph nodes (Figure 4.2.25 [A] and [B]).

To address the possibility that \(\alpha_{E2}\)-integrin might be expressed as an activation marker, expression of \(\alpha_{E2}\) was assessed separately on lymphocytes in the large cell gate. The proportion of large \(\alpha/\beta\) T cells from rNu/+ rats that expressed \(\alpha_{E2}\)-integrin was not very different (approximately 3-4%) from the proportion of positive cells in the total \(\alpha/\beta\) TCR\(^+\) population. This suggests that expression of this integrin is not associated solely with activation (data not shown).

4.2.6.2 Expression of UA002-antigen

Recently, a new mAb, (UA002), has been raised in this laboratory (Mayrhofer, unpublished). The surface antigen detected by this mAb is expressed by dendritic cells, macrophages, B cells, some NK cells, activated T cells (Concanavalin A blasts) and some endothelial cells. It has an apparent molecular weight of 95kD and the distribution is consistent with CD39 (Barclay et al., 1993). Because the \(\alpha/\beta\) T cells in rNu/rNu rats share a number of features with activated T cells, expression of the UA002-antigen by lymphocytes from rNu/rNu and age-matched rNu/+ rats was assessed by dual-
Figure 4.2.26  Expression of UA002-antigen by α/β T cells. Lymphocytes from 26 week old rNu/+ rats (A-D) and rNu/rNu rats (E-H) were first labelled indirectly with mAb UA002 (FITC), followed by direct labelling with mAb R73 (anti-αβ TCR; PE). (A and E) Thoracic duct lymphocytes falling within the small cell gate. (B and F) Lymph node cells falling within the small cell gate. (C and G) Spleen cells falling within the small cell gate. (D and H) Thoracic duct lymph (TDL) cells falling within the large cell gate.
fluochrome flow cytometry. As shown in Figure 4.2.26 [A], analysis of the α/β T cells from the TDL of rNu/+ rats revealed that those that fell into the small cell gate were essentially unstained by mAb UA002. The small α/β TCR° cells (mainly B cells) expressed low levels of the UA002-antigen. In comparison, the α/β TCR° and α/β TCR+ subpopulations within the small cells in TDL from rNu/rNu rats expressed similar levels of the UA002-antigen (Figure 4.2.26 [E]). This difference in expression of UA002-antigen between α/β TCR+ cells in TDL from rNu/+ and rNu/rNu rats was seen also on the small lymphocytes in cell preparations from lymph nodes (Figure 4.2.26 [B] and [F]). However, α/β TCR+ cells from spleens of rNu/rNu rats contained fewer cells that expressed UA002-antigen (Figure 4.2.26 [G]). Expression of the UA002-antigen by α/β T cells from rNu/+ rats was confined largely to the large cell population (Figure 4.2.26 [D]), whereas in rNu/rNu rats, the antigen was expressed by both large (Figure 4.2.26 [H]) cells in TDL and small cells in TDL and lymph nodes (Figure 4.2.26 [E] and [F] respectively). However, the antigen was expressed more uniformly by large cells in TDL (Figure 4.2.24 [H]), and lymph nodes and spleens (data not shown).

4.2.6.3 Expression of Thy-1 antigen (CD90)

Rat thymocytes, as well as recent thymic emigrants (RTE), express Thy-1 antigen (CD90). In young rats, aged 5-8 weeks, RTE are found in the spleen and lymph nodes, where they account for approximately 23% of the total α/β T cell population (Hosseinzadeh and Goldschneider, 1993). Mature α/β T cells do not express CD90. Because RTE express Thy-1, it was reasoned that the α/β T cells present in rNu/rNu might also be recent emigrants from sites of extra-thymic T cell maturation and express this antigen. To address this, mAb OX7 (anti-CD90) was used in dual-fluochrome flow
cytometry to examine CD90 expression by \( \alpha/\beta \) T cells in TDL, lymph nodes and spleens of age-matched rNu/+ and rNu/rNu rats. Approximately 18% of \( \alpha/\beta \) T cells in TDL from rNu/+ rats aged 13 weeks expressed CD90 (Figure 4.2.27 [A] and Figure 4.2.28 [A]). However, with increasing age, the proportion of \( \alpha/\beta \) T cells expressing this molecule in rNu/+ rats decreased and at 52 weeks of age, only approximately 5% of \( \alpha/\beta \) T cells were CD90\(^+\) (Figure 4.2.27 [A-C], Figure 4.2.28 [A]). This observation probably reflects the diminishing thymic output in older rats. However, it could also represent a contribution from extra-thymic sites. As shown in Figure 4.2.27 (E-G) and Figure 4.2.28 (A), a considerable proportion of the \( \alpha/\beta \) T cells in TDL from rNu/rNu rats expressed CD90. At 13 weeks of age, expression of CD90 by \( \alpha/\beta \) T cells in TDL from a group of rNu/rNu rats showed considerable variability (range: 8.5-33\%, \( n=4 \)) (Figure 4.2.27 [A] and Figure 4.2.28 [A]). However, expression of this molecule was less variable in older rats (aged 26, 39 and 52 weeks) and accounted for approximately 10-15% of total \( \alpha/\beta \) TCR\(^+\) cells (Figure 4.2.27 [F] and [G] and Figure 4.2.28 [A]).

It has been reported previously that activated mature \( \alpha/\beta \) T cells from normal rats can express CD90 (Paterson et al., 1987; Arthur and Mason et al., 1986). This raised the possibility that expression of CD90 by \( \alpha/\beta \) TCR\(^+\) cells in rNu/rNu rats could be acquired through activation. To address this, lymphoblasts (large cells) were analysed separately. As shown in Figure 4.2.27 (D) and Figure 4.2.28 (B), a considerable proportion (approximately 30-50\%) of the large \( \alpha/\beta \) T cells in TDL from rNu/+ rats expressed CD90. The level of expression of CD90 was typically higher than that seen on small \( \alpha/\beta \) T cells (Figure 4.2.27 [A-C] Vs [D]). For reasons that are not clear, few large \( \alpha/\beta \) T cells from 52 week old rNu/+ rats expressed CD90, although the proportion of large
Figure 4.2.27  Expression of Thy-1-antigen (CD90) by α/β T cells. Thoracic duct lymphocytes from rNu/+ rats (A-D) and rNu/rNu rats (E-H) were first labelled indirectly with either mAb 1B5 (negative control; FITC) or OX7 (anti-CD90; FITC), followed by direct labelling with mAb R73 (anti-αβ TCR; PE). Fluorescence of electronically gated α/β T cells is presented as histograms, where the dotted line represents the background fluorescence profile of cells stained with the negative control antibody, 1B5. (A and E) α/β T cells falling within the small cell gate from 13 week old rats. (B and F) α/β T cells falling within the small cell gate from 39 week old rats. (C and G) α/β T cells falling within the small cell gate from 52 week old rats. (D and H) α/β T cells falling within the large cell gate from 39 week old rNu/+ rats. The percentage of α/β T cells expressing CD90 is indicated above the histogram marker in the relevant panel.
Figure 4.2.28  Expression of Thy-1 antigen (CD90) by α/β T cells.

Thoracic duct lymphocytes from rNu/+ and rNu/rNu rats were first labelled indirectly with either mAb OX7 (anti-CD90; FITC), followed by direct labelling with mAb R73 (anti-αβ TCR; PE). Expression of CD90 by α/β T cells was assessed by dual parameter flow cytometric analysis and the proportions of α/β T cells expressing CD90 were calculated as a percentage of the total α/β T cells.

A. Expression of the CD90 by α/β T cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.

B. Expression of CD90 by large α/β T cells from thoracic duct lymph (TDL) of rNu/+ and Nu/rNu rats aged 13, 26, 39 and 52 weeks of age. Each data set represents the mean of 2-4 rats ± SD.
cells that were positive was higher than in the small cell population (Figure 4.2.28 [A] and [B]). The findings in rNu/+ rats suggest that the higher expression of CD90 by large α/β T cells is a result of activation. In contrast, the proportion of α/β T cells from rNu/rNu rats that expressed CD90 did not differ greatly between small and large cells (Figure 4.2.27 [F-H] and Figure 4.2.28 [A] and [B]). This is an interesting observation, consistent with the findings that expression of other activation and adhesion molecules by small and large α/β T cells from rNu/rNu rats did not differ greatly. It is of interest that the proportions of CD90+ α/β TCR+ cells (small and large) remained relatively constant throughout the adult lives of the rNu/rNu rats. The observations on expression of CD90 by α/β T cells in TDL were also reflected in analysis of lymphocytes prepared from spleens and lymph nodes (data not shown).

4.2.6.4 Expression of Mac-1 (CD11b)

In humans, a small subpopulation of CD4+ α/β T cells that does not express CD27 has been described recently (Baars et al., 1995; de Jong et al., 1992; Rotteveel et al., 1988). These cells constitute approximately 10-20% of the CD4+ CD45RA- cells. Characterisation of CD27- α/β T cells revealed that they expressed a pattern of adhesion molecules characteristic of an effector/memory status (high levels of CD18, CD49d, αββ7 and low levels of CD62L). In addition, approximately 7-19% of the cells expressed CD11b (Mac-1) and expression of this molecule was unique to this subset of T cells. The same subset has been shown to be potent in the production of cytokines (de Jong et al., 1992; Rotteveel et al., 1988). Because most CD4+ α/β T cells in rNu/rNu rats express the phenotype CD45RC- CD11ahi CD54hi CD49dhi CD62Llo, they were examined for expression of CD11b by the use of mAb WT-5 (anti-CD11b). A small
Figure 4.2.29 Expression of Mac-1 (CD11b) by α/β T cells isolated from lymph nodes. Lymph node cells from (A) a 26 week old rNu/+ rat and (B) a 26 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with mAb WT-5 (anti-CD11b; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). The proportion of gated cells in each quadrant is presented as a percentage.
Figure 4.2.30  Expression of Mac-1 (CD11b) by α/β T cells isolated from lymph nodes of individual rats. Lymph node cells from rNu/+ and rNu/rNu rats were first labelled indirectly with either mAb WT-5 (anti-CD11b; FITC), followed by direct labelling with mAb R73 (anti-αβ TCR; PE). Expression of CD11b by α/β T cells was assessed by dual parameter flow cytometric analysis and the proportion of α/β T cells expressing CD11b calculated as a percentage of total α/β T cells for two individual rNu/rNu rats and two individual rNu/+ animals.
CD11b expression by α/β LN T cells

- rNu/rNu #1
- rNu/rNu #2
- rNu/+ #1
- rNu/+ #2

Percentage of α/β T cells
proportion (approximately 3-5%) of α/β T cells in the lymph nodes from rNu/rNu rats was found to express detectable levels of CD11b (Figure 4.2.29 [B] and Figure 4.2.30). In comparison, CD11b+ α/β T cells comprised a very minor population (<0.5%) in the lymph nodes of rNu/+ rats (Figure 4.2.29 [A] and Figure 4.2.30). CD11b+ α/β TCR+ cells could not be detected in TDL from either rNu/+ and rNu/rNu rats (data not shown). Their presence has not been studied in inflammatory exudates but this would be of interest.

4.2.7 Characterisation of α/β T cells isolated from the livers of rNu/+ and rNu/rNu rats

Examination of lymphocytes isolated from the livers of normal mice has revealed two distinct α/β TCR+ populations, one of which appears to consist of classical T cells. The other population (TCRintermediate cells) characteristically expresses lower levels of the TCR on the cell surface and it is proposed to be of extra-thymic origin because these cells are present also in athymic mice (Sato et al., 1995; Iiai et al., 1992, Watanabe et al., 1992; Ohteki et al., 1992, Lawetzky and Hünig, 1988) and in thymectomized, irradiated, bone-marrow-reconstituted mice (Sato et al., 1995; Iiai et al., 1992).

Although in mice TCRintermediate cells have been identified in a number of organs, they are most abundant in the liver (Arai et al., 1995; Ohteki et al., 1992; Iiai et al., 1992).

Because α/β T cells from the TDL and peripheral lymphoid organs of rNu/rNu rats also express intermediate levels of surface TCR, the liver was examined to determine whether a corresponding population of TCRintermediate cells could be identified in rNu/+ rats. Phenotypic analyses were carried out on mononuclear cell populations prepared from the livers of rNu/+ and rNu/rNu rats, using a method adapted from Watanabe et al.
(1992). A yield of approximately $1 \times 10^7$ MNC per liver was obtained from both rNu/+ rats and rNu/rNu rats and the cells were then analysed by dual-fluochromet flow cytometry. The FSC Vs SSC dotplots generated from isolated liver MNC showed the characteristics of typical populations of MNC with little contamination of other cell types or debris (see Figure 2.1 [D] and [H]). A gating regime, similar to that used in the analysis of other lymphocyte populations, was employed and NK cells (identified as CD161hiCD8αlo cells) were shown to be the most abundant cell isolated by this method. They accounted for in excess of 60% of the total MNC (Figure 4.2.31 [C]), whilst B cells (labelled with mAb OX33) were few in number, even in preparations from rNu/rNu rats (Figure 4.2.31 [A]). Variable numbers of CD4lo cells were also present in the preparations and were presumed to be Kupffer cells (Figure 4.2.31 [B]). Cells expressing the α/β TCR were detected in rNu/rNu rats aged 13 weeks and the proportions of these cells increased to a maximum of approximately 10-15% of the mononuclear cells at 26-52 weeks of age (represented with examples from a 52 week old rNu/rNu rat, Figure 4.2.31 and a 26 week old rNu/rNu rat, Figure 4.2.32[A]). In rNu/+ rats, α/β TCR+ cells accounted for approximately 20% (mean = 20.3 ± 5.3) of mononuclear cells at all ages studied (illustrated with an example from a 26 week old rNu/+ rat, Figure 4.2.32 [A]). The proportions of γ/δ TCR+ cells rarely exceeded 1% of total MNC isolated from the livers of rNu/+ rats, or 2% in cells isolated from rNu/rNu rats at any age studied (illustrated with an example from a 52 week old rNu/rNu rat, Figure 4.2.31 [F]). Within the α/β TCR+ populations, the ratio of CD4+ cells to CD8α+ cells varied greatly from rat to rat. Although CD4+ α/β TCR+ cells were always more numerous, the ratio of CD4+ cells to CD8α+ cells ranged from approximately 2:1 to almost 5:1. This range was seen in MNC isolated from both rNu/rNu and rNu/+ rats and it was not dependent on age (illustrated in Figure 4.2.31 [B] and [E] with an example
Figure 4.2.31  Surface antigen phenotype of mononuclear cells isolated from rat liver. Mononuclear cells (MNCs) isolated from the liver of a 52 week old rNu/rNu rat were gated as shown in Figure 2.1 and characterised by dual fluochrome flow cytometry. (A) MNCs labelled first by the indirect immunofluorescence technique with mAb OX33 (anti-CD45RA/B; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). (B) MNCs labelled first by the indirect immunofluorescence technique with mAb W3/25 (anti-CD4; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). (C) MNCs labelled first by the indirect immunofluorescence technique with mAb 10/78 (anti-CD161; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). (D) MNCs labelled first by the indirect immunofluorescence technique with mAb R73 (anti-α/β TCR; FITC), followed by direct labelling with mAbs OX38 and OX8 (anti-CD4+CD8α; PE). (E) MNCs labelled first by the indirect immunofluorescence technique with mAb R73 (anti-α/β TCR; FITC), followed by direct labelling with a mixture of mAb OX8 (anti-CD8α; PE). (F) MNCs labelled first by the indirect immunofluorescence technique with mAb V65 (anti-γ/δ TCR; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). The proportion of gated cells in each quadrant is presented as a percentage and in (B) a second region is drawn to highlight the CD4dim population and the percentage of cells in this region indicated.
Figure A: 

- 

**α/β TCR**

CD45RA/B

Figure B: 

- 

**α/β TCR**

CD4

Figure C: 

- 

**CD8α**

CD161

Figure D: 

- 

**CD4**

**CD8α**

α/β TCR

Figure E: 

- 

**CD8α**

α/β TCR

Figure F: 

- 

**CD8α**

γ/δ TCR
from a 52 week old rNu/rNu rat). DN cells rarely exceeded 2% of the total α/β TCR⁺ cells in preparations from either rNu/+ or rNu/rNu rats (illustrated in Figure 4.2.31 [D] with an example from a 52 week old rNu/rNu rat).

The α/β TCR⁺ populations identified by flow cytometric analysis of MNC isolated from the livers of 26 week old rNu/+ and rNu/rNu rats were gated for further analysis. As shown in Figure 4.2.32 (B), a clear distinction was seen in the intensity of staining of α/β T cells from rNu/+ (MFI = 342.5) and rNu/rNu (MFI = 209.8) rats with mAb R73. Closer examination of the histogram generated from rNu/+ cells shows that it displays a relatively normal distribution. There is little evidence of a bimodal pattern of staining, which if present, would indicate the presence of two distinct α/β TCR⁺ populations expressing different levels of TCR on the cell surface. Similar results were obtained with rats aged 13, 39 and 52 weeks of age (data not shown). The results do not support the presence of a major TCRintermediate component of the α/β T cells in the livers of euthymic rats, although all of the α/β T cells in the livers of rNu/rNu rats express this phenotype.

Because there is only an approximately 1.5-fold difference in the level of TCR expressed by α/β T cells from rNu/+ and rNu/rNu rats, the presence of a distinct TCRintermediate population may be overlooked by flow cytometry. Since α/β T cells from rNu/rNu rats exhibit a characteristic pattern of adhesion molecule expression, this phenotype might be a useful marker for identifying a putative thymus-independent component among α/β T cells in the livers of rNu/+ rats. Figure 4.2.33 compares the expression of a number of activation and adhesion molecules by α/β T cells isolated from the livers of 26 week old rNu/+ and rNu/rNu rats. Approximately 60% of the α/β
Figure 4.2.32  Surface expression of the $\alpha/\beta$ TCR by T cells isolated from the livers of rats  

(A) Mononuclear cells (MNCs) isolated from the livers of 26 week old rats were stained by directly with mAb R73 (R73-PE) and analysed by flow cytometry. The fluorescence profiles of cells from a rNu/+ rat (teal line) and a rNu/rNu rat (purple line) are superimposed and the percentage of the $\alpha/\beta$ TCR$^+$ population in each preparation is shown above a region marker.  

(B) Direct comparison of the gated $\alpha/\beta$ TCR$^+$ cells in ‘A’ after scaling to give similar peak heights. The fluorescence profiles of the gated cells from a rNu/+ rat (teal line) and rNu/rNu rat (purple line) are superimposed and the mean fluorescence intensity (MFI) of each peak indicated.
T cells in MNC prepared from the livers of rNu/rNu rats were MHC class II+ (a proportion similar to that obtained from TDL and lymph node cell preparations), while only 13% of the cells prepared from the livers of rNu/+ rats were positive (a level similar to that observed among α/β T cells prepared from TDL and lymph nodes (4.2.17 [A] and [B]). The majority of α/β T cells (60% CD4+) in the cells prepared from rNu/rNu livers did not express CD45RC (73.5%), in accordance with results obtained from TDL and lymph node cell preparations (Figure 4.2.20 [A] and [B]). In comparison, approximately half of the α/β T cells (79% CD4+) from the livers of Nu/+ rats expressed CD45RC and this proportion was considerably lower that seen among α/β T cells from TDL and lymph nodes (70-90%, Figure 4.2.20 [A] and [B]).

Examination of CD25 and CD134 proved interesting (Figure 4.2.33 [D] and [E]). The proportions of α/β T cells that expressed CD25 in preparations of MNC from the livers of rNu/rNu and rNu/+ rats were similar (approximately 23% and 19% respectively) and in turn, these were similar to the proportions observed in TDL and lymph node cell preparations from rNu/rNu rats. As presented earlier (section 4.2.4), only 5-10% of α/β T cells in TDL and lymph node preparations from rNu/+ rats expressed CD25. The proportions of α/β T cells in TDL and lymph node preparations that express CD25 is generally higher than the proportion of CD134+ cells (see section 4.2.4). However, this difference was greater in preparations of liver MNC. Only approximately 9% of liver α/β T cells from rNu/rNu rats and 6.5% of liver α/β T cells from rNu/+ rats expressed CD134 (Figure 4.2.33 [E]). In the case of the rNu/rNu rats a higher proportion of CD8+ α/β T cells (39%) could account partly for the difference in the proportion of cells expressing CD25 and CD134, given that C134 is expressed by CD4+ cells but by few CD8+ cells (Paterson et al., 1987). However, because only 20% of the α/β T cells in the
preparation from the rNu/+ rat were CD8+, this is unlikely to account for the difference in this case. Furthermore, the proportions of CD134+ cells in the liver are similar to those seen in TDL and lymph node preparations from rNu/+ rats (see section 4.2.4). Thus while a higher proportion of α/β T cells isolated from the livers of rNu/+ rats express CD25 compared to α/β T cells present in the TDL, lymph nodes and spleens of these animals, the proportion of α/β T cells expressing CD134 are similar in all of these cell preparations.

As in TDL, lymph nodes and spleens from rNu/rNu rats (Figure 4.2.24 [D] and Figure 4.2.25), a high proportion (23.5%) of liver α/β T cells expressed the αE2+-integrin (Figure 4.2.33 [F]). In comparison, only 6% of α/β T cells from the livers of rNu/+ rats expressed this integrin (Figure 4.2.33 [F]). Nevertheless, this proportion of αE2+ α/β T cells was considerably higher than that observed in TDL from 26 week old rNu/+ rats, where only approximately 1% of the total α/β T cells expressed the αE2-integrin (Figure 4.2.24 [C] and Figure 4.2.25 [A]).

With respect to other adhesion molecules, most α/β T cells from the livers of both rNu/rNu and rNu/+ rats expressed CD54 and most were either CD62L or CD62Llo (Figure 4.2.33 [G] and [H]), a phenotype indicative of activation or memory. However, the entire population of liver α/β T cells from rNu/rNu rats expresses CD54, while expression of CD54 by α/β T cells from the rNu/+ rats is more heterogeneous. In comparison, as shown in Figure 4.2.22 [B], only a very small population of α/β T cells in the TDL from rNu/+ rats expressed detectable levels of CD54. Only 14% of the α/β T cells isolated from the liver of the rNu/rNu rat expressed detectable levels of CD62L
This is similar to the result obtained from α/β T cells in TDL from rNu/rNu rats (Figure 4.2.22 [H]). In contrast, while only approximately 24% of liver α/β T cells expressed detectable levels of CD62L in the rNu/+ rat (Figure 4.2.33 [H]), greater than 50% of α/β T cells in TDL were CD62L⁺ (Figure 4.2.22 [D]). Thus, while the expression of activation and adhesion molecules by α/β T cells in rNu/rNu rats is similar at all sites (TDL, spleen, lymph node and liver cell preparations), there is a distinct difference between α/β T cells from the livers of rNu/+ rats and α/β T cells present at other sites.
Figure 4.2.33  Surface antigens expressed by \( \alpha/\beta \) T cells isolated from the livers of rats. Surface antigen expressed by \( \alpha/\beta \) T cells isolated from the livers of a 26 week old rNu/+ rat (teal lines) and a 26 week old rNu/rNu rat (purple lines) were compared by gating electronically on \( \alpha/\beta \) TCR\( ^+ \) cells which had also been labelled indirectly with the following mAbs: (A) 1B5 (negative control; FITC), (B) OX6 (anti-MHC class II; FITC), (C) OX22 (anti-CD45RC; FITC), (D) OX39 (anti-CD25; FITC), (E) OX40 (anti-CD134; FITC), (F) OX62 (anti-\( \alpha_E \)-integrin; FITC), (G) 1A29 (anti-CD54; FITC) and (H) OX85 (anti-CD62L; FITC). The percentage of \( \alpha/\beta \) T cells that expressed each antigen is indicated above the relevant marker and in the relevant colour.
4.3 Discussion

4.3.1 Appearance of α/β T cells in rNu/rNu rats: A Summary

Work in this laboratory had shown previously that small numbers of cells expressing the α/β TCR could be detected in the TDL of young rNu/rNu rats, prior to their appearance of substantial numbers of these cells in peripheral lymphoid organs. Moreover, flow cytometric analysis revealed that the α/β T cells present in the TDL from 15 w/o rNu/rNu rats were large lymphocytes (lymphoblasts) on the basis of their large forward- and side-light scatter characteristics (Lee, 1993). These observations led to the generation of the hypothesis that the large α/β T lymphocytes appearing in the TDL in young rNu/rNu rats could be newly formed cells, which have a gut origin. This hypothesis was based on the observation that approximately 90% of the lymphocytes in the TDL have a gut origin (Mann and Higgins, 1950; Mayrhofer, unpublished observations and Spargo et al., 1996).

Previous studies in the rat have shown that thoracic duct drainage results in the removal of small recirculating lymphocytes, whereas the output of large lymphocytes remains relatively constant (Gowans and Knight, 1964, Mann and Higgins, 1950). Depletion of the recirculatory pool involves both B cells and T cells (Westermann et al., 1994). Administration of tritiated thymidine (³H-TdR) in vivo for 24 hours labels between 90-100% of the large lymphocytes recovered in TDL. This led to the proposal that the constant output of large lymphocytes from the thoracic duct was maintained by a continuous production of new cells in the secondary lymphoid tissues, notably those of the intestinal tract (Gowans and Knight, 1964).
The results presented in this section show that the small numbers of \( \alpha/\beta \) T cells that are present in the TDL from 9-10 w/o rNu/rNu rats are essentially all large cells. In contrast, the great majority of \( \alpha/\beta \) T cells in age-matched rNu/+ rats are small lymphocytes, although thoracic duct drainage leads to a rapid reduction in the numbers of these cells. Analysis of the large lymphocytes present in the TDL from 9-10 w/o rNu/rNu rats showed that an unexpectedly high number expressed the \( \alpha/\beta \) TCR. As shown in Figure 4.2.3 (A), up to 30% of the large lymphocytes in a freshly cannulated rNu/rNu rat were T cells. Of particular interest was the observation that the number of \( \alpha/\beta \) TCR\(^+\) cells in the TDL of 9-10 w/o rNu/rNu rats increased over the initial stages of thoracic duct drainage. In this respect they were unlike either large \( \alpha/\beta \) TCR\(^-\) cells (B cells) in TDL from rNu/rNu and rNu/+ rats or large \( \alpha/\beta \) TCR\(^+\) cells in the TDL from rNu/+ rats of the same age. This observation suggests that the source of these large \( \alpha/\beta \) T cells in young athymic rats is responsive to depletion of thoracic duct lymphocytes, perhaps through effects on the immunological homeostasis of the gut. Because \( \alpha/\beta \) TCR\(^+\) cells are virtually undetectable in the peripheral lymphoid tissues of 9-10 w/o rNu/rNu rats (data not shown), it is possible that the appearance of large lymphocytes expressing the \( \alpha/\beta \) TCR in the TDL of these young rats represents the release of newly formed cells, which could then seed the peripheral lymphoid organs. The gut is the likely source of these cells and LFV are likely candidates for the anatomical site of production. This speculation is supported by the finding that essentially all of the \( \alpha/\beta \) T cells detected in the TDL from 9-10 w/o rNu/rNu rats express CD4, a feature of the major population of \( \alpha/\beta \) TCR\(^+\) cells present in LFV.
The origin of the α/β T cells in the TDL of young rNu/rNu rats is an important issue that needs to be resolved. Continuous infusion of $^3$H-TdR into young rNu/rNu rats could confirm that the α/β TCR$^+$ cells present in the TDL of these rats are newly formed. However, given that all of the large lymphocytes in the TDL from freshly cannulated normal rats can be labelled after the administration of $^3$H-TdR, these experiments would not distinguish whether the α/β TCR$^+$ cells present in the TDL of young rNu/rNu rats are newly formed naive cells or the progeny of antigen-activated cells in secondary lymphoid organs. It is vital to devise ways of labelling cells in LFV selectively, or to discover markers that are restricted to the lineage of T cells that arises from these structures.

### 4.3.2 Extended phenotype of α/β T cells in rNu/rNu rats: A Summary

Although previous studies have examined the appearance of cells expressing T cell markers in athymic rats (Sarawar et al., 1991; Vaessen et al., 1986), there have been only limited studies on the extended surface antigen phenotype of these cells (Torres-Nagel et al., 1992; Sarawar et al., 1991). One aim of this study was to examine whether phenotypic differences exist which can distinguish the α/β T cells which arise extrathymically in rNu/rNu rats from T cells which develop in euthymic littermates. One important aspect of this study was to examine α/β T cells with respect to their expression of the CD4 and CD8 co-receptors. Although the experiments described above indicated that essentially all of the α/β T cells which appeared in the TDL of cannulated 9-10 w/o rNu/rNu rats were CD4$^+$, the CD4 to CD8 ratios in the spleens and lymph nodes from rats aged 13-52 weeks of age revealed no significant differences between rNu/rNu and rNu/+ rats. However, an increased ratio of CD4$^+$ to CD8α$^+$ was a feature of α/β T cells in TDL of older rNu/rNu rats aged 39-52 weeks of age. The
findings on the ratio of CD4\(^+\) to CD8\(^+\) \(\alpha/\beta\) T cells from the lymph nodes of CBH rNu/rNu rats confirms and extends previous observations on \(\alpha/\beta\) T cells isolated from the lymph nodes of PVG rNu/rNu rats (Sarawar et al., 1991). However, Sarawar et al., 1991 did not report the presence of significant numbers of DN \(\alpha/\beta\) T cells. In the present study in CBH rNu/rNu rats, cells with this phenotype have been detected in all of the tissues examined and also in the TDL. DN cells comprised 5-15\% of the \(\alpha/\beta\) T cells in the tissues and the TDL of rNu/rNu rats and less than 1\% of the \(\alpha/\beta\) T cells in the tissues and the TDL of rNu/+ rats. The proportion of DN \(\alpha/\beta\) T cells varied from animal to animal in rNu/rNu rats and cells with this phenotype were not enriched in any particular tissue, including the liver (data not shown). The identification of DN \(\alpha/\beta\) T cells in this study may reflect a strain difference between CBH and PVG rats or possibly a difference between animal house environments. The existence of significant numbers of DN \(\alpha/\beta\) T cells in rNu/rNu rats is interesting, given that DN T cells are a rare subset in normal mice and humans. Cells with this phenotype have been proposed to have important regulatory roles, distinct from classical T cell functions (reviewed by Vicari and Zlotnik, 1996; Bix and Locksley, 1995).

Expression of the CD8\(\alpha\alpha\) homodimer by a significant number of TCR\(^+\) cells, in particular those expressing the \(\gamma/\delta\) TCR, in rNu/rNu rats supports the view that T cells expressing this form of the CD8 co-receptor are less thymus-dependent than those T cells expressing the CD8\(\alpha\beta\) heterodimer. The CD8\(\alpha\alpha\) homodimer appears to be a marker of the thymus-independent subset of IEL, as discussed above. However, the observation that there is animal to animal variation in the proportions of the \(\alpha/\beta\) T cells in rNu/rNu rats that express the CD8\(\alpha\alpha\) homodimer and the CD8\(\alpha\beta\) heterodimer
highlights the fact that the form of CD8 co-receptor expressed by T cells is also dependent on factors other than the presence or absence of a functional thymus. It would be interesting to examine CD8αα expression by α/β T cells in the TDL of 9-10 week old rNu/rNu rats. Because most of the CD8α+ cells in LFV do not express the CD8β chain (see Chapter 3), it would be interesting to investigate whether CD8αα+ cells appear in the TDL at this age, when T cells are rare in secondary lymphoid organs. The appearance of small numbers of α/β T cells expressing the CD8αα homodimer in the TDL at this age would suggest LFV as a possible source.

4.3.3 α/β T cells in rNu/rNu rats are larger, express reduced levels of surface TCR but most exhibit 2N levels of DNA: A Summary

As discussed above, the α/β T cells that appear in the TDL of young nude rats aged 9-10 weeks are all large lymphocytes. At 13 weeks of age (as shown in Figure 4.2.12), the mean size of the α/β T cells that are present in the TDL is larger than that of the corresponding cells in the TDL of rNu/+ rats. Essentially all of the α/β T cells from the rNu/rNu rats had FSC and SSC measurements larger than those of small lymphocytes from euthymic rats. With increasing age, there was a decrease in the size of the α/β T cells in rNu/rNu rats but they remained larger than the small α/β T cells present in rNu/+ rats. These observations were not restricted to the TDL, as α/β T cells in the lymph nodes and spleens of rNu/rNu rats were also larger than the corresponding populations in rNu/+ rats, irrespective of the age of the animal.

The question therefore arises as to why the α/β T cells in rNu/rNu rats have a larger mean size than the conventional α/β T cells in euthymic rats. Does this larger size reflect differences in maturation state or perhaps different functional characteristics?
Recent thymic emigrants (RTE) in the rat have a larger mean than their more mature α/β T cell counterparts (Hosseinzadeh and Golschneider, 1993) and in addition these cells are CD45RC⁺. These features indicate that the large mean size of α/β T cells in rNu/rNu rats could reflect an immature phenotype. However, unlike RTE, most α/β T cells in rNu/rNu rats do not express the Thy-1 antigen (Figures 4.2.27 and 4.2.28). Furthermore, although the α/β T cells in rNu/rNu rats have a larger mean size than their counterparts in rNu/+ rats at all ages, the α/β T cells in older rNu/rNu rats are smaller than those that emerge in animals 13 weeks of age and younger. This raises the possibility that the α/β T cells present in young rNu/rNu rats are largely immature and that these give rise to the smaller α/β T cells which are more abundant in older rNu/rNu rats. The availability of congenic rats on the rNu background would enable studies in which α/β T cells from rNu/rNu rats aged less than 13 weeks of age could be transferred adoptively into recipient animals and their phenotype, in particular their size characteristics, could be monitored in host animals.

The greater size of the α/β T cells in the TDL and peripheral lymphoid organs from rNu/rNu rats is not due simply to the presence of a high proportion of dividing cells. Examination of DNA content by staining with propidium iodide indicated that the proportions of α/β T cells that contained 2N and 4N multiples of the haploid DNA content were similar in cells isolated from rNu/rNu and rNu/+ rats. It was noted consistently that more of the α/β TCR⁺ cells from rNu/rNu rats were in S-phase (DNA content >2N<4N) than was observed in the corresponding population from rNu/+ rats. However, the difference was small and it appears unlikely to account for the larger mean size of the cells from rNu/rNu rats. The presence of a small sub-population of the α/β
T cells from rNu/rNu rats that exhibited a DNA content less than 2N (see Figure 4.2.13) was of interest. These cells were not evident in either the TDL, spleen or lymph nodes from rNu/+ rats and their presence may indicate that apoptosis is more common in thymus-independent T cells.

Close examination of the α/β T cells from rNu/rNu rats by flow cytometry revealed that despite a larger cell size, the level of expression of the TCR by these cells was only 65% of that observed for the corresponding population in age-matched rNu/+ rats. This difference was evident in T cells obtained from TDL and from all of the tissues studied. This observation is important, because lower expression of the TCR may prove to be a useful marker for the thymus-independent lineage of T cells. This has been suggested also from studies in athymic mice (Sato et al., 1995; Iiai et al., 1992; Lawetzky and Hünig, 1988) and in thymectomized, irradiated, bone marrow reconstituted mice (Iiai et al., 1992). It is fascinating that an intermediate level of TCR is also a characteristic of NKT cells in mice (reviewed by Vicari and Zlotnik, 1996; MacDonald 1995). These cells express the α/β TCR at a level approximately 3-fold less than that expressed by conventional α/β T cells. However, reduced surface expression of the TCR could also reflect antigen-induced down-regulation of the TCR-CD3 complex. This phenomenon, which can be long lasting, has been observed in vitro (Valitutti et al., 1997). Activation of the α/β T cells by exogenous antigen or by autoantigen could account for both their large mean size and for the lower levels of α/β TCR that they express.
4.3.4 Comparison of activation marker expression by $\alpha/\beta$ TCR$^+$ cells in rNu/rNu and rNu/+ rats: A Summary

In normal rats, almost all of the CD8$^+$ subset of $\alpha/\beta$ T cells expresses the CD45 isoform (CD45RC) that is detected by mAb OX22 (Woollett et al., 1985). Only a subpopulation of activated CD8$^+$ blasts does not express CD45RC (Sarawar et al., 1993). However, only 50-70% of the CD4$^+$ subset of $\alpha/\beta$ T cells expresses this isoform (Woollett et al., 1995 and reviewed by Powrie and Mason, 1990). The remainder, which do not express CD45RC, are believed to be either activated effector cells or memory cells (Powrie and Mason, 1989 and reviewed by Powrie and Mason, 1990). Whereas most $\alpha/\beta$ T cells in rNu/+ rats were shown to express the CD45RC isoform, the majority of $\alpha/\beta$ T cells in rNu/rNu rats had the CD45RC$^-$ phenotype. At 13 weeks of age, only 10-20% of the $\alpha/\beta$ T cells in the TDL and lymph nodes from rNu/rNu rats expressed CD45RC. While expression of this isoform by $\alpha/\beta$ T cells increased with age in rNu/+ rats, it did not exceed 30% in most animals aged 52 weeks. Although expression of the CD45RC$^-$ phenotype by the CD4$^+$ subset has been associated with memory phenotype in normal rats, recent evidence suggests that CD45RC$^-$ cells revert to the CD45RC$^+$ phenotype following adoptive transfer to irradiated syngeneic hosts (Bunce and Bell, 1997; Hargreaves and Bell, 1997). Furthermore, it appears that continuing exposure to antigen is required for memory CD4$^+$ cells to maintain expression of the CD45RC$^-$ phenotype. In the absence of antigen, memory cells appear to revert to a CD45RC$^+$ phenotype (Bunce and Bell, 1997). The observation that the $\alpha/\beta$ TCR$^+$ cells from rNu/rNu rats have the CD45RC$^-$ phenotype is of interest because it implies that these cells are either in a state of continued activation or that they encounter stimulating antigen regularly. It is significant that the CD45RC$^-$ and the CD45RC$^+$ subsets of cells are distinct functionally. The CD45RC$^+$ cells produce more IL-2 after stimulation and respond well
to alloantigens (Arthur and Mason, 1986), while CD45RC− cells provide help for B cells in secondary responses (Arthur and Mason, 1986) but are poor mediators of allograft rejection (Yang and Bell, 1992). If the antigenic repertoire of the α/β TCR+ cells in rNu/rNu rats is limited to persisting (perhaps self) antigens, it could help explain the absence of classical cell-mediated immune responses to exogenous antigens in these animals. Alternatively, like some γ/δ T cells and NKT cells, they may have a restricted antigen receptor repertoire and recognise only a limited range of novel antigens (possibly of microbial origin).

Only a small population of α/β T cells in the TDL and lymphoid organs from rNu/+ rats expressed activation markers. This confirmed previous descriptions of the expression of CD25, CD134 (Paterson et al., 1987) and MHC class II molecules (Seddon and Mason, 1996; Broeren et al., 1995; Reizis et al., 1994) by α/β T cells in the peripheral lymphoid organs and TDL from normal rats. The cells that express these markers in TDL and in the lymphoid organs from rNu/+ rats were concentrated in the lymphocytes with the largest FSC and SSC (lymphoblasts) (see Figure 4.2.15). In contrast, most of the α/β T cells from rNu/rNu rats expressed MHC class II molecules. In excess of 90% of α/β T cells in the TDL from 13 week old rNu/rNu rats expressed MHC class II. The proportion cells expressing MHC class II decreased steadily with age, such that at age 52 weeks, approximately 50% of the α/β T cells expressed MHC class II molecules. Interestingly, expression of CD25, CD71 and CD134 by α/β T cells in TDL and lymph nodes from rNu/rNu rats did not follow this trend. Although the proportion of cells expressing these activation markers was higher (approximately 20-30%) than in α/β T cells from rNu/+ rats (approximately 5-10%), the proportions of α/β T cells from rNu/rNu expressing these markers of did not vary greatly with age. It is possible that
after activation, expression of MHC class II molecules is maintained while expression of CD25 and CD134 molecules is down-regulated. However, analysis of the activation markers expressed by mitogen activated splenocytes from normal rats after activation in vitro with Con A (Murphy, data not shown) indicate that over a 96 hour period, MHC class II is down-regulated more quickly than CD25. Sustained expression of MHC class II by α/β T cells in rNu/rNu rats raises the interesting possibility that these cells may have a number of non-classical functions. Recently, it has been shown that activated peripheral γ/δ T cells in cattle express MHC class II molecules and that these cells can present antigen to CD4⁺ T cells. It is conceivable that a non-classical function of α/β T cells in rNu/rNu rats (and their likely counterparts in normal animals) is to perform a similar function in antigen presentation and immunoregulation.

It has been argued above that the large mean size of α/β T cells in the TDL from young (10-13 weeks) rNu/rNu rats is not due to constant cell division, while most of the cells do not express the activation markers CD25, CD134 or CD71. It is possible, therefore, that the large cells in the TDL from these young rNu/rNu rats are newly differentiated naive T cells. The increase in the numbers of smaller α/β T cells that appearing in the TDL of rNu/rNu rats with age could then represent cells of greater maturity. In previous studies (Murphy, Honours thesis, 1994) it was found that approximately 25% of rat thymocytes expressed MHC class II molecules. Essentially all thymocytes were CD45RC⁻ and among the larger thymocytes (approximately 20% of total thymocytes), 25% of these cells expressed CD71, 7% expressed CD25 and 8% expressed CD134. These observations on thymocytes raise the possibility that the expression of activation markers and the mainly CD45RC⁻ phenotype is a reflection of immaturity of the α/β T cells in TDL from rNu/rNu rats, rather than an indication of memory/activated status.
An interesting addendum to the expression of activation markers by α/β T cells in TDL from rNu/rNu rats concerns the expression of CD71. In those 13 week old rNu/rNu rats which had very few α/β TCR$^+$ cells (<5%), the mean size of the T cells was larger than those present in TDL from rats of the same age where α/β T cells exceeded 5% of the total lymphocytes. In those 13 week old rats in which in excess of 5% of total lymphocytes expressed the α/β TCR, the size of these α/β T cells was intermediate in size between the large and small lymphocytes in TDL from rNu/+ rats (data not shown and Figure 4.2.12). Although expression of CD25 and CD134 by the small numbers of T cells was similar in both groups of rats, expression of CD71 by the α/β T cells from the rats with few T cells was as high as 50% (data not shown). This suggests that during the earliest appearance of T cells in the TDL in rNu/rNu rats, a large proportion of the cells express a marker that is associated with cell division. Thirteen weeks may be close to the critical time for the emigration of the first wave of newly-formed thymus-independent T cells into the circulation. Future studies on a larger number of rats at this age could provide a solution to the dilemma of the nature and origin of the T cells in nude rats and studies on rats in the age range 9-13 weeks would assist in the interpretation of these findings. As a corollary, the replacement of CD71$^+$ T cells by CD71$^-$ cells, both with time and with accumulating numbers of T cells, during this critical period would suggest a maturational shift towards a dominant population in TDL that is no longer in cell cycle.

4.3.5 Expression of adhesion molecules by α/β T cells in rNu/rNu and rNu/+ rats: A Summary

The pattern of adhesion molecule expression by α/β T cells in the TDL and peripheral lymphoid organs from rNu/rNu rats is relatively homogeneous and therefore distinct
from that expressed by α/β T cells from rNu/+ rats. Most α/β T cells from rNu/+ rats express CD62L, have only low levels of CD11a and CD49d and most do not express detectable levels of CD54. In contrast, few α/β T cells from rNu/rNu rats express CD62L but they express the phenotype CD11a<sup>hi</sup>, CD54<sup>hi</sup>, CD44<sup>hi</sup> and CD49d<sup>hi</sup>. This phenotype is consistent with an effector or memory phenotype in normal animals (reviewed in Sprent, 1997; Bradley et al., 1993). It is also a feature of TCR<sub>intermediate</sub> cells (including NKT cells) (Ohteki et al., 1992, Arai et al., 1995) and of the α/β T cells found in athymic mice (Ohteki et al., 1992; Arai et al., 1995) and thymectomized, irradiated and bone marrow reconstituted mice (Arai et al., 1995). The pattern of adhesion molecules, together with the reduced level expression of the α/β TCR by the cells from rNu/rNu rats may prove to be useful in identifying related cells in euthymic animals, especially as this phenotype is restricted largely to a population of large lymphocytes. Furthermore, this pattern of adhesion molecules may reflect the functional characteristics of thymus-independent T cells and it may influence the tissue distribution and localisation of such cells in both athymic and euthymic rats. In this respect, others have postulated that elevated expression of CD44, the hyaluronate receptor, mediates the adhesion of TCR<sub>intermediate</sub> cells to sinusoidal walls in the liver and perhaps the spleen. As discussed later in Chapter 5, α/β T cells from rNu/rNu rats demonstrate different tissue distribution characteristics to those of α/β T cells found in rNu/+ following adoptive transfer. These differences in tissue distribution may be due to differences in adhesion molecule expression.
4.3.6 Other differences in the expression of surface molecules by α/β T cells in rNu/rNu and rNu/+ rats: A Summary

In addition to the differences discussed above, this study identified a number of other surface molecules whose expression differed between α/β T cells from rNu/+ and rNu/rNu rats. It has been reported previously (Brenan and Rees, 1997 and Brenan and Puklavec, 1992) that expression of αE2 (the rat homologue of CD103) in the rat is restricted largely to mucosal T cells (namely IEL and lamina propria lymphocytes), while it is not expressed by most peripheral α/β T cells. In this study, a small proportion (1-2%) of the cells in the lymph nodes, spleen and TDL from rNu/+ rats expressed αE2. CD4+ cells accounted for approximately half of these cells. In contrast, a significant population (up to 20%) of α/β T cells in the lymph nodes, spleen and TDL from rNu/rNu rats expressed this molecule and most of these cells co-expressed the CD4 co-receptor. Because only 3-4% of the large T cells in TDL from rNu/+ rats expressed αE2, it is more likely that the expression of this integrin by a considerable proportion of the α/β T cells from rNu/rNu rats is related to their origin or their lineage, rather than to recent activation. It is an interesting possibility that the αE2+ T cells in euthymic rats belong to the extra-thymic lineage. Collectively, expression of αE2, CD49d and CD8αα by a considerable population of α/β T cells in rNu/rNu rats is consistent with an origin from a mucosal site such as the gut.

The UA002-antigen, recognised by mAb UA002, is expressed constitutively by dendritic cells, macrophages and B cells. However, it is not expressed by resting (small) α/β T cells in normal rats. Preliminary immunoprecipitation studies indicate that the antigen is a single polypeptide with an apparent size of approximately 85kD. The size and the distribution of the antigen are consistent with the human CD39 molecule.
Stimulation of splenocytes in vitro with concanavalin A leads to the rapid expression of this molecule by T cells, with maximal levels reached after 24-48 hours of stimulation (Murphy and Mayrhofer, unpublished). The antigen is still expressed by most α/β T cells after 96 hours of stimulation but at lower levels. These findings suggest that expression of the UA002 antigen by T cells is associated with activation and that its expression is maintained for a considerable period. It is interesting to note, therefore, that many of the α/β T cells in the TDL and lymph nodes from rNu/rNu rats express this molecule constitutively. Expression of both the UA002 antigen and MHC class II molecules by α/β T cells from rNu/rNu rats makes these cells distinct from the majority of α/β T cells in normal rats. If the UA002 antigen primarily is an activation marker, it may have an important function in the regulation of α/β T cells in rNu/rNu rats. However, its expression should also be examined among immature lymphocytes in the thymus and by lymphocytes in LFV using sensitive detection methods. Its presence on these cells was not detected by the indirect immunoperoxidase technique (Mayrhofer, unpublished).

Another molecule which was expressed by some α/β T cells from rNu/rNu rats was CD11b (Mac-1). Expression of CD11b has not been reported previously on rat α/β T cells and indeed, very few α/β T cells from rNu/+ rats were shown to express this molecule (<0.5%). Although expression of this molecule was examined in only a small number of rNu/rNu rats, in all of these animals a small proportion of α/β T cells expressed CD11b (up to approximately 5%). As a proportion, CD11b expressing lymphocytes are also rare in humans (Baars et al., 1995; de Jong et al., 1992; Rotteveel et al., 1988). Nevertheless, the CD11b+ subset of CD27+ T cells in humans are potent cytokine secreting cells (Baars et al., 1995). In the mouse, CD11b+ cell make up a small
subpopulation of the α/β T cells which co-express NK1.1 and they are believed to have an extra-thymic origin (reviewed by Taniguchi et al., 1996). It is therefore of interest that such cells are more numerous in athymic rats. It is intriguing that CD11b-expression by α/β T cells was detected only in lymph nodes of rNu/rNu rats, raising the possibility that they may represent differentiated effector cells delivered to the lymph nodes via afferent lymph.

The Thy-1 antigen (CD90) is expressed by thymocytes in rats but not by most of the peripheral α/β T cells. It has been reported, however, that small numbers of the CD90+ α/β T cells that are present in the periphery are emigrants from the thymus (Hosseinzadeh and Goldscheider, 1993). As expected, the proportions of peripheral α/β T cells that expressed CD90 in rNu/+ rats decreased with age, probably reflecting the involution of the thymus in these animals. In comparison, the proportions of α/β T cells from rNu/rNu rats that expressed this molecule remained relatively constant throughout life. If CD90 is expressed by recent emigrants from sites of extra-thymic T cell maturation, this raises the possibility that a constant number of new α/β TCR+ cells enter the periphery of rNu/rNu rats throughout life. Given that LFV are a likely site of extra-thymic T cell differentiation, expression of CD90 by lymphocytes in these structures was examined. However, it has been difficult to ascertain whether lymphocytes in LFV express CD90 because this antigen is expressed widely by non-lymphoid components of the gut lamina propria. Extra-thymic differentiation is also a possible source of the CD90+ T cells that are still present in considerable numbers in rNu/+ rats aged 39-52 weeks.
Examination of cells included in the large lymphocyte gate yielded interesting results. A greater proportion (30-50%) of large \( \alpha/\beta \) T cells in the periphery of rNu/+ rats expressed CD90 than was expressed by the whole \( \alpha/\beta \) T cell population (<10% in rats aged older than 26 weeks). Expression of CD90 by these large cells was not influenced by age (in contrast to the proportion of small cells that expressed CD90) and the intensity of staining was greater than that seen on small \( \alpha/\beta \) T cells (see Figure 4.2.27). Others have shown that CD90 can be expressed by mature \( \alpha/\beta \) T cells following activation in vitro (Paterson et al., 1987; Arthur and Mason et al., 1986) and it is likely, therefore, that CD90 expression by the large lymphocytes is as a result of activation. In contrast, CD90 was expressed by a similar proportion of the large and small \( \alpha/\beta \) T cell populations from rNu/rNu rats. This finding does not support the hypothesis that expression of CD90 by \( \alpha/\beta \) T cells in rNu/rNu rats is the result of activation. Rather, the CD90* \( \alpha/\beta \) T cells of all sizes may be recently immigrated cells and size may not be a reliable indicator of maturity in these cells. Alternatively, thymus-independent T cells may exhibit different patterns of activation marker expression compared with conventional thymus-derived T cells.

Studies should be undertaken in the future on T cells from rNu/+ and rNu/rNu rats with respect to expression of the molecules investigated in this study after activation in vitro. Activation can be achieved in vitro with anti-CD3 antibodies or by mitogen stimulation. Studies of this sort could provide interesting information about the activation phenotypes of the thymus-independent \( \alpha/\beta \) T cells from rNu/rNu rats and assist in the interpretation of the surface antigen phenotype of the cells in vivo. Further studies are needed also on the expression of surface molecules such as \( \alpha_{E2} \), MHC class II, CD49d, UA002-antigen and CD90 by lymphocytes in LFV. These will require the use of confocal microscopy.
and immuno-electronmicroscopy to resolve the localisation of antigens to lymphocyte and stromal components of LFV.

4.3.7 Characterisation of α/β T cells isolated from the livers of rNu/+ and rNu/rNu rats: A Summary

T cells that express intermediate levels of TCR (TCR\textsuperscript{intermediate} cells) are present in the livers of normal mice. Cells with an identical phenotype are found in the livers and spleens of athymic mice (Arai \textit{et al}., 1995, Iiai \textit{et al}., 1992; Watanabe \textit{et al}., 1992; Ohteki \textit{et al}., 1992). They are believed to differentiate locally in the liver, because transcripts of the genes encoding RAG-1 and RAG-2 have been detected in isolated liver mononuclear cells (Narita \textit{et al}., 1998, Kimura \textit{et al}., 1995; Sato \textit{et al}., 1995). \textit{rag-1} mRNA transcripts were also detected in mononuclear cells isolated from rat livers (Figure 3.2.4). Furthermore, the surface antigen phenotype of α/β T cells from rNu/rNu rats resembles that of TCR\textsuperscript{intermediate} cells in mice (intermediate level of TCR expression, low expression of CD62L and high levels of expression of CD11a and CD44). For these reasons, α/β TCR\textsuperscript{+} cells in mononuclear cell preparations from rat livers were investigated further. Staining of mononuclear cells (MNC) isolated from livers of rNu/+ rats with anti-α/β TCR antibodies revealed a level of TCR expression consistent with that seen in lymphocytes prepared from TDL, spleen and lymph nodes. That is, a distinct population of α/β T cells expressing an intermediate level of TCR was not detected. This could be because the difference in level of TCR expression by α/β T cells from rNu/+ and rNu/rNu rats is only 1.5-fold, whereas the difference in levels of TCR expression by α/β T cells in athymic and euthymic mice is 3-5 fold (Lawetzky and Hünig, 1988). However, this is unlikely to be the explanation and it may be the case that T cells in the livers of euthymic rats are dominated by thymus-derived classical T cells.
In contrast, α/β T cells in the livers of rNu/rNu rats, as in other sites in these animals, expressed intermediate levels of TCR. While cells of this type could be present in small numbers in euthymic rats, they are the dominant population in the livers of rNu/rNu rats. Furthermore, although α/β T cells in MNC prepared from livers of rNu/+ rats consisted of greater proportions of cells with the phenotype CD45RC−, CD25+, CD54+ and CD62Llo, the flow cytometric histogram profiles were still quite distinct from those of α/β T cells in preparations from rNu/rNu rats, especially with regard to the level of expression of CD54 (Figure 4.2.33). This raises the possibility that the liver may be a site where activated α/β T cells accumulate, perhaps as a result of the adhesion molecules that they express. In conclusion it would appear that a unique population of cells that expresses reduced levels of surface TCR is not present in the livers of normal rats. However, there should be a note of caution because studies in mice have shown that some α/β TCR+ populations are not recovered by Ficoll separation (Huang et al., 1994). This raises the possibility that a unique population of α/β T cells, corresponding in phenotype to those present in rNu/rNu rats, may not be recoverable from the livers of rNu/+ rats using this technique. The recovery of similar cells from livers of rNu/rNu rats by the same technique makes this explanation unlikely.
Chapter 5

Recirculation and redistribution characteristics of thymus-independent T cells
5.1 Introduction

In normal animals, recirculating lymphocytes in the TDL are mainly small resting cells (Gowans and Knight, 1964). Small lymphocytes migrate from the blood to secondary lymphoid organs. Migration into lymph nodes and Peyer’s patches is mediated by homing receptors and adhesion molecules that comprise selectins, members of the immunoglobulin superfamily and integrins (Springer, 1994). Large lymphocytes consist of blasts and activated cells. In TDL from normal animals, the blasts demonstrate a tropism for the gut (Gowans and Knight, 1964) and most large lymphocytes do not recirculate (Ford, 1975). Activated T cells generated in peripheral lymph nodes traffic mainly to sites of inflammation (Ford, 1975), although some may reappear in afferent lymph (reviewed by Mackay, 1993).

It has been shown recently in the rat that lymphocyte subsets demonstrate distinct recirculation kinetics. Following thoracic duct cannulation, CD4⁺ CD45RC⁺ cells are found predominantly in the early-mobilised population, while B cells and increased numbers of CD4⁺ CD45RC⁻ cells are dominant in populations that are mobilised later (Westermann et al., 1994). This suggests that naive T cells recirculate more quickly than either memory T cells or B cells, perhaps reflecting the specific pathways of traffic that these cells take through the extravascular tissues.

Naive, effector and memory T cells have distinct recirculation characteristics and these appear to be determined by the different patterns of adhesion molecules that they express (reviewed by Sprent 1997; Swain et al., 1996; Mackay 1993). Naive T cells express L-selectin (CD62L). Through the administration of blocking anti-CD62L monoclonal antibodies in the mouse, it has been shown that expression of L-selectin is very
important for lymphocyte entry into lymph nodes and Peyer's patches (Bradley et al., 1994 and reviewed by Swain et al., 1996). Expression of the α4β7 integrin is important for lymphocyte entry into gut associated lymphoid tissues (GALT). This is mediated through its counter-receptor, mucosal vascular addressin (MadCAM-1), which is expressed on the high endothelial venules (HEVs) of Peyer's patches and mesenteric lymph nodes and on the endothelium of venules in the gut mucosa (Berlin et al., 1993; Briskin et al., 1993; Schweighoffer; 1993). Following activation, a number of phenotypic changes accompany the transition from a naive T cell to an effector cell. CD62L is down-regulated, but a number of other adhesion molecules (discussed in section 1.6.2) are up-regulated (reviewed by Swain et al., 1996). The nature of the adhesion molecules that are up-regulated appears to be determined by the sites at which the cells are activated, enabling effector T cells to distribute to particular anatomical sites where their functions are required. Studies in sheep by Mackay et al. (1992) suggest that naive T cells traffic mainly through HEV of secondary lymphoid tissues while memory T cells enter lymph nodes via the afferent lymphatics. However, this may be an over-simplification, because Westermann et al. (1997) have shown directly in the rat that CD45RC+ lymphocytes also traffic through HEV.

Reflecting their high expression of CD62L, naive CD4+ T cells accumulate in large numbers in the lymph nodes and Peyer's patches 12 hours after adoptive transfer. In contrast, few T cells with an effector/memory phenotype are recovered at these sites (reviewed by Swain et al., 1996). Effector/memory T cells are, however, recovered in large numbers in the spleen and peripheral blood at this time and far outnumber naive T cells in non-lymphoid tissues such as the liver and small intestine. Within a few days, adoptively transferred memory (CD62L+) T cells are recovered in the lymph nodes and
This delayed accumulation is believed to reflect their dependence on afferent lymphatics for entry to these tissues (Bradley et al., 1994 and reviewed by Swain et al., 1996). However, recent studies in the rat (Westermann et al., 1997) have shown that CD45RC+ ('naive') and CD45RC- ('memory') CD4+ T cells enter the endothelium of HEV in lymph nodes and Peyer's patches in comparable numbers, suggesting that in this species at least, L-selectin is not the only adhesion molecule that mediates migration into lymph nodes and Peyer's patches.

As outlined above, large lymphocytes in TDL from normal rats show a tropism for the gut. Large lymphocytes consist of blasts and activated cells and hence it would be expected that many of the large T cells ion this population would express the low molecular weight form of CD45. Indeed, it has been shown in sheep that CD45RO+ (low molecular weight isoform) T cells, both small (memory) and large (blast/activated), home to the gut (Mackay et al., 1992). Also, gut homing T cells in humans and mice express α4β7 (Briskin et al., 1993) and another markers associated with a memory phenotype (Schweighoffer et al., 1993). In addition, the β7-integrin is upregulated on T cells following in vitro activation and this may also explain why activated and memory T cells distribute to the gut.

The site of activation appears to influence the tissue homing characteristics of blasts. Blasts isolated from peripheral blood, spleens and lungs of pigs demonstrated distinct tissue homing characteristics upon adoptive transfer (Binns et al., 1992). Similar percentages of blasts from each of these sources were recovered in the gastrointestinal tract (approximately 15% of blasts) but blood-derived blasts tended to stay more in the blood, splenic blasts homed to muscle and lung-derived blasts homed to the liver and
lung (Binns et al., 1992). Therefore, it would appear that blasts do not home predominantly to mucosal sites but rather to sites determined by the tissue in which they were activated. Similarly, the site of activation may influence the tissue homing characteristics of human T cells (Salmi et al., 1992). Human T cell lines isolated from peripheral blood and mucosal sites bound significantly better to mucosal and synovial HEV than to peripheral lymph node HEV \textit{in vitro}. In contrast, T cell lines from peripheral lymph nodes bound preferentially to lymph node HEV.

Given that the $\alpha/\beta$ T cells in rNu/rNu rats express MHC class II molecules and a pattern of adhesion molecules consistent with an effector/memory phenotype, it was examined whether these cells would be found in a different tissue distribution after adoptive transfer compared with the majority of $\alpha/\beta$ T cells present in the TDL from rNu/+ rats.
5.2 Results

5.2.1 Recirculation of thymus-independent α/β T cells

To examine whether the α/β T cells from rNu/rNu rats recirculate, TDL was collected from four 39 week old rNu/rNu rats. The cells were pooled and labelled with the fluorescent vital dye carboxyfluorescein diacetate succinimidy ester (CFSE). Aliquots of $5 \times 10^7$ cells were then introduced intravenously (i.v.) into two 10 week old rNu/+ recipients whose thoracic ducts had been cannulated. As a control, cells collected from the TDL of four 39 week old rNu/+ rats were also labelled with CFSE and injected i.v. into a further two 10 week old rNu/+ recipients. TDL from each of the rNu/+ recipient rats was then collected over next 72 hours in six separate 12 hour collections. The surface antigen phenotype of the CFSE-labelled cells in each collection was then determined by surface labelling with PE-conjugated monoclonal antibodies against the α/β TCR, CD4 and CD8. Two parameter flow cytometric analysis is shown in Figure 5.2.1 (rNu/+ donor cells) and Figure 5.2.2 (rNu/rNu donor cells). Because of the different proportions of α/β TCR$^+$ and α/β TCR$^-$ cells (B cells) in the thoracic duct lymphocytes obtained from the rNu/rNu and rNu/+ donors, the percentage recovery of each subset was calculated (see section 2.6.6) to enable direct comparisons to be made.

The data in Figure 5.2.3 show that the CFSE-labelled α/β TCR$^+$ cells from both rNu/+ and rNu/rNu rats recirculate through the thoracic duct with similar kinetics. In the early collections, α/β TCR$^+$ cells are the population recovered in greater abundance, with α/β TCR$^-$ cells (B cells) becoming the dominant population in the later collections. However, the percentage recovery of α/β TCR$^+$ cells from the rNu/rNu donor pool has considerably less than that from the rNu/+ donor pool, although the difference was less
Figure 5.2.1 Recovery and characterisation of CFSE-labelled cells of rNu/+ origin in recipient rats. Representative data showing the recovery of labelled cells in the fourth of six sequential 12 hour collections of thoracic duct lymph from a 10 week old recipient. The rNu/+ recipient had received $5 \times 10^7$ CFSE-labelled thoracic duct lymphocytes from 39 week old rNu/+ donor rats. Before analysis, the cells were labelled directly with (A) PE-conjugated mAb 107.3 (negative control), (B) PE-conjugated R73 (anti-$\alpha$/β TCR), (C) PE-conjugated mAb OX38 (anti-CD4) or (D) PE-conjugated OX8 (anti-CD8α). The proportion of cells in each quadrant is presented as a percentage of gated cells.
Figure 5.2.2  Recovery and characterisation of CFSE-labelled cells of rNu/rNu origin in recipient rats. Representative data showing the recovery of labelled cells in the fourth of six sequential 12 hour collections of thoracic duct lymph from a 10 week old recipient. The rNu/+ recipient had received $5 \times 10^7$ CFSE-labelled thoracic duct lymphocytes from 39 week old rNu/rNu donor rats. Before analysis, the cells were labelled directly with (A) PE-conjugated mAb 107.3 (negative control), (B) PE-conjugated R73 (anti-$\alpha/\beta$ TCR), (C) PE-conjugated mAb OX38 (anti-CD4) or (D) PE-conjugated OX8 (anti-CD8$\alpha$). The proportion of cells in each quadrant is presented as a percentage of gated cells.
Figure 5.2.3  Sequential recovery of CFSE-labelled donor cells in thoracic duct lymph. Thoracic duct lymph (TDL) was collected over six sequential 12 hour periods from 10 week old rNu/+ recipients which had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred α/β TCR+ and α/β TCR− cells in each collection was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in the 12 hour collection by the percentage (+100) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). Each data set represents the mean of 2 rats ± SD.
Recovery of CFSE-labelled cells in TDL

Recovery (± SD)

12 hr collections

rNu/+ TCR+  rNu/rNu TCR+

rNu/+ TCR-  rNu/rNu TCR-

1  2  3  4  5  6
marked in later collections (collections 4-6). Overall however, the numbers of α/β TCR+ cells collected from the TDL over 72 hours was about two-fold less in the case of rNu/rNu donor cells (i.e. approximately 35% of injected CFSE-labelled rNu/+ α/β TCR+ cells were recovered, compared with approximately 21% of CFSE-labelled rNu/rNu α/β TCR+ cells). In contrast, the percentages of the α/β TCR- (B cells) recovered over all time points was very similar, irrespective of the source of donor cells. The percentage of α/β TCR- cells recovered was in the order of 35% for both rNu/+ and rNu/rNu donors (Figure 5.2.4 [A]).

Analysis of the recovery of the CD4+ and CD8+ subsets revealed some interesting differences between cells from rNu/+ and rNu/rNu donors. The ratio of CFSE-labelled CD4+ cells to CFSE-labelled CD8+ cells in the lymphocyte pools from both rNu/+ and rNu/rNu donors that were injected into recipient rats was approximately 6:1. In the case of cells from rNu/+ donors, the ratio of CD4+ cells to CD8+ cells in the TDL collected from the recipients declined gradually (from >10:1 at collection 1 to <6:1 at collection 6, Figure 5.2.5). Because similar percentages of injected CD4+ and CD8+ cells from rNu/+ donors were recovered (approximately 35% for both subsets, Figure 5.2.4 [B]), this indicates that CD4+ cells recirculate faster than CD8+ cells. In contrast, the ratio of CD4+ to CD8+ cells recovered after transfer of TDL from rNu/rNu donors increased over the first 12-24 hours and then remained unchanged with a ratio in excess of 11:1 (Figure 5.2.5). Approximately 27% of the CD4+ cells that were injected were recovered in 72 hours, compared with approximately 11% of CD8+ cells (Figure 5.2.4 [B]). It appears, therefore, that CD8+ T cells from rNu/rNu donors have either a shorter life-span than CD4+ cells after adoptive transfer or that they become sequestered from the recirculating pool.
Figure 5.2.4  Total recovery of CFSE-labelled subsets in thoracic duct lymph. Thoracic duct lymph (TDL) was collected over six sequential 12 hour periods from 10 week old rNu/+ recipient rats which had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ donors or 39 week old rNu/rNu donors. The percentage recovery of adoptively transferred cells in each collection was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in the 12 hour collection by the percentage (+100) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). The individual recoveries were then summed to give the total recovery over the 72 hour collection period.

A.  Total Recovery of \(\alpha/\beta\) TCR\(^+\) and \(\alpha/\beta\) TCR\(^-\) cells after 72 hours of collection

B.  Total Recovery of CD4\(^+\) and CD8\(^+\) cells after 72 hours of collection

Each data set represents the mean of 2 rats ± SD.
Recovery of CFSE-labelled Subsets in TDL

A

Recovery of CFSE-labelled Subsets in TDL

![Bar graph showing percentage recovery of TCR+ and TCR- subsets with error bars.]

B

Recovery of CFSE-labelled Subsets in TDL

![Bar graph showing percentage recovery of CD4+ and CD8+ subsets with error bars.]

Legend:
- rNu/+  
- rNu/rNu
Figure 5.2.5  Ratio of donor CD4* cells to CD8* cells recovered in thoracic duct lymph. Thoracic duct lymph (TDL) was collected over six sequential 12 hour periods from 10 week old rNu/+ recipient rats which had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ donor rats or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred cells in each collection was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in the 12 hour collection by the percentage (+100) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2).

Ratio: Percentage of recovered CD4* cells + Percentage of recovered CD8* cells

Each data set represents the mean of 2 rats ± SD.
CD4:CD8 Ratio in Recovered TDL

- rNu/+ (lighter bars)
- rNu/rNu (darker bars)

12 hr collections

0 2 4 6 8 10 12 14 16 18
CD4:CD8 (+/- SD)
Figure 5.2.6  Sequential recovery of CFSE-labelled donor cells in thoracic duct lymph of rNu/rNu rats. Thoracic duct lymph (TDL) was collected over six sequential 12 hour periods from 10 week old rNu/rNu rats which had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ donor rats or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred α/β TCR⁺ and α/β TCR⁻ cells from both rNu/+ and rNu/rNu donor rats origin in each collection was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in the 12 hour collection by the percentage (+100) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). Each data set represents the mean of 2 rats ± SD.
Recovery of CFSE-labelled cells in TDL
This experiment was repeated using 26 week old donor rats and in this case the recipients were 10 week old rNu/rNu rats (Figure 5.2.6). As observed with rNu/+ recipients, similar proportions of \( \alpha/\beta \) TCR\(^+\) cells from rNu/+ and rNu/rNu donors (approximately 15\%) were collected over the 72 hours. This recovery was, however, less than half that observed in rNu/+ recipients of the same age (approximately 35\%). With respect to \( \alpha/\beta \) TCR\(^+\) cells, approximately 20\% of the injected \( \alpha/\beta \) TCR\(^+\) cells from rNu/+ donors were recovered from the rNu/rNu recipients, in comparison with approximately 6\% of \( \alpha/\beta \) TCR\(^+\) cells from rNu/rNu donors. These recoveries were considerably lower than those obtained when rNu/+ rats were used as recipients (35\% and 21\% respectively for \( \alpha/\beta \) TCR\(^+\) cells obtained from rNu/+ and rNu/rNu donors). Although fewer labelled cells are recovered, the appearance of \( \alpha/\beta \) TCR\(^+\) cells and \( \alpha/\beta \) TCR\(^-\) cells in the TDL from rNu/rNu recipients (Figure 5.2.6) followed a similar pattern to that observed in rNu/+ recipients (Figure 5.2.3). It appears that the tempo of recirculation is slower in rNu/rNu recipients, perhaps reflecting the less developed HEV that have been described in the lymph nodes of these animals (Fossum et al., 1983).

### 5.2.2 Distribution of thymus-independent \( \alpha/\beta \) T cells

To compare the tissue distribution of \( \alpha/\beta \) TCR\(^+\) cells from rNu/rNu and rNu/+ donor rats after intravenous transfer, aliquots of CFSE-labelled cells that were prepared from the TDL from 39 week old rats (described above) were transferred adoptively into normal (no thoracic duct cannula) 10 week old rNu/+ and rNu/rNu recipient rats. The recipient rats were bled 24 hours later, sacrificed and their tissues harvested. Lymphocytes prepared from various organs were labelled with PE-conjugated mAbs and analysed by dual-fluochrome flow cytometry as described above. As shown in Figure 5.2.7 and Figure 5.2.8, the tissue distribution of \( \alpha/\beta \) TCR\(^+\) cells (CFSE-labelled) from rNu/rNu
donor rats was different to that observed when the donor cells were obtained from rNu/+ rats. In contrast, the distribution of α/β TCR cells (B cells) of rNu/+ and rNu/rNu origin was very similar, thus providing a useful internal control. As summarised in Figure 5.2.10 (A) and shown individually in Figures 5.2.7 (A), 5.2.7 (B) and 5.2.8 (A) respectively, the α/β TCR+ cells of rNu/+ origin distributed in approximately equal proportions to the peripheral lymph nodes, mesenteric lymph nodes and the spleen, while very few were recovered from the liver (Figure 5.2.8 [B]) or Peyer’s patches (Figure 5.2.7 [C]). In contrast, a larger proportion of the α/β TCR+ cells of rNu/rNu origin accumulated in the spleen (rNu/+ : rNu/rNu = 1:2-2.5) but smaller proportions were recovered in the peripheral (rNu/+ : rNu/rNu = 2:1) and mesenteric (rNu/+ : rNu/rNu = 3:2) lymph nodes. A far greater proportion of α/β TCR+ cells of rNu/rNu origin were recovered in the liver (Figure 5.2.8 [B]) compared to those of rNu/+ origin (rNu/+ : rNu/rNu = 1:6). Indeed, the numbers of α/β TCR+ cells from rNu/rNu donors that were recovered in the liver were similar to those recovered from the peripheral lymph nodes (Figures 5.2.8 [B] and 5.2.10 [A]). Furthermore, a larger proportion of α/β TCR+ cells from rNu/rNu donors were recovered from the Peyer’s patches (Figure 5.2.7 [C]) compared with those from rNu/+ donors (rNu/+ : rNu/rNu = 1:4).

Studies in transgenic mice (reviewed by Swain et al., 1996) have shown that 12 hours after injection, memory (low molecular weight isoform of CD45) CD4+ cells fail to localise in peripheral and mesenteric lymph nodes and Peyer’s patches. They are, however, identified in the blood, spleen and to a larger extent than naive CD4+ cells, in the small intestine and the liver. The distribution of α/β TCR+ cells from rNu/rNu rats has, therefore, similarities with that described for memory cells. However, the appearance of considerable numbers of α/β TCR+ cells of rNu/rNu origin in the lymph
nodes and Peyer's patches suggests that their memory/effector phenotype does not prevent them from entering these sites. Furthermore, the proportions of α/β TCR+ cells that were recovered in the blood were similar whether they originated from rNu/+ or rNu/rNu donors (Figure 5.2.8 [C]).

In all tissues except the Peyer's patches, the proportion of CD4+ cells that was recovered was considerably greater than the recovery of CD8+ cells, leading to a ratio of greater than one (Figure 5.2.9). However, the ratio of CD4+ to CD8+ cells varied from tissue to tissue. The CD4+ and CD8+ cells obtained from rNu/+ and rNu/rNu donors were distributed approximately equally to the peripheral lymph nodes and Peyer's patches. However, greater numbers of CD4+ cells relative to CD8+ cells were distributed to the spleen, liver and mesenteric lymph nodes when the donors were rNu/rNu rats than was the case when the donors were rNu/+ rats.

Comparison of the distributions of CFSE-labelled cells from rNu/+ and rNu/rNu donors in rNu/rNu recipient rats (Figure 5.2.10 [B]) revealed a pattern that has some similarities to those seen in rNu/+ recipients (Figure 5.2.10 [A]). However, some differences were evident. Of the injected cells, only approximately half as many α/β TCR+ cells of rNu/rNu origin were recovered in the spleens and livers of rNu/rNu recipients as were recovered in rNu/+ recipients. Also, two to three times as many CFSE-labelled cells (particularly of rNu/+ origin) were recovered in the peripheral lymph nodes of rNu/rNu recipient rats. As mentioned above, this observation could reflect the fact that the post-capillary venules (PCV) in the lymph nodes of rNu/rNu rats are flatter than those present in rNu/+ rats. As a result, lymphocytes may not be selected from the circulation as efficiently by these PCV (Fossum et al., 1983). A consequence maybe that at 24 hours
Figure 5.2.7 Recovery of CFSE-labelled donor cells in lymphoid tissues of recipient rats. Cells were prepared from tissues of 10 week old rNu/+ recipient rats 24 hours after they had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ donor rats or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred $\alpha/\beta$ TCR$^+$ and $\alpha/\beta$ TCR$^-$ cells in each tissue preparation was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in each tissue preparation by the percentage ($\pm 100$) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). Each data set represents the mean of 2 rats $\pm$ SD.

A. Recovery of CFSE-labelled $\alpha/\beta$ TCR$^+$ and CFSE-labelled $\alpha/\beta$ TCR$^-$ cells of rNu/+ and rNu/rNu origin from peripheral lymph nodes

B. Recovery of CFSE-labelled $\alpha/\beta$ TCR$^+$ and CFSE-labelled $\alpha/\beta$ TCR$^-$ cells of rNu/+ and rNu/rNu origin from mesenteric lymph nodes

C. Recovery of CFSE-labelled $\alpha/\beta$ TCR$^+$ and CFSE-labelled $\alpha/\beta$ TCR$^-$ cells of rNu/+ and rNu/rNu origin from Peyer's patches
A. Recovered cells from Peripheral Lymph Nodes

B. Recovered Cells from Mesenteric Lymph Nodes

C. Recovered Cells from Peyer's Patches

- TCR+ and TCR- CFSE-Labelled Cells
- Percentage Recovery (±SD)
- rNu/+ and rNu/rNu
Figure 5.2.8 Recovery of CFSE-labelled cells in other tissues and blood of recipient rats. Cells were prepared from tissues and blood of 10 week old rNu/+ rats 24 hours after they had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred $\alpha/\beta$ TCR$^+$ and $\alpha/\beta$ TCR$^-$ cells in each tissue preparation was determined using the following formula:

$$\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100$$

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in each tissue preparation by the percentage (+100) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). Each data set represents the mean of 2 rats ± SD.

A. Recovery of CFSE-labelled $\alpha/\beta$ TCR$^+$ and CFSE-labelled $\alpha/\beta$ TCR$^-$ cells of rNu/+ and rNu/rNu origin from spleens of recipient rats

B. Recovery of CFSE-labelled $\alpha/\beta$ TCR$^+$ and CFSE-labelled $\alpha/\beta$ TCR$^-$ cells of rNu/+ and rNu/rNu origin from livers of recipient rats

C. Recovery of CFSE-labelled $\alpha/\beta$ TCR$^+$ and CFSE-labelled $\alpha/\beta$ TCR$^-$ cells of rNu/+ and rNu/rNu origin from peripheral blood of recipient rats
**A**

Recovered Cells from Spleen

<table>
<thead>
<tr>
<th>CFSE-Labelled cells</th>
<th>rNu/+</th>
<th>rNu/rNu</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCR-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

Recovered Cells from Liver

<table>
<thead>
<tr>
<th>CFSE-Labelled cells</th>
<th>rNu/+</th>
<th>rNu/rNu</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCR-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

Recovered Cells from Peripheral Blood

<table>
<thead>
<tr>
<th>CFSE-Labelled cells</th>
<th>rNu/+</th>
<th>rNu/rNu</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCR-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.2.9  Ratio of donor CD4\(^+\) and CD8\(^+\) cells recovered from tissues of recipient rats. Cells were prepared from tissues of 10 week old rNu/\(^+\) rats 24 hours after they had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/\(^+\) donor rats or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred CD4\(^+\) cells and CD8\(^+\) cells in each tissue preparation was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in each tissue preparation by the percentage (\(\times 100\)) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). Each data set represents the mean of 2 rats ± SD.

\[
\text{Ratio: Percentage of recovered CD4}^+\text{ cells} \div \text{Percentage of recovered CD8}^+\text{ cells}
\]

Each data set represents the mean of 2 rats ± SD.
CD4:CD8 Ratio of Recovered Cells

- Spleen
- pLN
- mLN
- Liver
- Blood
- PPs

Tissue

CD4:CD8 (± SD)

- rNu/+
- rNu/rNu
Figure 5.2.10  Comparison of the total recoveries of CFSE-labelled donor cells in rNu/+ and rNu/rNu recipients. Cells were prepared from the tissues of 10 week old rNu/+ and 10 week old rNu/rNu recipient rats 24 hours after they had been administered CFSE-labelled thoracic duct lymphocytes from either 39 week old rNu/+ or 39 week old rNu/rNu donor rats. The percentage recovery of adoptively transferred α/β TCR⁺ cells and α/β TCR⁻ cells in each tissue preparation was determined using the following formula:

\[
\text{Percentage recovery} = \frac{\text{Number of CFSE-labelled cells recovered}}{\text{Number of CFSE-labelled cells injected}} \times 100
\]

The number of CFSE-labelled cells recovered was calculated by multiplying the total number of lymphocytes in each tissue preparation by the percentage (+100) of recovered CFSE-labelled cells (see Figures 5.2.1 and 5.2.2). Each data set represents the mean of 2 rats ± SD.

A. Recovery of CFSE-labelled α/β TCR⁺ and CFSE-labelled α/β TCR⁻ cells of rNu/+ and rNu/rNu origin from the tissues of rNu/+ recipient rats.

B. Recovery of CFSE-labelled α/β TCR⁺ and CFSE-labelled α/β TCR⁻ cells of rNu/+ and rNu/rNu origin from the tissues of rNu/rNu recipient rats.
Recovered CFSE-labelled cells (rNu/+ recipients)

- rNu/+ TCR+
- rNu/rNu TCR+
- rNu/+ TCR-
- rNu/rNu TCR-

Percentage Recovery (+/SD)

Spleen, pLN, mLN, Liver, Blood, PPs

Recovered CFSE-labelled cells (rNu/rNu recipients)

- rNu/+ TCR+
- rNu/rNu TCR+
- rNu/+ TCR-
- rNu/rNu TCR-

Percentage Recovery (+/SD)

Spleen, pLN, mLN, Liver, Blood
after adoptive transfer, more lymphocytes may still be in transit through the lymph
nodes of rNu/rNu recipients than would be the case in rNu/+ rats with normal PCV.
This observation could help explain why fewer CFSE-labelled cells recirculated and
were recovered in the TDL of rNu/rNu recipients (see above).

As discussed above, increased numbers of memory CD4+ cells have been reported to
distribute to non-lymphoid tissues such as the small intestine, compared with secondary
lymphoid tissues (Mackay et al., 1992 and reviewed by Swain et al., 1996; Mackay
1993). Since most α/β TCR+ cells in the TDL from rNu/rNu rats do not express
CD45RC and many express CD49d, it was anticipated that more T cells would distribute
to the small intestine when the donors were rNu/rNu rats than when the cells were
obtained from rNu/+ donors. Attempts were made to isolate lamina propria lymphocytes
from the intestines of recipient rats but these were unsuccessful. Therefore, fluorescence
and confocal microscopy were employed to identify CFSE-labelled cells in tissue
sections prepared from the small intestines and from other tissues of recipient rats. As
shown in Figure 5.2.71, CFSE-labelled cells could be detected readily using confocal
microscopy in most tissues examined. By the use of dual immunofluorescence (anti-α/β
TCR-PE) in combination with the fluorescence of CFSE, it was observed that most
donor cells from rNu/rNu rats that were identified in T-dependent areas of the spleen
and lymph nodes expressed the α/β TCR (illustrated in Figure 5.2.11). CFSE-labelled T
cells were detected in the small intestine but their low frequency in sections made
counting to measure differences in the distribution patterns of cells derived from rNu/+ and
rNu/rNu donors impractical.
Figure 5.2.11  Confocal microscopic images of CFSE-labelled rNu/rNu donor cells identified in a section of mesenteric lymph node. Mesenteric lymph nodes were removed from a 10 week old rNu/+ rat 24 hours after it had received 5×10^7 CFSE-labelled thoracic duct lymphocytes from 39 week old rNu/rNu donor rats. Freshly cut 5μm tissue sections were air dried before fixation in 95% ethanol. The tissue sections were then incubated with mAb W3/25 (anti-CD4) and bound antibody was detected indirectly with PerCP.

A. CFSE-labelled cells

B. CD4^+ cells

C. Merged image

Bar = 40μm
5.2.3 Purification of CD4$^+$ T cells from rNu/rNu rats results in cell death

To obviate the need to identify CFSE-labelled cells as T cells by dual fluochrome fluorescence and therefore improve detection of donor CD4$^+$ T cells in the small intestine of recipient rats, CD4$^+$ α/β TCR$^+$ cells were purified prior to labelling with CFSE and adoptive transfer. TDL from four 52 week old rNu/+ and four 52 week old rNu/rNu rats was used to obtain donor cells for transfer into 10 week old rNu/+ recipients.

Pooled thoracic duct lymphocytes from each group of donors were incubated with a cocktail of mAbs against CD8β, the γ/δ TCR, CD161 (NKR-P1), CD11b, surface immunoglobulin and the B cell isoform of CD45, before incubating with anti-mouse immunoglobulin antibodies coupled to magnetic beads. The purity of the resultant populations of CD4$^+$ T cells selected negatively in this way from the pooled thoracic duct lymphocytes from rNu/+ and rNu/rNu donors was approximately 99% and 96% respectively. The CD4$^+$ cells were then labelled with CFSE before administration of 2×10$^7$ labelled cells i.v. into each of the recipient rats. Twenty-four hours after adoptive transfer, cells were prepared from the various tissues, as outlined above.

Surprisingly, no CFSE-labelled cells were recovered from any of the tissues obtained from animals which had received purified CD4$^+$ cells from rNu/rNu rats. Furthermore, CFSE-labelled cells were not detected by fluorescence microscopy in tissue sections. In contrast, CFSE-labelled CD4$^+$ cells from rNu/+ donors could be recovered in all tissues, although the recovery was considerably lower (approximately half of that expected) than that obtained after transfer of unseparated cells (data not shown).
Failure to detect CFSE-labelled CD4\(^+\) cells of rNu/rNu origin suggested that these cells do not survive after transfer to the recipient animals. During purification of CD4\(^+\) cells from rNu/rNu donors, it was observed that the final yield was lower than expected and that the viability assessed by Trypan-blue exclusion was only 80\% (typically, CFSE-labelled TDL had a viability of >99\%). To address whether the purification procedure had affected the viability, cells from the TDL of a 26 week old rNu/rNu rat and an age-matched rNu/+ rat were subjected to the purification protocol outlined above. The purity of the CD4\(^+\) cells from the rNu/+ donor was approximately 98\% (Figure 5.2.12 [A]), while those prepared from the rNu/rNu animal had purity of 95\% (Figure 5.2.12 [B]). Dual-fluochrome analysis revealed that essentially all of the CD4\(^+\) cells expressed the \(\alpha/\beta\) TCR. Following purification, which was conducted on ice, the CD4\(^+\) cells were incubated at room temperature in culture medium (RPMI+5\%FCS) for three hours and then examined for the presence of apoptotic and pre-apoptotic cells. Annexin V conjugated directly to FITC binds to phosphatidylserine present on the surface of apoptotic and pre-apoptotic cells and exposed in necrotic cells due to loss of membrane integrity, while propidium iodide (PI), which stains the DNA of permeable cells, was used to identify necrotic and late stage apoptotic cells. When labelled cells were examined by flow cytometry after three hours of culture, it was found that many more (27.6\%) of the CD4\(^+\) cells from the rNu/rNu rat had low FSC and SSC characteristics (dead cells) (Figure 5.2.13 [B]) compared with the preparation from the rNu/+ donor (13.6\%) (Figure 5.2.13 [A]). When the events with reduced light scatter were gated and analysed, it was found that the cells in this gate were PI\(^+\) and that they bound Annexin V (Figure 5.2.13 [E]; rNu/+ and [F]; rNu/rNu). When the cells with the scatter characteristics of viable cells were gated electronically, only a few (2.7\%) PI\(^+\) cells were evident among purified CD4\(^+\) cells from the rNu/+ donor (Figure 5.2.13 [E]).
Surprisingly, a high proportion of rNu/+ donor cells displaying viable scatter characteristics bound Annexin V (18.5%) and therefore were pre-apoptotic by definition. Considerable numbers of the purified CD4+ cells from the rNu/rNu donor that fell within the viable scatter gate bound Annexin V and were PI (12.1%), hence were also pre-apoptotic. However, a large proportion also stained with PI with varying intensity (20.3%) (Figure 5.2.13 [D]). This variable level of PI staining may reflect ongoing apoptosis among purified CD4+ cells from the TDL of the rNu/rNu rat.
Figure 5.2.12  Immuno-magnetic bead purification of CD4+ cells. CD4+ cells were purified by negative selection from the thoracic duct lymphocytes of a 26 week old rNu/+ rat and a 26 week old rNu/rNu rat. The purity of the cells from (A) the rNu/+ rat and (B) the rNu/rNu rat was assessed by dual parameter flow cytometry. Cells were labelled first by the indirect immunofluorescence technique with mAb R73 (anti-α/β TCR; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). The proportion of gated cells in each quadrant is presented as a percentage.
Figure 5.2.13  Detection of apoptosis and cell death by Annexin V binding and staining with propidium iodide. Purified CD4\(^+\) cells from a rNu/+ rat (A, C and E) and a rNu/rNu rat (B, D and F) were cultured for 3 hours. After this time, the cells were incubated with Annexin V-FITC and propidium iodide (PI) to assess cell viability.  (A and B) Forward scatter (FSC) and side scatter (SSC) dotplots of purified cells 3 hours after culturing. The cells lying in the ‘viable’ cell gate have the FSC and SSC characteristics of freshly obtained thoracic duct lymphocytes while those lying in the ‘dead’ cell gate have the reduced FSC and SSC characteristics of dead cells.  (C and D) Annexin V and PI staining of cells lying in the ‘viable’ cell gate.  (E and F) Annexin V and PI staining of cells lying in the ‘dead’ cell gate. The proportion of gated cells in each quadrant is presented as a percentage.
As a result of the experimental conditions, the percentage of dead cells was determined to be 13.6% and the viable cells were found to be 79.2%. Conversely, in a different condition, the dead cell percentage increased to 27.6% while the viable cell percentage decreased to 47.6%.

The data was further analyzed using Annexin V-FITC measurements, which revealed different PI values (0.1, 2.7, 18.5, 20.3, and 12.1) indicating the progression or status of cell death.
5.3 Discussion

5.3.1 Recirculation of thymus-independent α/β T cells: A summary

It has been shown previously that CD45RC⁺ α/β T cells recirculate through TDL more quickly than CD45RC⁻ α/β T cells and B cells (Westermann et al., 1994). The results presented in this chapter are therefore of interest as they show that many of the predominantly CD45RC⁻ α/β TCR⁺ cells present in rNu/rNu rats recirculate with a pattern similar to the α/β TCR⁺ cells (predominantly CD45RC⁺) derived from rNu/+ rats. However, a lesser proportion of these thymus-independent α/β TCR⁺ cells were recovered in the TDL during the 72 hour collection period, suggesting that an increased number are retained at various anatomical sites and that they either enter the recirculating pool more slowly than conventional T cells or they have a lower survival. However, even after 72 hours the TDL from recipients of cells from rNu/rNu rats contained fewer donor-derived α/β TCR⁺ cells than TDL from recipients of cells from rNu/+ rats. When the thoracic ducts of normal rats are cannulated the proportion of CD4⁺ T cells that have a memory phenotype increases steadily while the proportion of cells with naive phenotype decreases over a 60 hour collection period (Westermann et al., 1994). This suggests that memory T cells recirculate more slowly than naive T cells in normal animals. Because most of the T cells of rNu/rNu origin recovered in the TDL of recipient rats were CD4⁺ (approximately 90%) and were most likely CD45RC⁻ (given that greater than 80% of the injected CD4⁺ cells had this phenotype) it is interesting to note that these cells recirculated with similar kinetics to the predominantly CD45RC⁺ α/β T cells derived from rNu/+ rats. However, because rat CD45RC⁻ CD4⁺ T cells have been shown to express CD45RC rapidly after adoptive transfer (Bell and Sparshott, 1990) and in vitro (Sarawar et al., 1993), it is interesting to speculate that at
least some of the recirculating $\alpha/\beta$ T cells of rNu/rNu origin might express CD45RC. It would be of interest to further examine the phenotype of the recirculating $\alpha/\beta$ T cells of rNu/rNu origin.

It is of interest that fewer $\alpha/\beta$ T cells of rNu/rNu origin were recovered in the TDL from recipient rats compared to T cells of rNu/+ origin. This may reflect the fact that after adoptive transfer, a higher proportion of thymus-independent T cells migrate to specific tissues and do not recirculate, at least initially. If this is the case, then it would be predicted that increased numbers of these cells would be observed in the TDL of recipient rats at later time points, reflecting the increased length of time these cells spend at specific anatomical sites. However, this was not the case. An alternative explanation is that the $\alpha/\beta$ T cells obtained from rNu/rNu rats are short-lived cells. This would account for the reduced recovery of these cells in recipient rats, compared to the $\alpha/\beta$ T cells obtained from rNu/+ rats. The finding that increased numbers of $\alpha/\beta$ T cells from the TDL and tissues of rNu/rNu rats have an activated phenotype (as detailed in section 4.2.4) may account for this increase in cell death. This is because activated T cells have specific requirements for survival and are hence less stable following adoptive transfer.

Also of interest was the finding that CD8$^+$ cells from rNu/rNu rats appear to have different characteristics compared to the corresponding cell population from rNu/+ rats, with respect to recirculation. In support of data published previously (Westermann et al., 1994), the ratio of CD4$^+$ cells to CD8$^+$ cells from rNu/+ donors remained relatively stable (approximately 6:1) in the TDL collected from recipients and this is
reflected in the finding that the percentage recovery of the subsets was similar following adoptive transfer. In contrast however, the recovery of CD8+ cells derived from rNu/rNu rats in cannulated recipients was considerably less than the recovery of CD4+ cells. Indeed, donor CD8+ cells remained an extremely minor population throughout the collection period. This indicates that CD8+ cells in rNu/rNu rats, like CD4+ subset, demonstrate different recirculation characteristics compared to the corresponding populations in phenotypically normal rNu/+ littermates. Two main possibilities could account for these observations. As discussed above in respect to the recovery of CD4+ cells, perhaps the CD8+ cells from rNu/rNu rats are short-lived cells and the observation that fewer of these cells are recovered in the TDL of recipient animals may reflect their increased rate of cell death. Alternatively, these cells could distribute to distinct sites where they are detained and are only slowly released into the recirculatory pool. Mucosal tissues are likely sites for the retention of α/β T cells derived from rNu/rNu rats, as these cells express high levels of the α4-integrin (see section 4.2.5) and a considerable proportion express αE2, a gut-associated integrin (see section 4.2.6.1).

A potential area of investigation that arises from these studies concerns the fate of the α/β T cells present in the TDL of rNu/rNu rats aged 13 weeks. These cells are larger in size than the majority of α/β T cells present in the TDL of rNu/rNu rats aged greater than 26 weeks (Figure 4.2.12) and it is interesting to hypothesise that this large size corresponds to an immature phenotype. Recent thymic emigrants (RTE) are larger than the majority of α/β T cells and are distributed throughout the peripheral lymphoid tissues of young rats. If the large α/β T cells present in the TDL of rNu/rNu rats represent newly formed cells, then it is feasible that they migrate to the peripheral lymphoid organs of these animals, where they become more evident with increasing age.
To examine the life-span of α/β T cells in rNu/rNu rats aged approximately 13 weeks and their ability to seed peripheral lymphoid tissues, a congenic model would be an advantage. α/β T cells isolated from the TDL of 13 week old rNu/rNu rats could be transferred adoptively into thymectomised and irradiated recipients to determine whether they can give rise to a population of more mature (cells of smaller size) α/β T cells. It would also be of interest to compare the ability of α/β T cells obtained from the TDL of rNu/rNu rats aged greater than 26 week and those procured from euthymic littermates to reconstitute irradiated recipients. These experiments would also address the life-span and turnover of α/β T cells obtained from rNu/rNu rats in comparison to those obtained from phenotypically normal rNu/+ rats.

5.3.2 Distribution of thymus-independent α/β T cells: A summary

Following adoptive transfer, α/β T cells from rNu/rNu rats were found to have a different distribution when compared with α/β T cells obtained from TDL of rNu/+ rats. The recovery of CFSE-labelled α/β T cells obtained from the thoracic ducts of rNu/rNu rats aged greater than 26 weeks of age in the livers and spleens of recipient rats 24 hours after adoptive transfer was considerably higher than that observed for α/β T cells derived from age-matched rNu/+ rats. Cells from rNu/rNu donors were also recovered in higher numbers in the Peyer's patches but fewer were recovered in the lymph nodes.

The expression of high levels of CD49d by α/β TCR+ cells isolated from TDL of rNu/rNu rats, together with the finding that a significant proportion of these cells express the αE2-integrin (discussed in Chapter 4), suggests that these cells may distribute preferentially to mucosal tissues. This would be anticipated as both mouse (Hamann
et al., 1994) and human (Briskin et al., 1993) gut-tropic T cells have a memory phenotype (shared by the $\alpha/\beta$ TCR$^+$ cells of rNu/rNu rats) and express high levels of CD49d. MadCAM-1, a receptor for the $\alpha_4$-integrins (VLA-4 [$\alpha_4\beta_1$] and $\alpha_4\beta_1$), is expressed by the HEV of Peyer’s patches and mesenteric lymph nodes and also by venules in the mucosa (Briskin et al., 1993). This could explain why a greater proportion of the $\alpha/\beta$ T cells of rNu/rNu origin, compared to those isolated from rNu/+ rats, were recovered from Peyer’s patches (Figure 5.2.7 [C]). It may also account for the finding that more $\alpha/\beta$ TCR$^+$ cells from the TDL of rNu/rNu rats accumulated in the mesenteric lymph nodes, rather than in lymph nodes at peripheral sites. Because of these findings, it is anticipated that increased numbers of $\alpha/\beta$ T cells from TDL of rNu/rNu rats would also be recovered in the lamina propria of the small intestine of recipient rats. This proved hard to demonstrate by fluorescence microscopy on tissue sections, because of the sparsity of labelled cells. The development of a reliable method for isolating rat lamina propria lymphocytes, coupled with flow cytometry, should overcome this and provide important information on the recirculation characteristics of T cells that develop independently of the thymus.

It has been established using TCR-transgenic mice that the tissue distribution of CD4$^+$ T cells expressing a memory phenotype is different from that of naive CD4$^+$ T cells after adoptive transfer (reviewed in Swain et al., 1996; Bradley et al., 1993; Mackay 1993). Cells with a memory phenotype are recovered in low numbers in Peyer’s patches and lymph nodes, possibly reflecting their CD62L$^-$ phenotype (Bradley et al., 1994). They are, however, recovered in considerable numbers in the spleen and peripheral blood (reviewed by Swain et al., 1996) and in greater proportion than naive cells in the liver (reviewed by Swain et al., 1996) and small intestine (reviewed by Swain et al., 1996,
Mackay 1993). Based on these criteria, \(\alpha/\beta\) T cells derived from TDL of rNu/rNu rats do not distribute in a manner typical of memory T cells. Firstly, after adoptive transfer, the numbers of \(\alpha/\beta\) T cells that were recovered from the blood of recipients were similar whether the donors were rNu/rNu rats or rNu/+ rats. In contrast to reports that twice as many naive cells as memory cells are recovered from the spleen after adoptive transfer (reviewed by Swain et al., 1996), the proportions of donor \(\alpha/\beta\) T cells recovered from the spleens of recipients of TDL from rNu/rNu donors were twice those of recipients of rNu/+ TDL. Furthermore, although the majority of \(\alpha/\beta\) T cells from TDL of rNu/rNu rats are CD62L', significant numbers of these cells were recovered in the lymph nodes. Although \(\alpha/\beta\) T cells from rNu/+ rats distributed in higher numbers to these sites compared with cells from rNu/rNu donors (twice as many in the peripheral lymph nodes and one and a half times as many in the mesenteric lymph nodes), the recovery of \(\alpha/\beta\) T cells from rNu/rNu donors was, nevertheless, considerable. Some of these cells may have entered the lymph nodes via afferent lymphatics, as has been proposed for memory T cells. However, given their CD62L' phenotype (Bradley et al., 1994), it is noteworthy that others have shown that both the CD45RC- and CD45RC+ subsets of CD4+ T cells from normal rats can transverse the HEV of lymph nodes and Peyer’s patches (Westermann et al., 1997). It is possible, therefore, that the predominantly CD45RC- T cells from rNu/rNu rats enter lymph nodes and Peyer’s patches via HEV. Furthermore, some transverse the lymph nodes and enter the efferent lymph with kinetics similar to CD4+ T cells from normal donors (see above). The majority of adoptively transferred \(\alpha/\beta\) T cells obtained from rNu/rNu rats exhibited a CD45RC- phenotype. It would, nevertheless, be of interest to compare the surface phenotypes of those thymus-independent \(\alpha/\beta\) T cells that recirculate quickly through TDL with those that do not. It might be anticipated that those that recirculate rapidly through the TDL
might have a different surface phenotype and that these differences might not correlate with the expression of CD45 isoforms. With respect to the distribution of thymus-independent T cells, it is apparent that although these cells share surface antigen phenotypes similar to those of memory T cells, they are quite distinct from the memory population in normal animals. If a similar thymus-independent population exists in normal animals the surface antigen phenotype, recirculation and tissue distribution characteristics of these cells may be integral to their functions and are distinct from most conventional T cells.

The distribution characteristics of $\alpha/\beta$ TCR$^+$ cells from rNu/rNu rats were different compared with those from normal rNu/+ littermates. Because they have a memory/effector phenotype with respect to expression of adhesion molecules, it would be of interest to examine the recruitment of these cells to inflammatory sites. Information regarding the recruitment of $\alpha/\beta$ TCR$^+$ cells from rNu/rNu rats to such sites and whether this recruitment is influenced by whether the inflammation is induced by $T_{H1}$-type or $T_{H2}$-type mechanisms, would be of interest. It might offer insights into the role of thymus-independent $\alpha/\beta$ TCR$^+$ cells in euthymic individuals in health and disease.

5.3.3 Purification of CD4$^+$ T cells from rNu/rNu rats results in cell death: A Summary
An interesting observation was made when CD4$^+$ cells were purified from TDL. When the cells were prepared from rNu/rNu rats, rapid cell death was noted in vitro, while after adoptive transfer, few cells could be detected in recipients. When the purified CD4$^+$ cells were examined for the binding of Annexin V and staining by PI, those from rNu/rNu donors contained a larger proportion that were either pre-apoptotic or apoptotic.
As discussed in Chapter 4, the α/β T cells from rNu/rNu rats have a large mean cell size, reduced levels of TCR and they express surface molecules that are consistent with an effector phenotype. Furthermore, a small proportion contain less DNA than normal diploid cells. It has been shown that activated T cells co-express the Fas ligand (FasL) and its receptor Fas (Suda et al., 1995). It is possible, therefore, that when purified, CD4+ cells that express FasL and Fas could induce apoptosis among themselves. The finding that CD4+ cells from rNu/rNu rats died during purification and that among the ‘viable’ cells, over 35% bound Annexin V, suggests that some of these cells are undergoing programmed cell death. This phenomenon was not limited to CD4+ T cells from rNu/rNu rats. A considerable proportion of the CD4+ cells purified from the TDL of an rNu/+ rat also bound Annexin V three hours after purification. Although these cells were ‘viable’ (PI), Annexin V binding indicates that they are pre-apoptotic and this could explain the finding that less CFSE-labelled CD4+ cells were recovered from recipient rats than was expected. This finding presents a warning that the presence of FasL expressing T cells, especially when enriched, could induce the death of other cells (e.g. resting lymphocytes) that express Fas (Suda et al., 1995).

The question arises as to why the thymus-independent α/β T cells in rNu/rNu rats might express FasL. With the knowledge that a large proportion of α/β T cells in rNu/rNu rats express MHC class II molecules (a marker of activation), it is conceivable that expression of FasL might reflect the activated status of these cells. However, the question remains as to whether the expression of this ligand serves any function. If these cells have a regulatory function in normal animals, then expression of FasL might regulate tissue or cellular expansion. For example, at the end of an immune response the
presence of FasL expressing cells could induce apoptosis amongst potentially damaging effector cells.

Further investigations are needed to identify the mechanism that induces apoptosis in the CD4+ cells from rNu/rNu rats. The enhanced cell death in purified CD4+ cells could be due to removal of B cells or other 'blocking' cells, which otherwise reduce direct contact between the cells. Alternatively, it is possible that some factor(s), perhaps cytokine(s), are produced by other cell types and are necessary to maintain the viability of these cells. If a Fas-FasL or similar interaction is responsible for cell death, then detection of mRNA transcripts for FasL by RT-PCR or Northern analysis could be used to examine expression of FasL by α/β T cells in rNu/rNu rats. If the α/β T cells from rNu/rNu rats do express FasL, this may provide clues to the function of thymus-independent α/β T cells in euthymic individuals. NKT cells, which are proposed to have immunoregulatory roles in normal mice (reviewed by Vicari and Zlotnik, 1996), have been shown to express FasL. It has been shown that NKT cells in the mouse can kill Fas-expressing targets by the Fas-FasL apoptotic pathway (Arase et al., 1994). It is possible, therefore, that thymus-independent α/β T cells could also mediate this process. Such a function may be important in regulating immune responses where there is an accumulation of large numbers of activated cells. Alternatively, thymus-independent α/β T cells may themselves be powerful effector cells and, therefore, require stringent regulation. Accompanying their activated status, thymus-independent α/β T cells may be programmed for apoptosis. Unless these cells receive positive stimulation, potentially through cognate antigen, specific cytokines, or perhaps cell-cell contact at appropriate effector sites, these cells undergo programmed death.
Chapter 6

Relationship between thymus-independent T cells and NKT cells
6.1 Introduction

A subset of T cells that expresses the NK1.1 (CD161 [NKR-P1]) antigen has been described recently in mice. These cells (referred to herein as NKT cells) possess a number of distinctive features. Most express CD4 or have a DN phenotype. They have cytoplasmic granules similar to those found in NK cells, some IEL and some activated T cells; a restricted use of TCR Vα and Jα genes; a level of surface TCR that is lower than that found on classical T cells; a mean size that is larger than resting classical T cells; expression of adhesion molecules associated usually with memory/effector T cells; and the ability to produce large amounts of cytokines (in particular IL-4 and IFN-γ) rapidly in response to stimulation (reviewed in Vicari and Zlotnik, 1996; Taniguchi et al., 1996; Bendelac, 1995; MacDonald, 1995). T cells with a similar phenotype have also been described recently in humans (Davodeau et al., 1997, Porcelli et al., 1993). T cells which express CD161 have been identified in rats but unlike those found in mice and humans, most are CD8α+ while only a small population have a DN or CD4+ phenotype (Brissette-Storkus et al., 1994). Because rat NK cells and some non-classical T cells express CD8α chains in the absence of CD8β chains (Torres-Nagel et al., 1992) it will be of interest, therefore, to investigate expression of CD8 dimers (in addition to other surface molecules) by rat NKT cells. To date, there have been few studies on these cells in the rat.

As opposed to classical T cells which recognise antigen in the context of MHC class I and class II molecules, mouse and human NKT cells appear to be restricted by non-classical MHC molecules such as CD1 (Bendelac et al., 1995). Recently it has been shown that mouse and human NKT cells can recognise similar antigens (glycolipids) presented by CD1 molecules. Furthermore, mouse and human NKT cells can recognise
antigen presented in the context of the respective xenogeneic CD1 molecules (Brossay et al., 1998; Spada et al., 1998). This indicates that NKT cells, the antigens that they recognise and the functions of the cells, have been conserved across species.

It is of particular interest that NKT cells appear in foetal mice prior to the development of a functional thymus (Makino et al., 1996) and that they are present also in nude mice (Hashimoto et al., 1995, Kikly and Dennert, 1992; Ohteki et al., 1992) and in the livers of ATXBM mice (Sato et al., 1995). Although some NKT cells appear to arise by an intra-thymic differentiation pathway (reviewed by Vicari and Zlotnik, 1996; Bendelac, 1995; MacDonald, 1995), their presence in athymic mice indicates that others have an extrathymic origin. This suggests that NKT cells in euthymic mice, like IEL, contain a subset that is derived extra-thymically.

Because the phenotype of the α/β T cells found in rNu/rNu rats resembles that of mouse NKT cells in a numbers of respects (reduced level of TCR expression, large modal size and pattern of adhesion molecule expression), it is possible that these cells possess similar functional characteristics. This chapter compares α/β T cells from rNu/rNu rats with both classical T cells and NKT cells from rNu/+ rats. In particular, special attention is paid to the abilities of these cells to produce cytokines. The rapid production of immunoregulatory cytokines is paramount to the in vivo functions of NKT cells in normal individuals. It is a great interest to know whether this is a particular function of NKT cells or whether it is shared by other T cells that are also thought to have an extra-thymic origin.
6.2 Results

6.2.1 Mean size and level of TCR expression by NKT cells in rNu/+ rats and α/β T cells isolated from rNu/rNu rats

T cells that co-express the α/β TCR and CD161 have been described previously in euthymic rats (Brissette-Storkus et al., 1994). As shown in Figure 6.2.1 [B], flow cytometric analysis of lymph node cell revealed that α/β TCR⁺ CD161⁺ cells (NKT cells) were present in rNu/rNu rats. At other sites (data not shown), NKT cells accounted for up to 30% of α/β T cells in the livers of rNu/rNu rats but only 10% of α/β T cells in the livers of 26 week old rNu/+ rats. They comprised a considerable proportion of the α/β T cells in the bone marrow (approximately 10% in both rNu/rNu and rNu/+ rats) and in the spleens of 26 week old animals (range: rNu/+ = 10.5-13.5, rNu/rNu = 15.5-19.0% [n=4]).

The proportions of α/β T cells that expressed CD161 were lower in either the TDL (range: rNu/+ = 1.5-3%, rNu/rNu = 2.5-6.5% [n=4]) or lymph nodes (range: rNu/+ = 2.5-5.0%, rNu/rNu = 6.0-16.0% [n=4]) from 26 week old rNu/+ and rNu/rNu rats. Figure 6.2.1 illustrates the detection of NKT cells in lymphocytes prepared from lymph nodes of rNu/+ and rNu/rNu rats aged 26 weeks. The mean level of TCR expression by CD161⁺ α/β T cells in rNu/+ rats was approximately 1.5-fold less than that expressed by the CD161⁻ α/β T cells (classical T cells) (Figure 6.2.1 [A] and [C]). In contrast, TCR was expressed at similar levels by both the CD161⁺ and CD161⁻ α/β T cells in rNu/rNu rats (Figure 6.2.1 [B] and [D]) and the mean level of expression by both subpopulations was similar to the level expressed by NKT cells from rNu/+ rats (Figure 6.2.1 [C]). This finding complements the data presented in chapter 4 and shown in Figure 4.2.11 [A], where the α/β T cells from rNu/rNu rats expressed the TCR at levels approximately
Figure 6.2.1  Expression of \( \alpha/\beta \) TCR by NKT cells from the lymph nodes of rNu/+ and rNu/rNu rats. Lymph node cell preparations from (A and C) a 26 week old rNu/+ rat and (B and D) a 26 week old rNu/rNu rat were labelled by the indirect immunofluorescence technique with mAb R73 (anti-\( \alpha/\beta \) TCR; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE) to identify NKT cells. (A and B) Identification of CD161\(^+\) \( \alpha/\beta \) TCR\(^+\) and CD161\(^-\) \( \alpha/\beta \) TCR\(^-\) subpopulations. (C and D) Expression of \( \alpha/\beta \) TCR by the CD161\(^+\) \( \alpha/\beta \) TCR\(^+\) (NKT cells, red line) subpopulation and CD161\(^-\) \( \alpha/\beta \) TCR\(^-\) (blue line) subpopulation. The mean fluorescence intensity is displayed beside each peak.
Figure 6.2.2  **Sizes of NKT cells and classical T cells.** Lymph node cell preparations from (A) a 26 week old rNu/+ rat and (B) a 26 week old rNu/rNu rat were labelled by the indirect immunofluorescence technique with mAb R73 (anti-α/β TCR; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE) to identify NKT cells. The CD161⁺ (NKT cells, red line) and CD161⁻ (classical α/β T cells, blue line) subpopulations were gated and the forward light scatter (FSC; proportional to cell size) and side scatter of light (SSC; a measure of cell complexity) characteristics of these cell populations are displayed. The mean is displayed beside each peak.
1.5-fold less than those expressed by T cells from rNu/+ rats. The α/β T cells in rNu/rNu rats exhibit, therefore, a level of TCR expression that is shared by the NKT cells present in both rNu/+ and rNu/rNu rats.

As presented in Chapter 4, the α/β T cells from rNu/rNu rats are larger than the corresponding population from age-matched rNu/+ rats. Analysis of the size (FSC and SSC characteristics) of NKT cells from the lymph nodes of rNu/+ rats revealed that they also have a larger mean size and greater complexity than the classical (CD161) α/β T cells (Figure 6.2.2 [A]). Both subpopulations of α/β T cells from rNu/rNu rats share similar size and complexity (Figure 6.2.2 [B]). Furthermore, they resemble closely the α/β TCR⁺ CD161⁺ cells from rNu/+ rats.

### 6.2.2 Similarities between the surface markers expressed by NKT cells from rNu/+ rats and α/β TCR⁺ cells from rNu/rNu rats

In the lymph nodes (Figure 6.2.3), as well as the TDL, spleens and livers (data not shown) of both rNu/+ and rNu/rNu rats of all ages, dual fluochrome flow cytometry showed that almost all CD161dim lymphocytes co-expressed the α/β TCR. NKT cells can be identified, therefore, on this basis. They are distinct from the CD161bright α/β TCR⁺ NK cells (Figure 6.2.3), as described by Brissette-Storkus et al. (1994).

Most (approximately 90%) rat NK cells from rNu/+ rats express CD8α (Figure 6.2.3 [C]), as reported by others (Torres-Nagel et al., 1992; Brissette-Storkus et al., 1994) and only a few express CD4 (less than 1%). These observations apply also to NK cells from rNu/rNu rats, where approximately 80% expressed CD8α. However, a somewhat larger proportion (approximately 20%) than was the case for rNu/+ rats expressed CD4 (Figure
6.2.3 [C-F]). This observation may point to developmental links that exist between NK cells and T cells and these may be highlighted in an athymic environment.

The majority of NKT cells present in the lymph nodes of rNu/+ and rNu/rNu rats express CD8α (Figure 6.2.3 [C] and [D]), although a higher proportion of CD161^{dim} CD8α⁻ are evident in cells from rNu/rNu rats. Dual fluochrome analysis shows clearly that only a few CD161^{dim} cells (NKT cells) from rNu/+ rats express CD4 (Figure 6.2.3 [E]). In contrast, a larger proportion of NKT cells in preparations from rNu/rNu rats express CD4 (Figure 6.2.3 [F]). This latter observation correlates with the higher proportion of NK cells (CD161^{bright} cells) described above that expressed the CD4 co-receptor in cell preparations from rNu/rNu rats (Figure 6.2.3 [E] and [F]).

The CD161^{dim} population was gated (Figure 6.2.3) using the criteria that NKT cells express CD161 at lower levels than NK cells. Expression of a number of surface antigens by cells within this gate was determined using dual fluochrome flow cytometry (Figures 6.2.4, 6.2.5 and 6.2.7). The data presented in Figure 6.2.4 extend the findings on expression of CD8α and CD4 by NKT cells. Very few CD161^{dim} cells (NKT cells) from the lymph nodes of rNu/+ rats expressed CD4 or had a DN phenotype (Figure 6.2.4 [C] and [D]), while approximately 90% of them expressed CD8α (Figure 6.2.4 [A]). However, only approximately 80% of the CD161^{dim} cells expressed CD8β (Figure 6.2.4 [B]), indicating that approximately 10% of NKT cells express CD8α chains in the absence of CD8β chains. It is likely, therefore, that these cells express CD8αα homodimers.

Analysis of the expression of CD8α and CD8β chains by NKT cells from rNu/rNu rats
(Figure 6.2.5 [A] and [B]) revealed that fewer expressed CD8 (45-55%). Here also, approximately 10% more of the CD161<sup>dim</sup> cells expressed CD8α than expressed CD8β. As mentioned earlier, a distinct population of CD161<sup>dim</sup> cells expressed CD4. Although generally comprising less than 20% of CD161<sup>dim</sup> cells (depicted in Figure 6.2.3 [F]), as illustrated in Figure 6.2.5 (C) a larger CD4<sup>+</sup> population was evident in a few rNu/rNu rats. In general, expression of CD4 and CD8α by the CD161<sup>dim</sup> cells appear to be mutually exclusive, although as many as 10% of the cells may express both molecules because the proportion labelled by a cocktail of anti-CD4 and anti-CD8α antibodies (Figure 6.2.5 [D]) is fewer than the sum of the proportion labelled by each of the antibodies separately (Figure 6.2.5 [A] and 6.2.5 [C]). Very few CD161<sup>dim</sup> cells from rNu/rNu rats did not express either CD4 or CD8 (i.e. DN).

Flow cytometric analysis of lymphocyte populations, prepared from lymph nodes and TDL from rNu/+ and rNu/rNu rats, revealed an interesting finding with respect to expression of CD161 and CD8β (Figure 6.2.6). As shown in Figure 6.2.6 [A] and [C], the levels of CD8β expressed by the NKT cells were clearly lower than those expressed by conventional CD8<sup>+</sup> (CD161<sup>+</sup>) T cells in rNu/+ rats. Given that NKT cells and conventional T cells express similar levels of CD8α (as shown in Figure 6.2.3 [C]) it is likely, therefore, that NKT cells express CD8α chains in excess of CD8β chains. It is likely, also, that some cells could co-express both CD8αα homodimers and CD8αβ heterodimers. CD8β was expressed at similar levels by both the CD161<sup>-</sup> and CD161<sup>+</sup> populations of CD8<sup>+</sup> T cells in rNu/rNu rats and at levels comparable to those expressed by CD161<sup>dim</sup> cells from rNu/+ rats (Figure 6.2.6 [B] and [D]). Some of the CD161<sup>-</sup> cells in rNu/+ rats expressed lower levels of CD8β (Figure 6.2.6 [A] and [C]), raising the
Figure 6.2.3  Phenotypic characterisation of CD161<sup>dim</sup> cells. Lymph node cells from (A-D) a 26 week old rNu/+ rat and (E-H) a 26 week old rNu/rNu rat were labelled first by the indirect immunofluorescence technique with the following mAbs: (A and B) R73 (anti-α/β TCR; FITC), (C and D) OX8 (anti-CD8α; FITC) and (E and F) W3/25 (anti-CD4; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE). Each plot is divided into 6 regions. The middle two define the CD161<sup>dim</sup> populations.
Figure 6.2.4  Surface antigen phenotype of rat NKT cells. Lymph node cells from a 26 week old rNu/+ rat were labelled indirectly with the following mAbs: (A) OX8 (anti-CD8α; FITC), (B) 341 (anti-CD8β; FITC), (C) W3/25 (anti-CD4; FITC) and (D) OX8 + W3/25 (anti-CD8α+CD4; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE). CD161<sup>dim</sup> cells (see Figure 6.2.3) were gated electronically and the expression of each of the surface antigens was examined. Labelling with the negative control mAb (1B5) is superimposed on each histogram (dotted line) and the percentage of cells positive for each surface antigen is indicated above the relevant marker.
Figure 6.2.5  Surface antigen phenotype of NKT cells from rNu/rNu rats. Lymph node cells from a 26 week old rNu/rNu rat were labelled indirectly with the following mAbs: (A) OX8 (anti-CD8α; FITC), (B) 341 (anti-CD8β; FITC), (C) W3/25 (anti-CD4; FITC) and (D) OX8 + W3/25 (anti-CD8α+CD4; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE). CD161\textsuperscript{dim} cells (see Figure 6.2.3) were gated electronically and the expression of each of the surface antigens was examined. Labelling with the negative control mAb (1B5) is superimposed on each histogram (dotted line) and the percentage of cells positive for each surface antigen is indicated above the relevant marker.
A

Counts

0 5 10 15 20

Counts

0 5 10 15 20

Counts

0 5 10 15 20

Counts

0 5 10 15 20

CD8α

CD8β

CD4

CD8α + CD4

54.8%

44.91%

47.9%

93.8%
**Figure 6.2.6** Expression of CD8β by CD161\(^+\) (NK) T cells and CD161\(^-\) T cells from rNu/+ and rNu/rNu rats. Lymph node cell preparations from (A and C) a 26 week old rNu/+ rat and (B and D) a 26 week old rNu/rNu rat were labelled by the indirect immunofluorescence technique with mAb 341 (anti-CD8β; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE) to identify CD161\(^+\) CD8β\(^+\) (NKT) cells and CD161\(^-\) CD8β\(^+\) T cells. (A and B) Identification and gating of CD161\(^+\) CD8β\(^+\) and CD161\(^-\) CD8β\(^+\) subpopulations. (C and D) Levels of expression of CD8β by the CD161\(^+\) (NKT cells, red line) and CD161\(^-\) (blue line) subpopulations of CD8β\(^+\) cells from rNu/+ and rNu/rNu rats. The mean fluorescence intensity is displayed beside the relevant peak.
A

NKT cells

T cells

CD161

CD8β

B

NKT cells

T cells

CD161

CD8β

C

93.5 143.0

D

72.4 77.5

Counts

CD8β

Counts

CD8β
Figure 6.2.7 Expression of adhesion molecules by rat NKT cells.

Lymph node cells from a 26 week old rNu/+ rat were labelled indirectly with the following mAbs: (A) WT-1 (anti-CD11a; FITC), (B) 1A29 (anti-CD54; FITC), (C) MRα4-1 (anti-CD49d; FITC) and (D) OX85 (anti-CD62L; FITC), followed by direct labelling with mAb 10/78 (anti-CD161; PE). CD161dim cells (see Figure 6.2.3) were gated electronically and the expression of each of the surface antigens was examined. Labelling with the negative control mAb (1B5) is superimposed on each histogram (dotted line).
possibility that these represent an extra-thymically-derived subpopulation of T cells that is present in euthymic animals.

Dual fluochrome flow cytometry was used to examine the adhesion molecule profile of NKT cells in rNu/+ rats, for comparison with T cells from rNu/rNu rats (Chapter 4, section 4.2.5). NKT cells in rNu/+ rats exhibited a profile of adhesion molecules suggestive of an activated/memory phenotype. LFA-1 (CD11a) was expressed in a bimodal distribution (Figure 6.2.7 [A]), the more brightly stained cells expressing levels comparable with those expressed by α/β T cells from rNu/rNu rats (Figure 4.2.20 [A]) and higher than those expressed by most α/β T cells from rNu/+ rats (Figure 4.2.20 [E]). The patterns of expression of ICAM-1 (CD54), α4-integrin (CD49d) and L-selectin (CD62L) (Figure 6.2.7 [B-D]) were also similar to those observed for α/β T cells in rNu/rNu rats (Figure 4.2.20 [F-H]) and distinct from the pattern expressed by most of the T cells present in rNu/+ rats (Figure 4.2.20 [B-D]). When gated separately, the NKT cells from rNu/rNu rats were found to express a pattern of adhesion molecules similar to NKT cells from rNu/+ rats although expression of CD11a was more homogeneous and at a level corresponding to the brighter CD11a+ population displayed in Figure 6.2.7 [A] (data not shown). The level of expression of these molecules was similar between the CD161dim T cells and the ungated T cell population in rNu/r Nu rats.

### 6.2.3 Production of cytokines by thymus-independent α/β TCR+ cells and NKT cells from rNu/+ rats

As described above, lymphocytes that express the α/β TCR in rNu/r Nu rats share a number of characteristics with NKT cells. Because murine NKT cells have been reported to produce high levels of cytokines after stimulation (reviewed in Vicari and
Zlotnik, 1996; Bendelac, 1995; MacDonald, 1995), the production of IFN-γ and IL-4 by α/β T cells from rNu/+ and rNu/rNu rats has been compared with the production of the same cytokines by NKT cells. Lymphocytes from TDL and lymph nodes were stimulated in vitro with PMA and ionomycin in the presence of Brefeldin A. After 5 hours of culture, the lymphocytes were washed and fixed. The mAbs OX81 (anti-IL-4) and DB-1 (anti-IFN-γ) were then used to detect intracellular cytokine after the cells had been made permeable by treatment with saponin. Cytokine-producing cells were investigated further by labelling with PE-conjugated mAbs against the surface molecules α/β TCR (R73), CD4 (OX38) and CD161 (10/73. Figure 6.2.8 and Figure 6.2.9 represent the results obtained by dual-colour analysis of cells labelled to detect intracellular IFN-γ and IL-4 respectively after in vitro stimulation.

As shown in Figure 6.2.8 [A] and summarised in Figure 6.2.10 [A], approximately 5-8% of all α/β T cells in lymph node preparations from rNu/+ rats produced detectable IFN-γ protein after stimulation in vitro. In contrast, up to 70% of the α/β T cells from the lymph nodes from rNu/+ rats (Figure 6.2.8 [C] and Figure 6.2.10 [A]) had produced IFN-γ during this period and the amounts of intracellular cytokine were higher than those observed in cells from rNu/+ rats. In preparations from both rNu/+ and rNu/rNu rats, most of the IFN-γ-producing cells expressed CD4 (Figure 6.2.8 [B] and [D], and Figure 6.2.10 [B]). Although fewer thoracic duct lymphocytes produced IFN-γ after stimulation in vitro, the proportion of IFN-γ-producing α/β T cells from rNu/rNu rats (approximately 27-40%) was on average 10-fold greater than observed with cells from rNu/+ littermates (approximately 3%) (Figure 3.2.11 [A]). As in the case of lymph node cells, most of the IFN-γ-producing cells expressed CD4 (Figure 6.2.11 [B]).
The proportion of α/β T cells that produced IL-4 was also greater than in lymph node cell preparations from rNu/rNu rats (approximately 3-4%) than in preparations from their heterozygous littermates (approximately 1%) (Figure 6.2.10 [C]). When CD4+ cells from lymph nodes of rNu/rNu rats were examined, up to 8% had produced IL-4 after stimulation, compared with approximately 1% of CD4+ lymph node cells from rNu/+ littermates (Figure 6.2.10 [D]). The proportions of thoracic duct lymphocytes that produced IL-4 were greater than those in preparations of lymph node cells. After stimulation, up to 6.5% of α/β T cells (Figure 6.2.9 [B] and Figure 6.2.11 [C]) and 8% of CD4+ cells (Figure 6.2.9 [D] and Figure 6.2.11 [D]) in TDL from rNu/rNu rats had produced IL-4. This compared with only approximately 1-1.5% of α/β T cells (Figure 6.2.9 [A] and Figure 6.2.11 [C]) and 1-2% of CD4+ cells (Figure 6.2.9 [B] and Figure 6.2.11 [D]) from TDL of rNu/+ littermates. An interesting pattern emerged for cytokine production by α/β T cells and CD4+ cells from TDL and lymph nodes of rNu/rNu rats. Comparison of the proportions of cells producing IFN-γ and IL-4 revealed that in individual rNu/rNu rats, there appeared to be a reciprocal relationship between the production of IFN-γ and IL-4 (Figure 6.2.10 and Figure 6.2.11). However, examination of a greater number of animals would be necessary to establish this relationship statistically.

By examining only the cells that were CD161dim, it was possible to examine the ability of the NKT cells to produce IFN-γ and IL-4 after in vitro stimulation. After 5 hours of stimulation, in excess of 60% of NKT cells in lymph node cells prepared from rNu/+ rats had produced detectable levels of IFN-γ (Figure 6.2.12 [C]). The pattern of production was very similar to that observed in the ungated α/β T cell population from rNu/rNu rats (Figure 6.2.12 [B]) and clearly distinct from that of the ungated α/β T cell preparation from rNu/rNu rats in TDL.
Figure 6.2.8  Detection of intracellular interferon gamma (IFN-γ) in α/β T cells after activation in vitro. Lymph node cells from (A and B) a 26 week old rNu/+ rat and (C and D) a 26 week old rNu/rNu rat were stimulated in vitro with PMA and calcium ionophore A23187 in the presence of Brefeldin A for 5 hours before fixing. Fixed cells were made permeable with saponin and stained by the indirect immunofluorescence labelling procedure with mAb DB-1 (anti-IFN-γ; FITC), followed by direct labelling with either (A and C) mAb R73 (anti-α/β TCR; PE) or (B and D) OX38 (anti-CD4; PE). The proportion of gated cells in each quadrant is presented as a percentage.
Figure 6.2.9  Detection of intracellular interleukin-4 (IL-4) in α/β T cells after activation *in vitro*. Thoracic duct lymphocytes from (A and B) a 26 week old rNu/+ rat and (C and D) a 26 week old rNu/rNu rat were stimulated *in vitro* with PMA and calcium ionophore A23187 in the presence of Brefeldin A for 5 hours before fixing. Fixed cells were made permeable with saponin and stained by the indirect immunofluorescence labelling procedure with mAb OX81 (anti-IL-4; FITC), followed by direct labelling with either (A and C) mAb R73 (anti-α/β TCR; PE) or (B and D) OX38 (anti-CD4; PE). The proportion of gated cells in each quadrant is presented as a percentage.
Figure 6.2.10  Production of IFN-γ and IL-4 by lymph node cells from rNu/+ and rNu/rNu rats after stimulation in vitro. The percentage of the α/β TCR⁺ (mAb R73) cells or CD4⁺ (mAb OX38) cells that produced IFN-γ or IL-4 after 5 hours of stimulation in vitro was determined by dual fluochrome flow cytometry (see Figures 6.2.8 and 6.2.9) in preparations from lymph nodes (LN) of two 26 week old rNu/+ rats and two 26 week old rNu/rNu rats. Higher proportions of α/β TCR⁺ and CD4⁺ cells from rNu/rNu rats produced IFN-γ and IL-4, compared with the corresponding populations from rNu/+ rats.

A. Percentage of LN α/β T cells producing IFN-γ
B. Percentage of LN CD4⁺ cells producing IFN-γ
C. Percentage of LN α/β T cells producing IL-4
D. Percentage of LN CD4⁺ cells producing IL-4
Figure 6.2.11  Production of IFN-γ and IL-4 by thoracic duct lymphocytes from rNu/+ and rNu/rNu rats after stimulation in vitro. The percentage of the α/β TCR+ (mAb R73) cells or CD4+ (mAb OX38) cells in thoracic duct lymph (TDL) that produced IFN-γ or IL-4 after 5 hours of stimulation in vitro was determined by dual fluochrome flow cytometry (see Figures 6.2.8 and 6.2.9) on cells from two 26 week old rNu/+ rats and two 26 week old rNu/rNu rats. Higher proportions of α/β TCR+ and CD4+ cells from rNu/rNu rats produced IFN-γ and IL-4, compared with the corresponding populations from rNu/+ rats.

A. Percentage of TDL α/β T cells producing IFN-γ
B. Percentage of TDL CD4+ cells producing IFN-γ
C. Percentage of TDL α/β T cells producing IL-4
D. Percentage of TDL CD4+ cells producing IL-4
population from rNu/+ rats (Figure 6.2.12 [A]). A much smaller percentage of NKT cells produced IL-4 during this time (Figure 6.2.13 [B]). However, the proportion of NKT cells that produced IL-4 (Figure 6.2.13 [B]) was greater than the proportion of IL-4-producing cells in the ungated population of α/β T cells from the lymph nodes of rNu/+ rats (Figure 6.2.10 [D]).

Cytokine production by NKT cells from rNu/rNu rats was also examined. The patterns of production of IFN-γ (Figure 6.2.13 [A]) and IL-4 (Figure 6.2.13 [B]) were similar, irrespective of whether the NKT cells were obtained from rNu/+ or rNu/rNu rats. Therefore, it is concluded that NKT cells (in phenotypically normal or athymic rats) and the α/β TCR+ CD161− cells that are found in rNu/rNu rats, share the ability to produce large quantities of IFN-γ and lesser amounts of IL-4 after short stimulation in vitro.
Figure 6.2.12  Histograms showing the detection of intracellular interferon gamma (IFN-γ) in α/β T cells after activation in vitro. Lymph node cells from (A and C) rNu/+ rats and (B) rNu/rNu rats aged 26 weeks were stimulated in vitro with PMA and calcium ionophore in the presence of Brefeldin A for 5 hours before fixing. Fixed cells were made permeable with saponin and stained by the indirect immunofluorescence labelling procedure with mAb DB-1 (anti-IFN-γ; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE) or mAb 10/78 (anti-CD161; PE). NKT cells were identified on the basis of their CD161dim phenotype (see Figure 6.2.3). In each histogram the dotted line represents cells labelled with negative control mAb lB5 and cells incubated with mAb DB-1 are superimposed in red. The percentage of labelled cells is indicated above the relevant marker. (A) Only a small percentage of electronically gated α/β TCR+ cells from the rNu/+ rats contain IFN-γ. (B) Many electronically gated α/β TCR+ cells from the rNu/rNu rat contain IFN-γ. (C) Many CD161dim (NKT) cells from the rNu/+ contain IFN-γ.
Figure 6.2.13  Production of IFN-γ and IL-4 by NKT cells from rNu/+ and rNu/rNu rats. Intracellular IFN-γ and IL-4 within CD161dim (mAb 10/78) cells was compared by dual fluochrome flow cytometry (see Figures 6.2.3 and 6.2.12) from the lymph nodes of two 26 week old rNu/+ rats and two rNu/rNu rats after stimulation in vitro. The pattern of production of these cytokines by NKT cells was similar in preparations from rNu/+ and rNu/rNu rats.
A

Percentage of LN NKT cells Producing IFN-γ

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</tr>
<tr>
<td>rNu/rNu #2</td>
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<tr>
<td>rNu/+ #1</td>
<td>70</td>
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<tr>
<td>rNu/+ #2</td>
<td>60</td>
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B

Percentage of LN NKT cells Producing IL-4

<table>
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<th>Rat</th>
<th>Percentage of NKT cells</th>
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<tbody>
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</tr>
<tr>
<td>rNu/rNu #2</td>
<td>6</td>
</tr>
<tr>
<td>rNu/+ #1</td>
<td>8</td>
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<tr>
<td>rNu/+ #2</td>
<td>10</td>
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</table>
6.3 Discussion

6.3.1 Mean size and level of TCR expression by NKT cells in rNu/+ rats and αβ T cells isolated from rNu/rNu rats: A Summary

The presence of NKT cells during early embryogenesis in mice (Makino et al., 1996) and their presence in athymic mice (Hashimoto et al., 1995; Kikly and Dennert, 1992; Ohteki et al., 1992) and in ATXBM mice (Sato et al., 1995) suggests that at least some of these cells have an extra-thymic origin. The results presented in this chapter show that in the rat also, NKT cells populations develop in a thymus-independent manner. Furthermore, it appears that the αβ T cell population in rNu/rNu rats shares a number of features with NKT cells. NKT cells (αβ TCR+, CD161dim cells), together with the CD161+ αβ T cells in rNu/rNu rats, have a larger mean size than the classical T cells. In addition, NKT cells and all thymus-independent αβ T cells from rNu/rNu rats express lower levels of the TCR on the cell surface. Among classical T cells in normal animals, these features are consistent with antigen-induced activation (Valitutti et al., 1997). This suggests that NKT cells and thymus-independent αβ T cells can be considered to be in a continually activated state. However, if they have a rapid turnover, the surface phenotype could be consistent also with recent activation during development at the site(s) at which they are generated and the constant release of newly formed cells. In the future, congenic rats could be employed to measure the turnover of NKT cells. Adoptive transfer of congenically-marked cells, which include NKT cells, into recipient animals would make it possible to measure the turnover of NKT cells. In addition, incubating these cells prior to transfer with CFSE would make it possible to measure the division rates of these cells relative to classical T cells and to the αβ T cells found in athymic rats.
Although reduced levels of TCR expression and large cell size are not specific indicators of α/β T cells that have a thymus-independent origin, they are characteristics that could prove to be useful in the identification and isolation of such cells from euthymic rats. It would be of interest to isolate cells with this phenotype from normal animals and to examine whether they share other phenotypic features in common with the thymus-independent α/β T cells in athymic rats and with NKT cells. It would be especially interesting to determine whether such cells exhibit a similar profile of cytokine production with thymus-independent T cells and NKT cells. If this was the case it would indicate that a thymus-independent T cell population is part of the T cell repertoire in normal animals and that it has functions distinct from classical T cells.

Other phenotypic differences will be necessary to distinguish thymus-independent α/β T cells in euthymic animals definitively as to enrich them specifically. Constitutive expression of the IL-2Rβ chain (CD122) by NKT cells and TCR intermediate cells (thymus-independent T cells), together with an intermediate level of TCR expression, has been used to distinguish these cell types from classical T cells in mice (Emoto et al., 1997, Watanabe et al., 1995). Recently, an antibody against rat CD122 has been produced (mAb L316). It has been used to show that all CD161dim cells (NKT cells) in normal rats express this molecule constitutively (Park et al., 1996). In contrast, only a minor subset of the CD161+ CD4+ α/β T cells express CD122. It is anticipated that the α/β T cells in rNu/rNu rats will also express CD122 constitutively.

6.3.2 Similarities between the surface markers expressed by NKT cells from rNu/+ rats and α/β TCR+ cells from rNu/rNu rats: A Summary
As well as sharing a larger mean size and expression of lower levels of surface TCR, α/β
T cells from rNu/rNu rats and NKT cells (from both rNu/+ and rNu/rNu rats) express a pattern of adhesion molecules consistent with an effector/memory phenotype. Both populations of α/β T cells express higher levels of CD11a, CD54 and CD49d than are expressed by α/β T cells from euthymic rats. Furthermore, neither cell-type expresses high levels of CD62L. Thus α/β T cells from rNu/rNu rats and NKT cells in euthymic rats share a number of features with NKT cells in mice (reviewed in Vicari and Zlotnik, 1996; Bendelac, 1995; MacDonald, 1995).

Although most NKT cells in mice and humans express CD4 or have a DN phenotype (Davodeau et al., 1997, Porcelli et al., 1993), a majority of NKT cells in rats have been shown previously to express CD8α (Brissette-Storkus et al., 1994). As presented in this chapter approximately 90% of NKT cells from euthymic rNu/+ rats were shown to express CD8α. As discussed in Chapter 4, most α/β T cells from rNu/rNu rats express CD4 but of those expressing the CD8 co-receptor, a substantial proportion were shown to express CD8αα homodimers. This observation is of interest in relation to the data presented in this chapter. With respect to those α/β T cells in rNu/rNu rats that are CD8α+, indirect evidence is presented which indicates that there is an excess of CD8α chains relative to CD8β chains on the cell surface. This suggests that these α/β T cells may co-express CD8αα homodimers and CD8αβ heterodimers. It is intriguing that a similar imbalance is also seen on the majority of NKT cells in euthymic rNu/+ rats. NKT cells may, therefore, belong to the same lineage as thymus-independent T cells in athymic rats.

The expression of CD8αα homodimers on the surface of CD8α+ αβ T cells in athymic
mice and by NKT cells in both athymic and normal mice is an interesting observation. A significant proportion of IEL in mice (Rocha et al., 1991; Mosley et al., 1990), humans (Latthe et al., 1994; Jarry et al., 1990) and rats (Helgeland et al., 1996; Torres-Nagel et al., 1992) express CD8\(\alpha\alpha\) on their cell surfaces. It is proposed that the development of these cells in the gut epithelium leads to the expression of the CD8\(\alpha\alpha\) homodimer (reviewed by Rocha et al., 1995). This raises the possibility that the development of the CD8\(^+\) \(\alpha/\beta\) T cells present in athymic rats and of the NKT cells in both athymic and normal rats is influenced by the intestinal epithelium. Also, given that the CD4\(^+\) \(\alpha/\beta\) T cells present in athymic rats share a number of features in common with NKT cells, it is possible that these cells also arise at similar sites. LFV (described and discussed in Chapter 3) are obvious candidate sites.

In a biological sense, expression of CD8\(\alpha\alpha\) by these unique T cells may in part account for the proposed different functional and developmental characteristics these cells exhibit in comparison to classical T cells. Some evidence suggests that the CD8\(\alpha\alpha\) coreceptor has a weaker affinity for MHC class I than the CD8\(\alpha\beta\) heterodimer (Cruz et al., 1998). Expression of the CD8\(\alpha\alpha\) homodimer during selection may lead to the differentiation of unique T cells with functions distinct from classical T cells. After maturation, expression of these weaker affinity coreceptors may influence the activation of these T cells. A downstream signalling pathway may arise from engagement of the CD8\(\alpha\alpha\) coreceptor which is distinct from that arising from signalling via the CD8\(\alpha\beta\) heterodimer. Alternatively, expression of CD8\(\alpha\alpha\) may be associated with other antigen presentation pathways. Antigen presentation through non-classical MHC class I molecules such as CD1 has been shown to stimulate both human and mouse NKT cells (Brossay et al., 1998; Spada et al., 1998). It is conceivable that in the rat, antigen
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presentation by CD1 molecules is associated with weak CD8\(\alpha\alpha\)/MHC class I interactions.

Although few NKT cells in rNu/+ rats express CD4 (and these may co-express CD8\(\alpha\)), a greater proportion of NKT cells from rNu/rNu rats expressed this co-receptor. Furthermore, in a few rNu/rNu rats, over 40% of the CD161\(^+\) \(\alpha/\beta\) T cells expressed CD4. If these CD4\(^+\) NKT cells are functionally equivalent to the majority population of CD8\(^+\) NKT cells, then these observations may be very significant. It could indicate that most \(\alpha/\beta\) T cells in rNu/rNu rats, although they do not express CD161, may share function and lineage with NKT cells. It is also worth noting that NKT cells stimulated \textit{in vitro} lose expression of CD161 (Chen \textit{et al.}, 1997), indicating that expression of this molecule is not a universal marker of NKT cells. Indeed, CD161 may be less a lineage marker of these cells than a marker of their stage of differentiation and/or activation. In this respect, it is significant that essentially all (immature) lymphocytes in LFV express CD161 (Chapter 3, section 3.2.3). Therefore, the CD4\(^+\) CD161\(^-\) \(\alpha/\beta\) T cells in rNu/rNu rats may be the counterpart of a population of CD62L\(^{low}\) CD4\(^+\) T cells that has been characterised recently in mice. These cells are as numerous as NKT cells in normal mice and despite being CD161\(^-\), are otherwise phenotypically and functionally indistinguishable from NKT cells (Chen and Paul, 1998).

6.3.3 Production of cytokines by thymus-independent \(\alpha/\beta\) TCR\(^+\) cells and NKT cells from rNu/+ rats: A Summary

Mouse (Chen and Paul, 1997; Chen \textit{et al.}, 1997) and human (Davodeau \textit{et al.}, 1997; Porcelli \textit{et al.}, 1993) NKT cells produce large amounts of IL-4 and IFN-\(\gamma\) after stimulation. The data presented in this chapter show that after stimulation \textit{in vitro}, a
high proportion of NKT cells from rNu/+ and rNu/rNu rats produced IFN-γ, while a lesser proportion produced IL-4. With respect to IL-4 production, the NKT cells in rats appear to differ from NKT cells in mice and humans. Recently, other investigators have generated clones of NKT cells from rats. While all of these clones produced IFN-γ, only a small number produced IL-4 (Knudsen et al., 1997). Surprisingly, all of these clones expressed CD4 or had the DN phenotype, despite being cloned from freshly isolated NKT cells (of which greater than 90% expressed CD8α). Cloned rat NKT cells have been shown by RT-PCR to accumulate mRNA encoding IFN-γ but little mRNA encoding IL-4 after stimulation with anti-CD3 (Badovinac et al., 1998). Although these findings support the results presented herein, the data presented in this chapter was obtained from freshly isolated cells, rather than from cloned NKT cells. The observation that rat NKT cells are different from NKT cells from mice and humans with respect to IL-4 production is interesting. A recent study in mice (Leite-De-Moraes et al., 1998) has shown that the presence of IL-12 in the microenvironment can bias NKT cells towards production of IFN-γ rather than IL-4. Perhaps, under the appropriate conditions, the response of rat NKT cells can also be dominated by production of IL-4. In this regard, it is worth noting that in this study an inverse relationship existed between IFN-γ and IL-4 production in individual rats (Figure 6.2.10 and Figure 6.2.11). Alternatively, the predominant CD8+ subset of NKT cells may produce mainly IFN-γ, as has been proposed for CD8+ classical T cells (reviewed by Carter and Dutton, 1996), while production of IL-4 might be a function of the CD4+ subset of NKT cells and their related CD4+ CD161− counterparts.

The data presented in this chapter provides strong evidence that the thymus-independent α/β T cells from rNu/rNu rats and the NKT cells (present in euthymic and athymic rats)
are related. Both express reduced surface levels of the α/β TCR, have a large cell size and express a pattern of adhesion molecules consistent with an effector/memory phenotype. With respect to those cells which express CD8β chains, the level of expression of this molecule on the cell surface is lower than that found on conventional CD8+ α/β T cells in euthymic rats. Furthermore, both NKT cells and thymus-independent α/β T cells from rNu/rNu rats share the ability to produce large quantities of IFN-γ and lesser amounts of IL-4 after stimulation in vitro. The latter features indicate that thymus-independent α/β T cells may posses important regulatory roles in normal individuals, as has been proposed for NKT cells.
Chapter 7

General Discussion
7.1 The appearance of \( \alpha/\beta \) TCR* cells in athymic rats and possible links to extra-thymic T cell development in Lymphocyte filled villi

This thesis extends earlier studies that showed that lymphocytes expressing the \( \alpha/\beta \) TCR are found in athymic rNu/rNu rats and that their numbers increase with age (Vos et al., 1980; Vaessen et al., 1986; Sarawar et al., 1991). Lee (1993), in this laboratory, showed that prior to 10 weeks of age, cells expressing the \( \alpha/\beta \) TCR are almost undetectable in the peripheral lymphoid organs of athymic rats. However, they are present in small numbers in the TDL of rats of this age and analysis of the \( \alpha/\beta \) TCR* population in TDL revealed that they were large lymphocytes. In the studies described herein, the numbers of these cells increased with age. Furthermore, during prolonged thoracic duct drainage not only the proportion but the output of these cells from young (13 weeks) rats increased. The latter observation suggested that these cells were recently formed, because others have shown in euthymic rats that 24 hours after infusion of tritiated thymidine (\(^3\)H-TDR), 90-100% of the large lymphocytes recovered in TDL are labelled (Gowans and Knight, 1964). It is likely that these large T cells arise from the gastrointestinal tract, because 90% of the cells recovered in TDL in the absence of deliberate stimulation are derived from gut tissues (Mann and Higgins, 1950; Mayrhofer, unpublished observations and Spargo et al., 1996). The almost complete absence of \( \alpha/\beta \) T cells among the small lymphocytes in the TDL from athymic rats aged 13 weeks and younger raises the question of whether these cells are part of the recirculating pool (Gowans and Knight, 1964), whether they are all newly-formed virgin cells and whether some mature further in the periphery and give rise to the smaller \( \alpha/\beta \) T cells seen in older animals. Alternatively, they could be activated T cells, akin to the large T cells found in the TDL of euthymic rats. In the latter case, it can be asked whether they are
released in an activated state from the primary site of generation or whether they have been activated in the periphery.

The origin of the α/β TCR+ cells in athymic rats is unknown. However, it is most likely that they have extra-thymic origin because the thymic rudiment in athymic rats is profoundly deficient in lymphocytes (Vos et al., 1980). Furthermore, the production of α/β T cells in athymic rats does not appear to be limited to the classical pre-pubertal phase of thymic T cell development. In contrast, there is a progressive increase in the numbers of these cells with age. Although the gut epithelium is now well established as a site of extra-thymic T cell development, it is unlikely that this represents the source of the major subpopulation of CD4+ α/β T cells in athymic rats because most IEL in both athymic and euthymic rats express the CD8 co-receptor and a considerable proportion express the γ/δ TCR (Lyscom and Brueton, 1982; Torres-Nagel et al., 1992).

The detection of rag-1 mRNA transcripts in RNA prepared from mononuclear cells isolated from livers raises the possibility that the liver is a site of extra-thymic T cell development in rats, as has been proposed for both mice (Narita et al., 1998; Kimura et al., 1995, Sato et al., 1995) and humans (Collins et al., 1996). However, the phenotype of the cells that express rag-1 mRNA was not addressed in the present study and there are no markers available with which to identify and characterise α/β T cells which may have developed at this site.

The numerous lymphocyte-filled villi (LFV) in the rat small intestine (approximately 1% of all villi) represent attractive sources of the CD4+ α/β T cells that appear in athymic rats. A population of CD4+ α/β TCR+ cells, plus a few CD8α+ cells, appear in these
structures in athymic rats at around 7-8 weeks of age (Mayrhofer and Brooks, 1995). Such cells are, therefore, present in LFV slightly before they are detected in the TDL and peripheral lymphoid tissues of athymic rats. This observation, and the fact that large \( \alpha/\beta \) TCR\(^+ \) cells are present in the TDL from 10 week-old athymic rats, raises the possibility that CD4\(^+ \) \( \alpha/\beta \) T cells are generated in LFV and that they leave these structures to enter the TDL. In athymic rats, these cells could then seed the periphery, leading to their accumulation and detection in peripheral lymphoid organs. Moreover, because LFV are present in normal rats, it is probable that a sub-population to the \( \alpha/\beta \) T cells present in normal rats consists of the thymus-independent T cells that predominate in athymic animals. It seems likely that the production of at least some \( \alpha/\beta \) T cells in LFV is an antigen-driven process, resulting in the production of newly-formed cells that have an activated effector function directed against local antigens in the gut microenvironment. Uptake of horseradish peroxidase from the gut lumen by modified epithelial cells in LFV (Mayrhofer, unpublished results) indicates that these structures can sample local antigen, suggesting that T cells in these structures could be selected by such antigens. Positive selection may, therefore, be a feature of what may be a primitive pre-thymic system of lymphopoiesis. It could result in the generation of T cells with a limited and idiosyncratic TCR repertoire, as is the case for IEL (reviewed in Rocha et al., 1995; Poussier and Julius 1994) and NKT cells, other populations which appear to have extra-thymic origin (Makino et al., 1996; Kikly and Dennert, 1992; Ohteki et al., 1992). A preliminary examination of this possibility did not reveal a predominant usage of V\( \beta8.2 \) among thoracic duct, lymph node and spleen \( \alpha/\beta \) T cells in rNu/rNu rats, as has been described in mouse NKT cells (reviewed in Vicari and Zlotnik, 1996; Taniguchi et al., 1996; Bendelac, 1995; MacDonald, 1995).
The majority of lymphocytes in LFV express CD44, CD43, CD25 and CD161. This phenotype resembles closely that expressed by early committed T cells in the thymus (Carlyle et al., 1997). A minor population expresses one or more markers of mature T cells, namely CD3, α/β TCR, CD5, CD2 and CD4. There is evidence that suggests that these TCR+ cells are derived from the much larger α/β TCR- CD44+ CD43+ CD25+ CD161+ population. Firstly, LFV contain a population of TCR/CD3- cells which are labelled over 1 hour of intravenous injection of 3H-TdR. However, after infusion of 3H-TdR over 6 days, label is found in cells that express surface TCR/CD3 (Mayrhofer and Brooks, 1995). Furthermore, LFV are excluded from the recirculating pool, suggesting that lymphocytes within LFV rearrange and express TCR in situ (Mayrhofer and Brooks, 1995). The detection of RAG-1 protein among lymphocytes within LFV supports further the notion that LFV represent a site of thymus-independent T cell development.

Work in this laboratory (Moghaddami and Mayrhofer, manuscript in preparation) has detected LFV in the intestinal mucosa of mice, while others (Kanamori et al., 1996; Saito et al., 1998) have identified structures called cryptopatches in the intestinal mucosa of mice which may be similar. Both of these structures are somewhat different from LFV in rats because they contain very few cells that express the α/β TCR and no RAG-1 protein has been detected. However, a minor subpopulation of cells expresses CD4. Recently, adoptive transfer of cells isolated from cryptopatches into SCID mice has been shown to reconstitute IEL and mesenteric lymph node T cell populations (Saito et al., 1998). Cells expressing both the γ/δ TCR and α/β TCR were generated, suggesting that cryptopatches contain precursors of mucosal and (perhaps peripheral) T cells but that they are not themselves sites at which mature T cells develop. The CD4+ cells in murine LFV and/or cryptopatches could represent T cell precursors, because
CD4⁺ TCR⁺ committed lymphoid progenitors have been identified in murine bone marrow (Fredrickson and Basch, 1994) and thymus (Wu et al., 1991), and also in adult human peripheral blood (Bruno et al., 1997). Although rare α/β TCR⁺ cells might be generated in murine LFV, it seems more likely that CD4⁺ α/β TCR⁻ cells leave and complete their maturation at other sites, including the intestinal epithelium. In rats, it appears that at least some TCR gene re-arrangement occurs within the LFV, giving rise mainly to CD4⁺ α/β TCR⁺ cells. However, it is also possible that as in mice, some precursors leave LFV with un-rearranged TCR genes. These could give rise to the thymus-independent compartment of the IEL population by rearrangement of the α/β and γ/δ TCR genes locally in the intestinal epithelialum.

7.2 Phenotypic and functional characteristics of thymus-independent α/β TCR⁺ cells
As described by others in both athymic mice (MacDonald et al., 1981; Lawetzky and Hünig, 1988; Kennedy et al., 1992) and athymic rats (Vos et al., 1980; Vaessen et al., 1986; Sarawar et al., 1991), the numbers of cells expressing T cell markers increases with age. In the present study, cells expressing the α/β TCR accounted for up to 30% of the total lymphocytes in the TDL and lymph nodes of rNu/rNu rats aged 39-52 weeks. Although a small population of cells expressing the γ/δ TCR (1-2% in TDL and secondary lymphoid tissues) were present in athymic rats, they were not examined in detail and will be excluded from this discussion. With respect to the α/β TCR⁺ population in rNu/rNu rats, cells expressing the CD4 co-receptor out-numbered those expressing either of the CD8 dimers by a factor of approximately 3-3.5:1 (a similar ratio to that seen in euthymic rNu/+ littermates). However, there was considerable diversity in the expression of these co-receptor molecules, ranging through CD4⁺CD8⁻ (DN),
CD4⁺ CD8α⁺, CD4⁺ CD8α⁺, CD4⁺ CD8α⁻β⁺ and (potentially) CD4⁺CD8α⁻β⁻. This variety of phenotypes raises a number of possibilities. The various subpopulations could represent different lineages or tissue sources of extra-thymically derived T cells, different states of activation within a common lineage, or differences in co-receptor requirement for selection in extra-thymic sites of differentiation. Although these questions cannot be resolved definitively using the information presented in this thesis, they have relevance to comparisons between the thymus-independent α/β T cells in athymic rats and NKT cells (see below).

The α/β T cells in athymic rats have a number of features that distinguish them from the majority of classical T cells. Recirculating lymphocytes in the TDL from euthymic rats are mainly small cells (Gowans and Knight, 1964 and reviewed by Mackay, 1993) and in this study approximately half were found to express the α/β TCR. While most CD8⁺ T cells in euthymic rats express the CD45RC isoform, only 60-70% of the CD4⁺ cells express this isoform. The remaining CD45RC⁻ population of CD4⁺ cells comprises memory cells and activated effector cells (Powrie and Mason, 1989). In the present study, only a small population of the T cells in the TDL of heterozygous littermates of rNu/rNu rats were shown to express activation markers. Furthermore, cells expressing these activation markers were limited largely to the lymphocytes with higher FSC and SSC. This confirms previous findings which show that expression of CD25, CD134 (Paterson et al., 1987) and MHC class II (Seddon and Mason, 1996; Broeren et al., 1995; Reizis et al., 1994) by T cells in peripheral lymphoid organs and TDL from euthymic rats is limited to less than 10% of T cells.

In contrast, the α/β T cells in the TDL from young adult (10-13 weeks) athymic rats
were shown to be large lymphocytes, most of which expressed MHC class II molecules. The proportion of the α/β T cells that expressed activation markers in TDL from these rats was approximately 3-fold higher than in TDL from euthymic T cells. Furthermore, most α/β T cells in athymic rats were shown to be CD45RC−. Memory (CD45RC+) T cells derived from normal animals have been shown to revert to the CD45RC+ phenotype in the absence of the relevant stimulating antigen, whereas in an environment where the stimulating antigen is present they maintain the CD45RC− phenotype (Bunce and Bell, 1997). Therefore, it is feasible that the CD45RC− phenotype of α/β T cells in athymic rats is due to constant activation and/or stimulation with antigen. This may reflect the inability of athymic rats to clear antigens as effectively as euthymic littermates. Alternatively, the antigens recognised by thymus-independent α/β T cells could be distinct from those recognised by most α/β T cells in normal rats and they may be self-antigens or antigens that are ubiquitous in the gastrointestinal tract. The larger cell size of α/β T cells in athymic rats, compared with those in euthymic littermates, may reflect this constant stimulation. However, only a slightly greater proportion of the α/β T cells in TDL from rNu/rNu rats compared with rNu/+ littermates was shown to be in S-phase of the cell cycle. The finding that the entire α/β T cell population in athymic rats expressed the phenotype CD11a^hi CD44^hi CD54^hi CD49d^hi CD2^hi CD62lo in a relatively homogeneous pattern provides further support to the notion that these cells are in regular contact with stimulating antigen. At the least, these differences suggest that thymus-independent T cells have population kinetics different from those of classical T cells.

Although the α/β T cells in rNu/rNu rats are larger than the α/β T cells in euthymic rats, at least some were shown to recirculate from the blood to the thoracic duct lymph. The
proportion of α/β T cells from athymic donors that was found to recirculate through the thoracic duct after adoptive transfer was approximately half that observed after transfer of α/β T cells from euthymic littermates. This may reflect the memory phenotype of these cells and in particular, their low expression of CD62L. In rats, CD4⁺ CD45RC⁻ cells have been shown to recirculate more slowly than CD4⁺ CD45RC⁺ cells (Westermann et al., 1994). However, although most α/β T cells from euthymic rats expressed the CD45RC⁻ phenotype, α/β T cells from athymic and euthymic rats recirculated with similar kinetics (albeit in lower numbers in the case of cells from athymic rats). This observation indicates that despite their activated phenotype, some thymus-independent α/β T cells recirculate. The precise phenotype of the recirculating population has not been determined. These studies have been performed using thoracic duct lymphocytes from older donors (26 to 39 weeks) and the recirculating capacity of the predominantly large lymphocytes from young athymic rats has not been examined.

In comparison with α/β T cells from euthymic animals, the α/β T cells from athymic rats demonstrated a different tissue distribution after adoptive transfer. This distribution was different also from that described for memory/effector T cells in mice after adoptive transfer (reviewed by Swain et al., 1996; Bradley et al., 1993; Mackay et al., 1993). Despite having a CD62L⁺ phenotype, α/β T cells from athymic rats distributed in considerable numbers to lymph nodes (especially the mesenteric lymph nodes). They were also recovered in higher numbers in the spleen and liver than α/β T cells from euthymic littermates. Accumulation of large numbers of thymus-independent α/β T cells in the spleen and liver might reflect high expression of CD44 (and perhaps other adhesion molecules), as has been proposed for TCR⁺ cells in mice (Arai et al., 1995). In addition to their greater accumulation at these sites, a higher percentage of α/β
T cells from athymic rats were recovered from the Peyer’s patches. Expression of high levels of CD49d could be responsible for this, because MadCAM-1, a receptor for the \( \alpha_4 \)-integrins, is expressed on the HEVs of Peyer’s patches and mesenteric lymph nodes (Briskin et al., 1993). Expression of the \( \alpha_{E2} \)-integrin by a significant proportion (15-20\%) of \( \alpha/\beta \) T cells in athymic rats may also influence the mucosal distribution of these cells, because expression of this integrin by T cells in normal rats is restricted largely to those which are found at mucosal sites (Brenan and Puklavec 1992; Brenan and Rees 1997).

Expression of CD49d, \( \alpha_{E2} \) and the CD8\( \alpha \alpha \) homodimer by a considerable population of \( \alpha/\beta \) T cells in athymic rats and by a smaller population in euthymic rats further implicates the gut mucosa as a potential site of origin for these cells. As discussed earlier, LFV represent a potential source for these cells in both athymic and euthymic rats. Most CD8\( \alpha^+ \) cells in LFV do not express CD8\( \beta \) and preliminary experiments indicate that \( \alpha/\beta \) T cells in LFV could co-express CD49d and \( \alpha_{E2} \)-integrin. However, the latter observation is difficult to interpret because DC in these structures also express CD49d and \( \alpha_{E2} \)-integrin. If LFV are a source of extra-thymically-derived T cells, these cells could be replenished constantly and the process could be regulated by mucosal antigens. The cells that are released could be mature effector cells. This model would account for the presence of \( \alpha/\beta \) T cells in the TDL from young athymic rats, the activated phenotype of the cells and the expression by them of mucosa-associated adhesion molecules.
7.3 Expression of the TCR by thymus-independent T cells and their relationship to NKT cells.

Another difference between the $\alpha/\beta$ T cells in athymic rats and those in euthymic littermates was the level of TCR expressed by these cells. Despite their larger size, the $\alpha/\beta$ T cells in athymic rats expressed less TCR than their counterparts in euthymic rats. This could be associated with an activated phenotype, given that antigen-induced activation can lead to down-regulation of the TCR by classical T cells (Valitutti et al., 1997). However, it is worth noting that the large $\alpha/\beta$ T cells from rNu/+ rats still express higher levels of TCR than cells of corresponding size in rNu/rNu rats. It has been suggested that the reduced level of the TCR could be used as a useful lineage characteristic with which to identify T cells (excluding IEL) that develop extra-thymically in nude mice (Lawetzky and Hünig, 1988; Sato et al., 1995; Iiai et al., 1992) and in ATXBM mice (Iiai et al., 1992). However, whereas the difference in TCR expression between T cells in athymic mice compared with those in euthymic mice is approximately 3-fold (Lawetzky and Hünig, 1988), the difference in the level of TCR expressed by $\alpha/\beta$ T cells from athymic rats compared with those from euthymic rats was found to be only approximately 1.5-fold. As a result, the level of TCR expression is a relatively unsatisfactory criterion with which to identify T cells of extra-thymic origin among classical T cells in normal rats.

Of great interest is the similarity between NKT cells and the thymus-independent $\alpha/\beta$ T cells in athymic rats. Firstly, CD161$^+$ $\alpha/\beta$ TCR$^+$ cells (NKT cells) are present in athymic rats. This indicates that, as in mice (Kikly and Dennert, 1992; Ohteki et al., 1992), at least some NKT cells have an extra-thymic origin. NKT cells in normal mice (reviewed in Vicari and Zlotnik, 1996; Taniguchi et al., 1996; Bendelac, 1995; MacDonald, 1995)
share with extra-thymically derived α/β T cells in athymic mice a reduced level of TCR expression (Lawetzky and Hünig, 1988; Sato et al., 1995; Iiai et al., 1992), low expression of CD62L (Arai et al., 1995) and high expression of other adhesion molecules such as CD44 and CD11a (Arai et al., 1995; Ohteki et al., 1992). Similarly, when compared to classical α/β T cells from euthymic rats, the findings presented in this thesis show that the NKT cells in phenotypically normal rats share with the α/β T cells in athymic rats a reduced level of expression of the TCR and CD62L and increased levels of expression of CD11a, CD54 and CD49d. Furthermore, NKT cells (from either athymic or euthymic rats) share with thymus-independent α/β T cells (mainly CD161+) from athymic rats the ability to produce large quantities of IFN-γ and lesser amounts of IL-4, in response to stimulation in vitro. Rat NKT cells appear to differ, therefore, from those found in mice (Chen and Paul, 1997 and Chen et al., 1997 and reviewed by Vicari and Zlotnik, 1996; Bendelac, 1995; MacDonald, 1995) and humans (Davodeau et al., 1997, Wilson et al., 1998), where NKT cells produce large quantities of IL-4 as well as IFN-γ. However, the rapid production of cytokines by large proportions of both NKT cells and thymus-independent α/β T cells from rats is a feature shared by both subsets as well as by NKT cells from mice and humans. The recent description of a subset of CD4+ T cells in normal mice which are phenotypically and functionally indistinguishable from NKT cells, except that they do not express CD161 (Chen and Paul, 1998), raises the possibility that the α/β T cells in athymic rats are the equivalents of this cell type. Furthermore it suggests that thymus-independent α/β T cells are present and functional in normal animals.

NKT cells in mice and humans differ from those in rats because in the latter species, most express CD8 (Brissette-Storkus et al., 1994). NKT cells in athymic (rNu/rNu) rats
and their euthymic (rNu+/) littermates also share this characteristic. However, it has been shown in the present study that although rat NKT cells express predominantly CD8α (approximately 90% in euthymic rats), approximately 10% of them express CD8α chains exclusively. In addition, it appears likely that NKT cells and the CD8α/β+ α/β T cells in athymic rats express an excess of CD8α chains relative to CD8β chains on the cell surface. This suggests that both of these cell types can co-express CD8αα homodimers and CD8αβ heterodimers on the cell surface and this highlights another similarity between NKT cells and the thymus-independent α/β T cells. It is possible, therefore, that T cells which develop by non-classical (including extra-thymic) pathways are present in euthymic animals.

7.4 Future prospects

Many of the findings presented in this thesis warrant further investigation. It would be most beneficial to develop a technique to isolate cells from LFV. The isolation of cells from cryptopatches (Saito et al., 1998) indicates that this should be feasible. Cells isolated from LFV could be phenotyped more accurately by multi-parameter flow cytometry, and the co-expression of molecules such as TCR, CD4, CD8, CD25, CD43, CD161, CD49d, αE2-integrin and others could be established. Furthermore, adoptive transfer to congenic (or thymectomised and irradiated) recipients could allow examination of the capacity of such cells to colonise the periphery. Isolated subsets of cells from LFV could also be examined by RT-PCR to detect mRNA transcripts from the genes encoding the enzymes RAG-1, RAG-2, TdT and also from the gene encoding pTα.

The adoptive transfer experiments described herein could be extended.
questions that could be addressed are whether $\alpha/\beta$ T cells from athymic rats are recruited to sites of inflammation, the turnover and life-span of $\alpha/\beta$ T cells in rNu/rNu rats and the recruitment of these cells to the gut mucosa. It would be of great interest to compare the behaviour of the earliest $\alpha/\beta$ T cells in TDL (10 week old rNu/rNu rats) with that of subsets of $\alpha/\beta$ T cells isolated from the TDL of older nude rats. Furthermore, it is important to compare the behaviour of the thymus-independent T cells from athymic rats with the behaviour of NKT cells. Transfer of subsets of thymus-independent T cells, defined by surface antigens, would allow investigation of the stability of these phenotypes and their inter-conversions.

CD1 has been shown to play an important role in the development of NKT cells and possibly some IEL (Balk et al., 1991). It would be most interesting, therefore, to determine whether CD1 protein (antibody staining) and/or mRNA (in situ hybridisation) is present in LFV. Furthermore, it is important to examine whether $\alpha/\beta$ T cells from athymic rats and NKT cells recognise, and/or are stimulated through interactions with CD1 molecules. NKT cells exhibit a limited TCR repertoire which has been shown to recognise CD1 molecules (Brossay et al., 1998; Spada et al., 1998 and reviewed by Vicari and Zlotnik, 1996; Taniguchi et al., 1996; MacDonald, 1995; Bendelac, 1995; Bix and Locksley, 1995). It would, therefore, be of interest to examine the TCR repertoire of the $\alpha/\beta$ T cells found in athymic rats and to compare these repertoires with those expressed by NKT cells in normal rats.

NKT cells are believed to have immunoregulatory functions and in some experimental autoimmune diseases they appear to exert a protective influence (Wilson et al. 1998; Godfrey et al., 1997; Baxter et al., 1997; Meiza et al., 1996). Since $\alpha/\beta$ T cells from
Athymic rats share a number of features with NKT cells, it will be of interest to examine whether they have regulatory functions in various models of autoimmunity in rats. Investigation of the recruitment of thymus-independent T cells to sites of inflammation is feasible, using RT7 congenic animals.

Another possible similarity between NKT cells and the thymus-independent α/β T cells in athymic rats that could be investigated is the expression of FasL. It would be interesting to examine whether these cell types express FasL (RT-PCR detection of mRNA transcripts) and whether they can induce apoptosis in Fas expressing cells. This may represent one mechanism by which non-classical α/β T cells could regulate immune responses in normal individuals.

Because NKT cells from euthymic rats and the α/β T cells from athymic rats appear to be related, it would be anticipated that the α/β T cells in athymic nude mice would also produce large quantities of cytokines following stimulation in vitro. NKT cells have been more widely studied in this species but their relationship to other thymus-independent T cells, such as those found in nude mice, has not been explored in detail.
7.5 Conclusions

The findings presented in this thesis indicate that both the CD161\(^{-}\) and CD161\(^{+}\) (NKT cells) subsets of \(\alpha/\beta\) TCR\(^{+}\) cells in athymic rats are heterogeneous with respect to expression of CD4, CD8\(\alpha\) and CD8\(\beta\). However, they have striking similarities with respect to size, levels of TCR expression, expression of adhesion molecules and cytokine production in response to stimulation in vitro. The phenotypic heterogeneity between these cells might reflect different extra-thymic origins, as discussed in section 7.2. However, the expression of \(\alpha_{E2}\), CD49d and CD8\(\alpha\alpha\) homodimers by significant proportions of \(\alpha/\beta\) T cells in athymic rats suggests that at least some of these cell may originate from mucosal sites such as the gut. It is interesting to note in this respect that the majority of lymphocytes in LFV express CD161. CD161 is a differentiation marker that is expressed by subsets of mouse (Carlyle et al., 1997) and human (Poggi et al., 1996) thymocytes and it could, therefore, also be expressed by T cells developing at extra-thymic sites. Although the majority of \(\alpha/\beta\) T cells in athymic rats are CD161\(^{-}\), this might reflect a particular stage in differentiation or activation. CD161 could be down-regulated as the cells mature, either in a programmed manner (as appears to happen in the mouse thymus [Carlyle et al., 1997]) or in response to activation (as occurs with mouse NKT cells in vitro [Chen et al., 1997]). In athymic rats, the variety of CD4 and CD8 phenotypes associated with the CD161\(^{-}\) and CD161\(^{+}\) subpopulations and the marked variations between individual rats favour the latter possibility. For this reason, it is important to note that some mouse CD4\(^{+}\) NKT cells down-regulate expression of both CD4 and CD161 following in vitro activation (Chen et al., 1997). It is feasible, therefore, that NKT cells in the rat express CD161 and CD4 molecules at particular stages in differentiation (and not others) and that they express CD8\(\alpha\alpha\) in response to activation.
These findings suggest that the extra-thymically derived (thymus-independent) $\alpha/\beta$ T cells in athymic rats are related closely to NKT cells and that they may belong to the same lineage. To the extent that extra-thymic T cell developmental pathways identified in athymic animals also exist in euthymic animals (e.g. in LFV), the cells produced by these pathways (including both NKT cells and NKT-like cells) can be expected to be present in both athymic and euthymic animals. As part of a more primitive immune system, cells that express the TCR and recognises antigen possibly in association with non-classical MHC molecules such as CD1 (as exhibited by mouse and human NKT cells [Brossay et al., 1998; Spada et al., 1998]), could lack classical T cell effector functions (such as graft-rejection and graft versus host response) which are based on recognition of polymorphisms in classical MHC molecules (Vos et al., 1980; Sarawar et al., 1991). Furthermore, like NKT cells (reviewed in Vicari and Zlotnik, 1996; Taniguchi et al., 1996; Bendelac, 1995; MacDonald, 1995), these cells may rely on relatively conserved TCR repertoires. They may also leave their sites of primary differentiation with fully developed capacity to elicit rapid effector functions, rather than with a reliance on memory, clonal expansion and differentiation after exposure to antigen. If this was the case, thymus-independent T cells would need to be produced continuously throughout life rather than during the pre-pubertal stage. In a layered immune system, thymus-independent T cells may have function(s) that are primarily regulatory, through the rapid deployment of cytokines in response to novel (perhaps self) antigens. In this way they could complement the effector functions of other cell types including macrophages, dendritic cells and classical T cells. Furthermore, it is possible that populations of thymus-independent T cells might also take part in normal immunological homeostasis. Like NKT cells (Wilson et al. 1998; Godfrey et al., 1997;
Baxter et al., 1997; Meiza et al., 1996), they could have important roles in the control of responsiveness to self-antigens and in the pathogenesis of autoimmune diseases. This aspect alone should ensure that thymus-independent T cells and the sites at which they are developed are worthy subjects for research in the future.
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