Chapter 1 Introduction

1.1 Introduction

The intricate process of signal transduction plays a vital role in the immune system by enabling cytokines to control leukocyte development and function. Cytokines are soluble factors which have pleiotropic roles in the control of leukocytes, acting during the development of cells from progenitors, the coordination of the immune response and during activation of mature cells at sites of infection. Cytokines exert their effects by binding to specific receptors on the surface of cells, resulting in the transduction of various signals within the cell. Dysregulation of cytokine signalling contributes to diseases such as leukaemia and asthma.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine which regulates both lineage determination during haemopoiesis, and effector cell functions, such as phagocystosis and the production of toxic reactive oxygen species (ROS), during immune responses. GM-CSF binds a cell surface receptor consisting of a ligand-specific alpha chain (GMRα) and a common beta subunit (βc), shared with the receptors for interleukin-3 (IL-3) and IL-5. Binding of GM-CSF to its receptor triggers intracellular signal transduction which results in the regulation of cell proliferation, survival, differentiation and effector functions. A number of signalling pathways mediated by the βc have been described and regions of the receptor necessary for cell proliferation, differentiation and survival of cell lines have been investigated. However, the signalling pathways and receptor-proximal events by which GM-CSF regulates the survival and effector functions of mature myeloid cells are unknown.
The overall aim of this thesis is to characterise these signalling pathways and determine the nature and mechanism of GM-CSF regulation of survival and effector cell functions.

1.2 The Roles of the Haemopoietic System

1.2.1 Steady-state haemopoiesis

Steady-state haemopoiesis is the tightly regulated process by which early haemopoietic cells (progenitors) are produced and differentiate into recognisable lineages (reviewed in Ogawa, 1994). It is a continuous process, constantly required to renew the vast numbers of leukocytes and erythrocytes required in humans to maintain homeostasis. Early in the development of mammals, haemopoiesis occurs in the foetal liver and then later in the post-natal bone marrow. Most blood cells mature in the bone marrow and are mobilized into the blood circulation, lymphatic system or tissues, as needed. There are two main lineages of differentiation (Figure 1.1); 1) the lymphoid lineage, which gives rise to T- and B-lymphocytes as well as natural killer cells, and 2) the myeloid lineage which gives rise to monocytes, macrophages and granulocytes. Erythroid cells, mast cells and platelets, are also derived from the common myeloid progenitor, CFU-GEMM (colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte).

Deriving from the lymphoid progenitor, CFU-L (colony forming unit-lymphocyte), are three types of lymphocytes with specialised functions: 1) T-cells, which mature in the
thymus, are central to the control of the immune system, 2) B-cells, which mature in the bone marrow, produce antibodies, and 3) Natural killer cells which recognise and destroy virally infected cells and some tumour cells (reviewed in Blach-Olszewska, 2005) (Figure 1.1).

Multiple cell types are derived from myeloid progenitors. These include monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils), erythrocytes and megakaryocytes which produce platelets (reviewed in Metcalf, 1991; Ogawa, 1994) (Figure 1.1). Of particular importance in this thesis are the functions of monocytes, macrophages and neutrophils. Neutrophils constitute the majority of blood leukocytes and directly fight invading microbial pathogens. They are short-lived cells which are able to migrate to areas of infection and release toxins as well as phagocytose foreign particles. Monocytes, like neutrophils, are able to migrate to sites of infection and directly fight microbial pathogens by the phagocytosis of foreign particles and release of toxins. They differentiate into macrophages upon migration into tissues, becoming long lived cells which are effective in the destruction of microbial pathogens.

As many different lineages of haemopoietic cells are required by the human body, there is a constant requirement for renewal of these populations. Tight control over the proliferation and differentiation of haemopoietic progenitors is required. A complex control system has evolved which is mediated, in part, by the action of many different soluble factors, termed cytokines. These soluble factors bind cell surface receptors and trigger signalling events which are necessary for the regulation of the proliferation, survival and differentiation of progenitors into mature cells of specific lineages. For example, *in vivo* differentiation of progenitors into neutrophils requires the action of the
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Figure 1.1 Schematic representation of the development of the haemopoietic system. Haemopoietic cells are derived from the pluripotent haemopoietic stem cell by a process which involves proliferation and differentiation under the control of soluble factors such as cytokines. The two basic lineages, lymphocytic and myeloid, are described here in terms of their colony forming ability in vitro. The final differentiation of the mature cells of the haemopoietic system often occurs outside of the bone marrow, e.g., macrophage differentiation occurs in tissues once from monocytes after migration out of the blood. Colony Forming Units (CFU) refer to immature cells with the restricted potential for differentiation, as designated by L: Lymphocyte, G: Granulocyte, E: Erythrocyte, M: monocyte, Meg: Megakaryocyte, Eo: Eosinophil, Bas: Basophil.
cytokine granulocyte colony-stimulating factor (G-CSF) (Liu et al., 1996). *In vitro* studies have shown that GM-CSF can stimulate the proliferation and survival of the precursors of many lineages including neutrophils, monocytes and eosinophils (Metcalf, 1986). GM-CSF can also act as a differentiation agent, promoting the maturation of both granulocytes and monocytes.

1.2.2 Reactive haemopoiesis and the activation of phagocytes

In addition to their roles in regulating steady-state haemopoiesis, cytokines are also important in the regulation of reactive haemopoiesis. In emergency situations such as infection, the haemopoietic system rapidly responds to restore homeostasis. This response includes accelerated haemopoietic cell production (reactive haemopoiesis), an increase in the capacity of specific haemopoietic cells to survive and the functional activation of circulating haemopoietic cells. Activation of mature phagocytic cells such as neutrophils, monocytes/macrophages and eosinophils includes the activation of effector functions such as adherence, migration to sites of infection (chemotaxis), degranulation, release of toxic reactive oxygen species (ROS) and phagocytic activity, i.e. the ability to recognise, completely engulf and kill large particles such as bacteria (described in greater detail in Sections 1.3.1 and 1.4.4.5). These cellular events are necessary in mounting an inflammatory response and constitute a frontline of defence against microbial pathogens.

1.3 The Immune System

The immune system has evolved to protect us from infections such as bacteria, viruses and parasites. The human immune system is capable of two broad types of response to such
infections: (1) the innate immune response, where pathogens are recognised in a non-specific way, and (2) the adaptive immune response, where specific antigens are recognised and remembered by components of the immune system. The activity of cytokines is essential to the implementation of these responses, as they mediate communication between leukocytes and activation of effector cells which fight infection, examples of which are described below.

1.3.1 Innate Immune Response

During the innate immune response, phagocytic cells such as neutrophils, monocytes and macrophages respond to invading microorganisms by releasing toxins such as ROS (Hampton et al., 1998) and antimicrobial factors from granules during “degranulation”, (Faurschou and Borregaard, 2003) to kill the pathogen, then internalise and destroy them via a process named phagocytosis (Kwiatkowska and Sobota, 1999). The recognition of microorganisms by these cells is not necessarily specific for particular antigens, for example, recognition of bacteria can occur via host receptors which bind to fMLP (a tripeptide, Met-Leu-Phe, chemotactic factor similar to bacterial peptides) and are present on the surface of neutrophils, monocytes and macrophages (reviewed in Panaro and Mitolo, 1999).

The activation of phagocytes by fMLP can be enhanced or “primed” by prior stimulation with cytokines such as GM-CSF or TNFα. Cytokine priming of effector functions enhances phagocyte killing of micro-organisms. For example, fMLP-stimulated ROS production in neutrophils and monocytes/macrophages is synergistically enhanced by priming of these cells with GM-CSF or TNFα (Lopez et al., 1986; Smith et al., 1990). Phagocyte production of ROS upon detection of bacteria was shown to make a major

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contribution to the killing of the invading organisms by these cells (Samuni and Czapski, 1978; Wolcott et al., 1994). Phagocytic activity of neutrophils and macrophages can also be primed by GM-CSF (Fleischmann et al., 1986; Coleman et al., 1988). Thus cytokines such as GM-CSF are critical regulators of the front-line defence against the invasion of foreign pathogens and therefore important regulators of innate immunity. It is the ability of GM-CSF to prime monocytes/macrophages to respond to foreign pathogens and either generate ROS or phagocytic activity that will be examined in this thesis.

The innate immune response is also mediated by the activation of the complement system (reviewed in Morgan et al., 2005). During the complement response, a group of serum proteins promotes inflammation, lysis of micro-organism cell membranes and activation of phagocytes. Neutrophils and macrophages have specific receptors for the complement fragment C5a, which is generated on the surface of the target micro-organism. Diffusion of C5a away from the target promotes chemotaxis of phagocytes to the site of infection. Production of GM-CSF or TNFα at the site of infection then inhibits C5a-stimulated chemotaxis (Binder et al., 1999), which results in the localisation of phagocytes to the site, where they promote the destruction of the invading micro-organism.

In summary, the innate immune response is a frontline defence against infection which is mediated by phagocytic cells and complement factors, and is enhanced by the action of cytokines such as GM-CSF.

1.3.2 Acquired Immune Response

The acquired (or adaptive) immune response is highly specific for a particular antigen and becomes more effective on subsequent encounters with the same antigen. During the
acquired immune response there is considerable interaction between lymphocytes and phagocytes. Lymphocytes are responsible for the recognition of foreign antigens, leading to various attacks on the infectious agent. B-cells produce soluble antibodies which recognise foreign antigens. Antibody binding to micro-organisms can lead to its phagocytosis by neutrophils, eosinophils, monocytes or macrophages, or to activation of the complement system which creates holes in the membrane of the micro-organism. Cytotoxic T-cells recognise target cells via their genetically variable T-cell receptors (TCRs). These cells destroy their targets by the action of cytotoxic granules or by triggering apoptosis in the target cell. Activated T-cells also release cytokines such as GM-CSF which activate phagocytes and promote their destruction of micro-organisms.

Regulation of the acquired immune response requires the action of many cytokines. Upon recognition of antigen by a type of T-cell known as T-helper cells (Th cells), specific cytokines are released. The type of cytokines released by the Th cell is a major determinant of the type of immune response which is mounted. The Th1 response is mediated by Th cell release of IL-2 and interferon γ (IFNγ) and leads to cell mediated inflammation. This response is important in the fighting of intracellular pathogens such as viruses and includes the action of macrophages. During the Th2 immune response, Th cells release IL-4, IL-5, IL-6 and IL-10, increase B-cell proliferation and, pathologically, can lead to allergic responses. The Th2 immune response functions primarily to protect against parasites, such as helminths, and can be mediated by activated eosinophils (reviewed in Shi, 2004).

Production of cytokines such as GM-CSF and TNFα by activated T-cells and macrophages at sites of infection, enhances the life-span and effector functions of phagocytes during the
acquired immune response (Flad et al., 1999; Wick, 2004). Cytokines therefore play an essential role in the regulation and implementation of the acquired immune response.

1.4 The roles of specific cytokines and their receptors in regulating steady-state haemopoiesis, reactive haemopoiesis and effector cell functions

Cytokines are soluble factors which regulate interactions between leukocytes as well as the cellular functions of individual leukocytes. Cytokines regulate such processes as haemopoiesis, the co-ordination of the immune response and the activation of effector cells. Some cytokines have also been found to act on non-haemopoietic cells such as epithelial cells, neurons and hepatocytes.

It is the distribution of specific cytokine receptors which defines the cellular targets of each cytokine. Many cytokines have multiple functions, a phenomenon due in part to the pattern of receptor expression on differing cell types at different stages of haemopoiesis, and also to multiple and distinct signalling pathways emanating from their receptors. In this Section is a description of such pleiotropic cytokines whose functions are pertinent to this thesis.

1.4.1 The GM-CSF/IL-3/IL-5 family of cytokines share a common receptor subunit, $\beta_c$

The cytokines GM-CSF, IL-3 and IL-5 share an important feature in that they all signal through a common subunit, designated $\beta_c$. The cellular expression of specific $\alpha$ chains for each cytokine, in combination with the $\beta_c$, permits signalling by individual cytokines. Each of these cytokines regulates specific stages of haemopoiesis, reactive haemopoiesis and the functions of specific mature haemopoietic cells. Table 1.1 describes the basic features of
GM-CSF, IL-3 and IL-5. From these descriptions we can see that each cytokine shares features with the others, but also has distinct roles in the control of haemopoietic cells. The broad range of target cells for IL-3 is consistent with the expression of the IL-3Rα subunit of the receptor on the widest variety of cell types. Likewise, the more restricted roles of GM-CSF and IL-5, are also a reflection of the expression pattern of their α subunit receptors. The functional roles of these cytokines are described in more detail in Sections 1.4.2 to 1.4.5.

Typical for secreted proteins, the cytokines GM-CSF, IL-3 and IL-5 are produced and released in heavily glycosylated forms. GM-CSF and IL-3 act as monomers but active IL-5 exists as a homodimer (McKenzie et al., 1987). The crystal structure of GM-CSF, IL-3 and IL-5 indicate they are composed of two pairs of antiparallel α helices, similar to numerous other haemopoietic cytokines (Diederichs et al., 1991). Despite the low levels of sequence identity between the mouse and human forms, genetic and biochemical studies have shown that these cytokines are functionally well conserved.

GM-CSF, IL-3 and IL-5 signal by binding cell surface receptor complexes which share the βc and specific α subunits GMRα, IL-3Rα and IL-5Rα respectively. In the mouse, IL-3 also signals via a complex of IL-3Rα and a separate β chain designated βIL-3. Specificity is conferred via the expression of the α subunit and intracellular signalling events are initiated by the βc subunit. The mechanism of signal transduction via GM-CSF, IL-3 and
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Length of mature hu peptide</th>
<th>Size (kDa, including glycosylation)</th>
<th>Homology (aa identity between hu and mu)</th>
<th>Main producers (normal conditions)</th>
<th>Receptor components</th>
<th>Main receptor expressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>127aa</td>
<td>23</td>
<td>56%</td>
<td>- Activated T-cell (major source)</td>
<td>GM-CSFα, βc</td>
<td>- Monocytes</td>
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<td></td>
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<td>- Activated macrophages</td>
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<td>- Neutrophils</td>
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<td></td>
<td>- Stromal cells</td>
<td>GM-CSFα, βc</td>
<td>- Eosinophils</td>
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<td></td>
<td></td>
<td></td>
<td>- Bone marrow vasculature</td>
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<td>- Dendritic cells</td>
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<td></td>
<td></td>
<td>endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>133aa</td>
<td>26</td>
<td>30%</td>
<td>- Activated T-cell (major source)</td>
<td>IL-3α, βc (in mu also β_{IL-3})</td>
<td>- Haemopoietic progenitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Activated eosinophils</td>
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<td>- Granulocytes</td>
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<td></td>
<td>- Mast cells</td>
<td>IL-3α, βc (in mu also β_{IL-3})</td>
<td>- Monocytes</td>
</tr>
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<td>- Megakaryocytes</td>
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<td></td>
<td></td>
<td></td>
<td>IL-3α, βc (in mu also β_{IL-3})</td>
<td>- Dendritic cells</td>
</tr>
<tr>
<td>IL-5</td>
<td>Homodimer of 134aa monomers</td>
<td>26 (monomer)</td>
<td>70%</td>
<td>- Activated T-cell (major source)</td>
<td>IL-5α, βc</td>
<td>- Eosinophils</td>
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<td>- Activated eosinophils</td>
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<td>- Mast cells</td>
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</table>

Table 1.1 Comparison between cytokines which signal through the $\beta_c$ receptor. Mouse: mu; human: hu, interleukin: IL; amino acid: aa.
IL-5 receptors is discussed in much greater detail in Sections 1.5.2 and 1.5.4.

1.4.2 GM-CSF, IL-3 and IL-5 share overlapping biological functions

Interestingly, a range of biological outcomes can result from signalling from members of this cytokine family, many of which are shared between the three cytokines. Each are able to stimulate the proliferation, differentiation, survival and effector cell functions of a range of haemopoietic cells during in vitro studies. As the βc is the major signalling subunit for GM-CSF, IL-3 and IL-5, it is not surprising that these cytokines share a number of overlapping functional activities.

1.4.2.1 GM-CSF, IL-3 and IL-5 stimulate the proliferation and differentiation of haemopoietic progenitors and cells line in vitro.

GM-CSF, IL-3 and IL-5 are all able to stimulate proliferation and differentiation of haemopoietic progenitor cells in colony forming assays and factor-dependent cell lines in vitro. In cultures of human bone marrow cells, huGM-CSF stimulates the formation of granulocyte and/or macrophage colonies at low cytokine concentrations, but erythroid, multi-lineage and megakaryocytic colonies can also occur with high GM-CSF concentrations (Sieff et al., 1985; Metcalf, 1986).

Since IL-3 receptors are expressed on a diverse range of haemopoietic progenitors (e.g. myelomonocytic stem cells, myelocytes, megakaryocytes and erythrocyte precursors) it is not surprising that IL-3 is able to stimulate in vitro proliferation and differentiation of the widest variety of cells. It can stimulate the generation of macrophages, neutrophils, eosinophils, basophils, mast cells megakaryocytes and erythroid cells from primary
cultures containing haemopoietic precursors, such as bone marrow (reviewed in Schrader, 1998). In addition, IL-3 shows strong synergistic activities with other cytokines during in vitro colony forming assays. For example, IL-3 synergises with CSF-1 in the production of macrophages (Koike et al., 1986). Unlike GM-CSF and IL-3, IL-5 has a narrow range of target cells during haemopoiesis: IL-5 stimulates the production of eosinophils in liquid bone marrow cultures (Sanderson, 1998).

GM-CSF, IL-3 and IL-5 are capable of stimulating the proliferation and survival of factor-dependent cell lines. GM-CSF and IL-3 are capable of stimulating the proliferation of factor-dependent cell lines such as M-O7e (a human megakaryoblastic leukaemia line) (Avanzi et al., 1988) and the premyeloid cell line TF1 (Kitamura et al., 1989). When the murine leukemic cell line M1 is transfected with huGMRa and huβ, huGM-CSF is able to stimulate the differentiation of these cells into macrophages (Smith et al., 1997). IL-5 is also able to stimulate the proliferation of cell lines such as the IL-5-dependent mouse myeloma line MOPC-104E (Mita et al., 1989).

The signal transduction mechanisms by which enable GM-CSF, IL-3 and IL-5 to promote the proliferation and differentiation of cells are discussed in Sections 1.5.4 and 1.5.5. The specific signalling pathways promoting proliferation are reasonably well understood, in contrast, the pathways effecting differentiation are only beginning to be unravelled.

1.4.2.2 GM-CSF, IL-3 and IL-5 stimulate the survival of haemopoietic progenitors and cell lines in vitro.

The specific intracellular signals which promote cell survival are essentially distinct from those which regulate proliferation (described in more detail in Sections 1.5.5.1 and
Therefore, although in many situations such as during colony-forming assays, survival and proliferation are simultaneously stimulated by the one cytokine, these two biological outcomes should be considered separately. Stimulation of survival is important during steady-state as well as reactive haemopoiesis. During the immune response to infection, mature cells such as neutrophils, monocytes and eosinophils need to be able to enter and function in environments where toxic factors such as ROS and nitric oxide (NO) are released to fight invading micro-organisms, therefore enhancement of the cell’s ability to survive will enhance their ability to function.

We know from primary culture experiments that GM-CSF is able to stimulate the survival of neutrophils (Brach et al., 1992) and eosinophils (Lopez et al., 1986b). IL-3 also promotes survival of a number of cell types, including mast cells (Schrader, 1981) and splenic haemopoietic progenitors (Spivak et al., 2006). GM-CSF and IL-3 promote the survival of cell lines such as M-O7e (a human megakaryoblastic leukaemia line) (Avanzi et al., 1988). Both IL-3 and IL-5 are capable of promoting the survival of eosinophils in vitro (Rothenberg et al., 1988; Begley et al., 1986), and IL-5 the survival of IL-5 dependent cell lines such as MOPC-104E (Mita et al., 1989).

1.4.3 The in vivo roles of GM-CSF, IL-3 and IL-5 as revealed by analysis of knockout and transgenic mice

1.4.3.1 The in vivo functions of GM-CSF

Surprisingly, despite its marked effects on the proliferation and differentiation of myeloid progenitors in vitro, GM-CSF$^{−/−}$ knockout mice display no major defect in the production of any haemopoietic cell type (Stanley et al., 1994; Dranoff et al., 1994). The numbers of
Peripheral haemopoietic cells, as well as the colony-forming ability of bone marrow and splenic cells and the myeloid:erythroid ratio were not significantly perturbed. The only minor change to steady-state haemopoiesis was a greater variety between mice of granulocyte subpopulation numbers and splenic masses in GM-CSF−/− mice. GM-CSF is therefore not required for steady-state haemopoiesis in vivo. Since GM-CSF is a promoter of haemopoiesis in vitro, these results indicate that redundancy exists amongst the cytokines necessary for the regulation of steady-state haemopoiesis.

Expression of GM-CSF is involved in the production of the antigen-presenting dendritic cells (DC). In transgenic mice which overexpressed GM-CSF, there was a small increase in DC numbers in thymus and spleen and a threefold increase in DC numbers in the lymph nodes. However, GM-CSF is not required for overall production of DC, as normal numbers of DC were found in GM-CSF−/− and muβ−/−,muβIL-3−− mice, excepting a significant reduction in the DC population of lymph nodes (Vremec et al., 1997).

Further analysis of the GM-CSF−/− knockout mice has implicated an essential role for GM-CSF in the regulation of effector cell functions of alveolar macrophages. Lungs from GM-CSF−/− exhibit peribronchovascular infiltration by lymphocytes, large numbers of intra-alveolar macrophages and evidence of surfactant accumulation in alveoli. This alveolar-proteinosis-like pathology is similar to the human condition pulmonary-alveolar proteinosis (PAP). It is thought that this lung defect in GM-CSF−/− mice is probably the result of impaired macrophage function, leading to the failure of surfactant clearance. Consistent with the hypothesis that GM-CSF is required for bronchial surfactant homeostasis, impaired surfactant clearance can be alleviated by delivery of GM-CSF to the lungs by various means (Reed et al., 1999).
A number of experiments using mouse models of inflammation have shown that GM-CSF⁻/⁻ mice are defective in inflammatory responses. In mouse model of collagen-induced arthritis GM-CSF⁻/⁻ mice were virtually disease free (Campbell et al., 1998). GM-CSF⁻/⁻ mice exhibited reduced clearance of the fungus *Cryptococcus neoformans* in a model of lung infection (Chen et al., 2007). Reduced Th1 and Th2 cytokine production, delayed monocyte recruitment and reduced eosinophil recruitment were observed in *C. neoformans* infected GM-CSF⁻/⁻ mouse lungs. Alveolar macrophage phagocytosis, killing, and H₂O₂ production were defective in GM-CSF⁻/⁻ mice with lung infections of the gram-negative bacteria *Pseudomonas aeruginosa* (Ballinger et al., 2006). GM-CSF⁻/⁻ mice were also protected from inflammatory disease in two models of experiments glomerular nephritis: neutrophil-mediated heterologous-phase anti-glomerular basement membrane glomerulonephritis (GMB) and T cell/macrophage-mediated crescentic autologous-phase anti-GBM glomerulonephritis (Kitching et al., 2002). Consistent with these studies, constitutive overexpression of GM-CSF in mice leads to massive macrophage expansion, which results in blindness and fatal tissue damage (Lang et al., 1987). GM-CSF is clearly involved in reactive haemopoiesis; it is necessary and required for the full activation of specific mature haemopoietic cells. The role of GM-CSF signalling in reactive haemopoiesis is discussed in further detail in Section 1.4.4.4.

1.4.3.2 The *in vivo* functions of IL-5

Transgenic mice in which T-cells overexpress IL-5 develop massive eosinophilia, indicating that IL-5 is sufficient to induce differentiation of eosinophils (Dent et al., 1990). Interestingly, these mice are otherwise healthy. These observations are in contrast to *in vivo* overexpression of IL-3 and GM-CSF where in both cases mice died after only a few
weeks due to massive tissue infiltration by myeloid cells (Johnson et al., 1989; Chang et al., 1989). In the case of IL-5, it seems that increased numbers of eosinophils themselves are not harmful. It is possible that the tissue damage seen in allergic responses as a result of eosinophilia requires other agents which activate eosinophils. In support of this possibility is the observation that IL-5 plays no role in the production of IgE antibodies (Coffman et al., 1989). It is seems likely that, despite its activation of eosinophils in \textit{ex vivo} experiments (Section 1.4.2), IL-5 may not be sufficient to activate eosinophils \textit{in vivo}.

In IL-5\(^{-/-}\) knockout mice, the numbers of circulating eosinophils in were 2 to 3 fold reduced (Foster et al., 1996). However, morphologically-normal eosinophils are consistently produced in IL-5 deficient mice, indicating a functional redundancy of IL-5 (Foster et al., 1996; Kopf et al., 1996). IL-5 deficient mice also lack eosinophil production in response to \textit{Mesocestoides corti} helminth infection or aero-allergen sensitization with ovalbumin (Kopf et al., 1996; Foster et al., 1996). Furthermore, the administration of anti-IL-5 antibody to mice infected with the parasites \textit{Nippostrongylus brasiliensis}, \textit{Schistosoma mansoni}, \textit{Heligmosomoides polygyrus} or \textit{Strongyloides venezuelensis} totally blocked the development of eosinophilia (reviewed in Sanderson, 1998). Therefore IL-5, like GM-CSF, plays a reactive haemopoiesis \textit{in vivo}, but not absolutely necessary for eosinophil production at basal levels.

\textbf{1.4.3.3 The phenotype of }\beta_c^{-/-}\textit{ mice}

Mice deficient for the \(\beta_c\) have useful in examining the effect on the haemopoietic system through the combined loss of GM-CSF and IL-5 responses (IL-3 is able to still signal through \(\beta_{IL-3}\)). The analysis of \(\beta_c^{-/-}\) mice has shown an alveolar proteinosis phenotype
similar to that of GM-CSF\(^{−/−}\) mice, deficiency in eosinophil numbers and the eosinophil response to the parasite *Nippostrongylus brasiliensis* (Nishinakamura et al., 1995). Whether the reduction of basal eosinophils in these mice is greater than the reduction of basal eosinophils in IL-5 deficient mice is not known. These defects represent a cumulative effect of the loss of both GM-CSF and IL-5 and is consistent with the phenotype described by both knockout lines described above.

Two findings indicate that the regulation of effector cell functions is the most important *in vivo* role of the \(\beta_c\): the alveolar proteinosis lung disease exhibited by \(\beta_c^{−/−}\) mice, and the inability of these mice to fight off parasitic infections such as *Nippostrongylus brasiliensis* (Nishinakamura et al., 1995). The defect in effector functions of neutrophils and macrophages from \(\beta_c\) deficient mice was confirmed in a detailed study by (Scott et al., 1998). These studies showed that GM-CSF priming of survival, phagocytosis and hydrogen-ion production were defective in \(\beta_c^{−/−}\) neutrophils, and alveolar macrophages displayed a phagocytic defect. It is then surprising that the molecular mechanisms by which signalling through the \(\beta_c\) controls effector cells functions are largely unknown.

1.4.3.4 The phenotype of \(\beta_c^{−/−};IL-3^{−/−}\) and \(\beta_c^{−/−};\beta_{IL-3}^{−/−}\) mice

\(\beta_c^{−/−}\) mice are not deficient in IL-3 signalling; the response of \(\beta_c^{−/−}\) bone marrow to IL-3 is normal. Furthermore, IL-3 signalling is also undisturbed in \(\beta_{IL-3}^{−/−}\) mice, which respond normally to IL-3 in bone marrow colony-forming assays (Nishinakamura et al., 1995). Therefore the \(\beta_{IL-3}\) receptor is able to completely compensate for the loss of IL-3 signalling through \(\beta_c\) and vice versa. To study the *in vivo* role of IL-3, mice were generated with deficiencies in \(\beta_c\) and IL-3 ligand (\(\beta_c^{−/−};IL-3^{−/−}\)). Surprisingly, despite the potent ability of
IL-3 to stimulate almost all phases of haemopoiesis, $\beta_c^{-/-};IL-3^{-/-}$ mice were capable of normal haemopoiesis, with the exception of a decrease in eosinophil numbers, consistent with the loss of IL-5 signalling. In addition, analysis of mice deficient for IL-3 only ($IL-3^{-/-}$) also demonstrated no perturbation to steady-state haemopoiesis (Mach et al., 1998). IL-3, like GM-CSF, is therefore is not required in vivo for steady-state haemopoiesis (Nishinakamura et al., 1996). IL-3 may play a redundant role in the regulation of steady-state haemopoiesis, which would become evident when mice are constructed carrying deficiencies for IL-3 signalling in combination with deficiencies of other cytokines such as CSF-1, which synergises with IL-3 during production of macrophages (Koike et al., 1986).

In vivo overexpression of IL-3 in the mouse leads to a non-neoplastic myeloproliferative syndrome and death in the majority of cases (Chang et al., 1989). Therefore, although loss of IL-3 signalling is not sufficient to perturb haemopoiesis in vivo, an increase in IL-3 is sufficient for massive myeloid cell expansion in vivo.

Mice lacking $\beta_c$ as well as $\beta_{IL-3}$ ($\mu\beta_c^{-/-};\mu\beta_{IL-3}^{-/-}$) exhibited essentially the same phenotype as $\beta_c$ knockout mice and the combination $\beta_c^{-/-};IL-3^{-/-}$ (Scott et al., 2000). These mice were capable of steady-state haemopoiesis and displayed normal white blood cell numbers, but with a reduction in eosinophils. Despite this reduction in eosinophil numbers, some mature eosinophils were present, leading to the conclusion that neither GM-CSF, IL-3 or IL-5 are absolutely required for the production of haemopoietic cells. An alternative mechanism must therefore exist which can account for the loss of these cytokines during steady-state haemopoiesis, although this mechanism cannot achieve normal numbers of mature eosinophils.
1.4.4 Roles of GM-CSF, IL-3 and IL-5 in reactive haemopoiesis and control of effector cell functions

When homeostasis is threatened by invading pathogens, the innate immune system responds in terms of reactive haemopoiesis (accelerated haemopoietic cell production) and an increase in survival and functional activation of mature cells such as neutrophils, eosinophils and monocytes/macrophages (Section 1.2.2). These responses are regulated by cytokines such as GM-CSF, IL-3 and IL-5. Although much is known of the outcomes of stimulation of haemopoietic cells by these cytokines, the mechanisms by which they activate mature cells remain mostly uncharacterized and are a focus of this thesis.

1.4.4.1 GM-CSF, IL-3 and IL-5 promote reactive haemopoiesis

GM-CSF, IL-3 and IL-5 stimulation of accelerated haemopoiesis in subsets of myeloid cells occurs upon their release in response to immune attack, leading to the promotion of proliferation and differentiation of haemopoietic cells bearing receptors. All three cytokines are primarily produced by activated T-cells, with activated mast cells and eosinophils common sources for IL-3 and IL-5, and activated macrophages a common source for GM-CSF. IL-3 release by activated T-cells during reactive haemopoiesis results in accelerated cycling of stem and progenitor cells and increases the numbers of mature cells of multiple lineages (reviewed in Schrader, 1998).

Although it has not been absolutely demonstrated that GM-CSF is necessary for reactive haemopoiesis in vivo, it seems likely since GM-CSF is released upon insult to the immune system (eg bacterial (endotoxin) (Burgess and Metcalf, 1980) or red algae (carrageenin) (Shikita et al., 1981) cell wall treatment) and overexpression of GM-CSF in vivo results in
massive expansion of macrophages (Metcalf et al., 1988). IL-5 promotes the expansion of eosinophil numbers during parasite infections, (Section 1.4.4.2) as demonstrated by studies showing that mice deficient for IL-5 are unable to increase production of eosinophils upon parasite infection. Interestingly, parasite infections, which result in IL-5 production, can lead to eosinophilia in the absence of increases in other cell types, eg neutrophils. This phenomenon is likely to be a reflection of the relatively restricted and specific effects of IL-5 on eosinophils.

1.4.4.2 GM-CSF, IL-3 and IL-5 prolong the survival of haemopoietic cells during reactive haemopoiesis

An enhanced capacity for survival is an important feature for cells which enter sites of inflammation, since these areas contain toxins such as ROS and NO, released to kill invading pathogens (reviewed in Kobayashi et al., 2005). In addition to promoting the rapid accumulation of newly-made myeloid cells, GM-CSF, IL-3 and IL-5 can prolong survival of specific myeloid cells. GM-CSF stimulates survival of primary cultured neutrophils, and the \( \beta_c \) is necessary for this effect (Scott et al., 1998). IL-3 has been shown to increase the survival of mast cells associated with mucosal surfaces (Crapper et al., 1984). IL-3 (Rothenberg et al., 1988) and IL-5 (Begley et al., 1986) are able to prolong survival of eosinophils isolated from peripheral blood. The \( \beta_c \) signalling pathways which influence survival are not well characterised and discussed further in Section 1.5.5.2.

1.4.4.3 GM-CSF and IL-5 increase (prime) ROS production by mature myeloid cells

As introduced in Section 1.3.1, some mature myeloid cells are able to produce toxic ROS in response to activation. ROS include superoxide ions and other free radicals which are
produced by the NADPH oxidase enzyme in a “burst” of oxygen consumption and released from neutrophils, eosinophils and macrophages. These toxic products act to indiscriminately kill cells in the near vicinity and therefore make an important contribution to the immune response against pathogens. In fact, this oxidative burst is essential for the killing of some invading pathogens, as demonstrated by individuals who are deficient in NADPH function, resulting in susceptibility to a number of infections (Hampton et al., 1998).

Full activation of macrophages, neutrophils and eosinophils in terms of ROS production in vitro can be achieved by “priming” (prior exposure to specific cytokines). GM-CSF primes neutrophil and macrophage ROS production in response to fMLP in vitro (Lopez et al., 1986b; Weisbart et al., 1985) and IL-5 can potentiate an increase in eosinophil superoxide production (Lopez et al., 1988). When these primary cells are incubated in the presence of GM-CSF prior to addition of fMLP, the superoxide response is synergistically greater than the responses to fMLP or cytokine alone. The mechanisms by which signalling through the βc primes ROS production by fMLP stimuli are essentially unknown and are addressed in this thesis.

1.4.4.4 GM-CSF, IL-3 and IL-5 increase (prime) the phagocytic activity of neutrophils and macrophages

Phagocytosis is the process by which cells such as neutrophils and macrophages recognize large foreign particles such as bacteria and completely engulf them into specialised compartments called phagosomes where they are killed by toxins such as ROS (reviewed in Kwiatkowska and Sobota, 1999). Similar to the requirements for maximal ROS production in vitro, enhanced phagocytic activity of primary cultured myeloid cells can be
promoted by exposure to GM-CSF, IL-3 or IL-5. Cultured neutrophils from $\beta_c^{-/-}$ mice were
deficient in GM-CSF stimulated phagocytosis, and uptake of colloidal carbon by alveolar
macrophages was reduced (Scott et al., 1998). Although the mechanisms by which GM-
CSF stimulates phagocytosis are poorly understood, GM-CSF stimulation has been shown
to increase the affinity of Fc receptors (FcR), which is predicted to increase FcR dependent
phagocytic activity (Graziano et al., 1989; Weisbart et al., 1988). Similar to GM-CSF, IL-3
stimulation of macrophages can result in enhanced phagocytosis of yeast and increased
levels of major histocompatibility complex antigens (Crapper et al., 1985; Chen et al.,
1988). IL-3 is also capable of increasing the phagocytic activity of purified eosinophils
towards antibody-coated tumour cells (Lopez et al., 1986b), and serum-opsonised yeast
particles (Sanderson, 1998). As for priming of ROS production, the mechanisms which
underlie the effect of $\beta_c$ signalling on phagocytic activity are essentially uncharacterised
and are addressed in this thesis.

1.4.4.5 GM-CSF, IL-3 and IL-5 increase of cytotoxicity and other effector
cell functions

GM-CSF, IL-3 and IL-5 are able to stimulate numerous specific functions of mature
myeloid cells, (including survival, ROS production and phagocytic activity) which
ultimately result in an increase in the cytotoxicity of these cells for invading pathogens.
Aside from stimulation of survival, ROS production and phagocytosis, which are central to
the issues of this thesis, a number of other effector functions of activated cells are
stimulated by cytokines via $\beta_c$ signalling. GM-CSF (Yong et al., 1991; Hirai et al., 1988a)
and IL-5 (Lopez et al., 1986a; Walsh et al., 1990) have been shown to increase the surface
expression of $\beta_2$-integrin adhesion molecules CD11b/CD18, a feature which is likely to
increase adhesion of these cells and enable them to migrate and invade sites of infection.
IL-5 is also a potent inducer of immunoglobulin-induced eosinophil degranulation (Fujisawa et al., 1990) which results in the release of the toxic contents of granules, enhancing the killing of pathogens. IL-5 is also reported to have specific chemotactic activity for eosinophils (Yamaguchi et al., 1988; Wang et al., 1989). Basophils express IL-5 receptors and have been shown in some studies to be primed for histamine production and leukotriene production by IL-5 (Bischoff et al., 1990; Hirai et al., 1990). An in vivo role for IL-3 in the response of the immune system to threat, was demonstrated by administration of IL-3 to mice infected with *Trichinella spiralis* which accelerated the expulsion of these worms (Korenaga et al., 1996). We know of many functional changes to neutrophils, macrophages and eosinophils, but nothing of the mechanisms by which signalling through the βc is able to regulate the transformation to an activated cell type.

1.4.5 GM-CSF, IL-3 and IL-5 in human pathologies and animal disease models

Important insights into the *in vivo* functions of GM-CSF, IL-3 and IL-5 can be gained from the study of human pathologies and mouse disease models involving dysregulated expression of these cytokines.

1.4.5.1 Pathologies involving GM-CSF

Consistent with its role in the promotion of inflammation and ability to promote expansion of certain populations of myeloid cells, abnormal expression of GM-CSF has been detected in disease states where inflammation or myeloid cell expansion have gone unchecked. Excess local endogenous GM-CSF has been found in chronic inflammatory diseases such as rheumatoid arthritis (Alvaro-Gracia et al., 1989), and aggravation of arthritis has been
reported in clinical trials in which patients are treated with GM-CSF (de Vries et al., 1991). In addition, aggravation of other autoimmune diseases such as thyroiditis or idiopathic thrombocytopenic purpura in patients treated with GM-CSF has been reported (Lieschke et al., 1989; Hoekman et al., 1991). GM-CSF is detected in skin scales of patients with the chronic inflammatory disease psoriasis (Takematsu and Tagami, 1990) and the disease phenotype is exacerbated upon GM-CSF therapy (Kelly et al., 1993). Autocrine secretion of GM-CSF has been detected cells from acute myeloid leukaemia (AML) (Young and Griffin, 1986), and the results of Young, (Young et al., 1987) further suggest that the dysregulated expression of this gene could contribute to the abnormal growth properties characteristic of AML. In subsets of patients with chronic myelomonocytic leukaemia (CMML), the presence of GM-CSF associated with malignant cells has been demonstrated (Everson et al., 1989). Treatment of these cells in vitro with antibodies to GM-CSF and IL-6 inhibited spontaneous colony formation. These results indicate that GM-CSF is necessary for the proliferation of these cancerous cells.

Pulmonary alveolar proteinosis (PAP), a rare disease in humans, is characterised by excessive accumulation of surfactant lipids and proteins in the alveolar space. Mice with homozygous deletions of GM-CSF, or the mμβ, have a PAP-like phenotype (see Section 1.4.3.1) (Reed et al., 1999; Stanley et al., 1994; Dranoff et al., 1994; Nishinakamura et al., 1995). A number of pieces of evidence strongly support a lack of available GM-CSF as a causative factor of human PAP. One patient exhibited intact GM-CSF gene expression but defective protein release (Tchou-Wong et al., 1997). Further, a number of PAP patients exhibit reduced huβc expression and/or function (Dirksen et al., 1997). A GM-CSF-neutralising agent was also detected in the bronchial-alveolar lavage of numerous patients (Tanaka et al., 1999). Most notably, treatment of patients with a GM-CSF aerosol
improved PAP symptoms, demonstrating the importance of the lack of GM-CSF to the disease phenotype (Kavuru et al., 2000).

GM-CSF plays an important role in arthritis, both in human pathologies and mouse models. In mice with collagen-induced arthritis (CIA), treatment with GM-CSF exacerbated the disease phenotype (Campbell et al., 1997) and lead to monocyte/macrophage infiltration (Bischof et al., 2000). Moreover, GM-CSF−/− mice had virtually no disease phenotype when treated with collagen to induced CIA (Campbell et al., 1998). A human monoclonal antibody targeting GMRα is currently undergoing trials for the treatment of patients with rheumatoid arthritis.

Experimental autoimmune encephalomyelitis (EAE) is an demyelinating inflammatory disease that can be induced in mice by the transfer of autoreactive T cells that recognise myelin basic protein (MBP), and is similar to multiple sclerosis. Local delivery of GM-CSF by retrovirally transduced T-cells lead to severe, chronic EAE in mice, demonstrating the potential importance of GM-CSF to the mouse disease phenotype (Marusic et al., 2002; Ponomarev et al., 2007). Importantly, GM-CSF−/− mice are resistant to induction of EAE (McQualter et al., 2001), therefore GM-CSF is a potential therapeutic target for treatment of multiple sclerosis.

To summarise, excess or ectopic GM-CSF can be involved in chronic inflammatory diseases and is likely to exacerbate these diseases. In addition GM-CSF has also been detected in some cancers where it might play a role in the proliferation of malignant cells.
1.4.5.2 Pathologies involving IL-3

The dysregulated activity of IL-3 is implicated in disease phenotypes such as leukaemias and inflammatory diseases. Munoz, (2001) found IL-3 receptor α subunit expression in malignant samples from patients with AML but not acute lymphocytic leukaemia (ALL). Gene rearrangements of IL-3 have been detected in bone marrow cells of some patients with AML (Schmetzer et al., 2000). In the mouse, a number of myeloid leukaemias have been described in which pathologic activation of the IL-3 gene was a key oncogenic event (Leslie and Schrader, 1989). In some instances, the growth of these leukaemias could be inhibited by anti-IL-3 antibodies (Schrader, 1998). IL-3 expression has been detected in Sjogren's syndrome (a B-cell lymphomagenesis disorder) and increased expression of IL-3 has been detected in the autoimmune disease multiple sclerosis (Baranzini et al., 2000).

1.4.5.3 Pathologies involving IL-5

As the influence of IL-5 on haemopoietic cells is more restricted than GM-CSF and IL-3, it is not surprising that the known pathologies involving IL-5 are restricted, so far, to those involving eosinophil dysregulation. Eosinophil recruitment into the upper or lower airways appears to be essential for the clinical manifestations of allergen inhalation during allergic asthma in rodents (reviewed in O'Byrne et al., 2001). The relationship between IL-5 and the development of airway eosinophilia has been well established in animal models: transgenic mice in which T-cells overexpress IL-5 exhibit eosinophilia in lung sections and other tissues (Dent et al., 1990), and in IL-5−/− mice, the eosinophilia, lung damage and airways hyper-reactivity normally associated with aeroallergen challenge were abolished (Foster et al., 1996). Airway eosinophilia and airway hyper-responsiveness were reduced in a mouse model of chronic asthma when mice were treated with a monoclonal anti-IL-5
antibody (Mathur et al., 1999). Additionally, the role of eosinophils in asthma was well defined by a study using mice devoid of eosinophils (Lee et al., 2004). During ovalbumin allergen challenge, these mice exhibited reduced airway epithelial hypertrophy and goblet cell mucus accumulation, compared to wildtype mice, indicating an essential role for eosinophils in the asthma pathology. In the human, the exact role that IL-5 plays in the asthma phenotype is not as well established. One indication of the contribution of IL-5 to the asthma phenotype comes from studies where monoclonal antibodies blocking IL-5 function were used in human trials. In four trials using anti-IL-5 treatment, reductions were seen in blood and sputum eosinophil numbers, but no significant changes in physiological parameters of airway hyper-responsiveness, the late asthmatic reaction or in lung function of the patients were seen (reviewed in Leckie, 2003). However, a further study using anti-IL-5 treatment depleted eosinophils from the airway mucosa of mild atopic asthmatics and significantly decreased the deposition of extracellular matrix proteins in the bronchial tubes (Flood-Page et al., 2003). The involvement of IL-5 in the human asthma phenotype is therefore likely to be complex and to differ from the mouse ovalbumin studies. From these experiments, however, it is clear that IL-5 is capable of stimulating eosinophilia and recruiting eosinophils into the lungs in the human, where further activation of these and other cells lead to a life-threatening pathology.

1.4.6 How do GM-CSF, IL-3 and IL-5 exert their influences on haemopoietic cells?

The focus of this thesis is the question of how GM-CSF, IL-3 and IL-5 exert their varied but related effects on different haemopoietic cells. The expression pattern of each receptor alpha chain is consistent with the pattern of cells affected by each cytokine, but the different responses (proliferation, differentiation, survival and numerous effector cell
functions) of specific cells to cytokine signalling through the $\beta_c$ is not well understood. In order to unravel this mystery, many groups have examined the signal pathways emanating from the $\beta_c$ in response the GM-CSF, IL-3 and IL-5. These experiments have used both primary cultured cells and cells lines which can be transfected with mutant forms of the $\beta_c$ for genetic analyses. As a result of these studies, the mechanisms by which $\beta_c$ signalling stimulates cell proliferation is reasonably well understood and described in Section 1.5.5.1. In contrast, the mechanisms involved in $\beta_c$ regulation of survival and differentiation are very poorly understood, and those involved in the stimulation of mature cell functions such as ROS production and phagocytic activity are essentially unknown. What is known of $\beta_c$ signal transduction is discussed in Section 1.5. The focus of this thesis is to decipher the $\beta_c$ signalling pathways involved in GM-CSF regulation of survival and effector cell functions.

### 1.5 The Receptors for GM-CSF, IL-3 and IL-5

The human receptors for GM-CSF, IL-3 and IL-5 are composed of ligand specific $\alpha$ subunits (GMR$\alpha$, IL-3R$\alpha$ and IL-5R$\alpha$, respectively) and a common $\beta$ subunit ($\beta_c$). Orthologous receptors exist in the mouse, with the addition of a second beta subunit for muIL-3, called mu$\beta_{IL-3}$, which is orthologous to mu$\beta_c$. These receptors belong to the Cytokine Receptor Superfamily which is responsible for the signal transduction of a vast number of cytokines. Described in this Section are the molecular compositions of the receptors for GM-CSF, IL-3 and IL-5, as well as mechanisms of receptor activation, the resulting signal transduction from these receptors and what we know of how these signals regulate cellular functions.
1.5.1 Molecular composition of the receptors for GM-CSF, IL-3 and IL-5

1.5.1.1 Structural features of the βc

Typical for cytokine receptors, the βc extracellular region is involved in ligand recognition and the intracellular region for signal transduction. Whereas the extracellular region of the huβc exhibits similarities to other Cytokine Receptor Superfamily members, the intracellular region exhibits little similarity to any known protein. A single polypeptide chain comprises the single-pass transmembrane domain. The domain composition and conserved elements of the huβc are represented in Figure 1.2 and described below.

The extracellular portion of the βc consists of 4 domains and, as for most cell surface proteins, is glycosylated, which protects the protein from degradation, contributes to the overall structure of the subunit and is essential for binding of huβc to the huGMRα:huGM-CSF complex (Niu et al., 2000). The huβc extracellular region contains four domains, each 100 aa in size, related to fibronectin-type III domains. These 4 domains were each predicted to consist of seven β strands and be organised into two cytokine receptor modules (CRMs) of 200 aa each (Gustin et al., 2001; Bagley et al., 1997; Lyne et al., 1995). Several sequence motifs present on the βc extracellular region are highly conserved amongst members of the cytokine receptor superfamily and therefore likely to
Conserved cysteine residue
Transmembrane domain
Conserved WSXWS motif
Box 1
Box 2
Fibronectin type III like domain
Intracellular region

huβc

huGMRα
huIL-3Rα
huIL-5Rα

CRM

CRM

Y577
Y612
Y695
Y750
Y806
Y866
Figure 1.2 Schematic representation of the receptor subunits for huGMR, huIL-3R and huIL-5R. A key to the conserved elements is shown on the right. Each receptor subunit is a member of the cytokine receptor superfamily, which is characterised by such conserved features as: cytokine receptor modules (CRM) consisting of two 100aa fibronectin III like domains, four conserved cysteine residues in each CRM, a conserved WSXWS motif in each CRM (huβc second CRM contains PSXWS) (described in Sections 1.5.1.1 and 1.5.1.2), transmembrane domain and intracellular domain which contains two conserved regions, Box 1 and Box 2 (described in Sections 1.5.1.1, 1.5.1.2, 1.5.4.1, 1.5.5.1 and Figure 1.3). Conserved tyrosine residues only are shown for the huβc (described in Sections 1.5.1.1, 1.5.4.1, 1.5.4.2, 1.5.5.1 and Figure 1.3 and 1.5).
make important contributions to the function of the receptor. As depicted in Figure 1.2 and also shown in the huβc sequence in Figure 1.3, these conserved extracellular motifs include an N-terminal tryptophan, four conserved cysteine residues predicted to be involved in disulphide linkages, conserved proline residues at the N-terminus and a conserved motif labelled WSXWS, near its C-terminus. The WSXWS motif, so named for its tryptophan-serine-random-tryptophan-serine aa sequence, may play a role in ensuring surface expression of the receptor protein, as mutation of the serine and tryptophan residues of the conserved WSXWS motif in the erythropoietin receptor resulted in lack of surface expression of this receptor (Hilton et al., 1996).

The transmembrane domain of the βc has been shown to contribute to the regulation of receptor activity by the study of huβc forms containing mutations in this region. Expression of huβc containing a single aa change of valine at huβc residue 449 to glutamate (huβc V449E), conferred factor-independent proliferation and differentiation of the primary murine haemopoietic cells (McCormack and Gonda, 1997). It is proposed the V449E mutation leads to constitutive huβc dimerisation and intracellular signal transduction (Jenkins et al., 1995). Most pertinent to this thesis is the cytoplasmic portion of the βc from which intracellular signalling is derived. Although most of this region has little similarity to other known proteins, the membrane proximal 50aa region contains proline-rich elements labelled “Box 1” and “Box 2” which are partially conserved among cytokine superfamily members. These elements are proposed to play a role in signal transduction via association of a the tyrosine kinase JAK2, (reviewed in Bagley et al., (1997) and described in more detail in Section 1.5.4.1). In addition, intracellular huβc contains eight tyrosine residues, six of which are conserved from mouse to human βc. The
Figure 1.3 The peptide sequence of huβc. On the left, numbers indicate the aa in the predicted mature protein (bold) and precursor polypeptide (listed second). The leader peptide is predicted to be cleaved at the * (Sakamaki K et al., 1992). The transmembrane domain is boxed. Box 1 is depicted in dark blue, Box 2 in light blue. All eight intracellular tyrosines are shown in magenta and the amino acid number for each tyrosine is indicated (numbers refer to predicted mature protein sequence). Tyrosines 577 to 866 are conserved from human to mouse. The putative 14-3-3 binding motifs are shown in red: motif 1 is positioned from amino acids 582 to 587, and motif 2 from amino acids 820 to 826. Putative phosphoserines in the 14-3-3 binding sites are underlined. (Sequence code: CYRB_HUMAN).
roles of signalling through these are other conserved motifs are discussed in Section 1.5.4.

1.5.1.2 Structural features of the GMRα, IL-3Rα and IL-5Rα

Each of the GMRα, IL-3Rα and IL-5Rα subunits shares some structural similarity to the βc, as depicted in Fig 1.2. These α subunits are smaller than the βc in both the extracellular portion (consisting of only one CRM) and the cytoplasmic portion, which is considerably shorter. Like the βc, they are glycosylated for expression on the cell surface (Shibuya et al., 1991; Murata et al., 1992; Kitamura et al., 1991). The extracellular region of each α subunit contains a conserved WSXWS motif, similar to the huβc, and membrane-proximal proline-rich elements similar to Box 1 and Box 2. The intracellular regions of the GMRα, IL-3Rα and IL-5Rα subunits are necessary for signal transduction via their respective receptor complexes (Ronco et al., 1995; Barry et al., 1997; Caldenhoven et al., 1995). The relatively short cytoplasmic portion and lack of early publications describing specific signalling pathways emanating from α subunits lead to speculation that the primary role of these subunits was to provide cellular specificity via the initial binding of cytokine. However, recent publications have described specific signalling events associated with IL-5Rα and GMRα (described in Sections 1.5.3, 1.5.4.1 and 1.5.5.3), introducing the possibility that uncharacterised signal transduction can also occur from the IL-3 and GMR alpha subunits. Aside from these few reports, the study of intracellular signalling from the βc constitutes the majority of our understanding, limited as it is, of how these cytokines effect many different cell functions. Signal transduction from the huβc is therefore the major focus of Section 1.5.
1.5.2 The mechanisms of activation of the GM-CSF, IL-3 and IL-5 receptor complexes

The process of receptor assembly upon ligand stimulation begins with recognition and binding of the ligand to its specific α subunit with low affinity (reviewed in Bagley et al., 1997). This ligand/α subunit complex is unable to signal or promote a biological effect. The recruitment of the βc to the ligand/α subunit complex converts the receptor to a high affinity complex capable of signal transduction and biological effects. It is reported that the βc cannot bind cytokine on its own, but is recruited to the ligand/α subunit complex where it makes contact with the α subunit, leading to the conversion of this complex into an active one which mediates signal transduction (reviewed in Guthridge et al., 1998). The intracellular portion of βc, as opposed to α subunits, is responsible for most of the signal transduction from the complex.

Dimerisation of the GM-CSF, IL-3 or IL-5 receptor subunits has been shown to be necessary for the formation of active receptor complexes. Experiments using dominant-negative and chimeric receptors as well as modelling studies indicate that at least two huβc subunits are required for receptor activation and signalling (Takaki et al., 1994; Jenkins et al., 1995)(reviewed in Guthridge et al., 1998). These studies imply that the active receptor complex is composed of a 2:2:2 stoichiometry of huGM-CSF:huGMRα; huβc. Other studies also support the notion that dimerisation of the βc is sufficient for receptor activation and intracellular signalling, without the necessity of ligand binding or α subunits (Muto et al., 1995a; Muto et al., 1995b; Sakamaki et al., 1993). Furthermore, there is evidence that two GMRα subunits are necessary for the formation of an active complex; a C-terminal truncated mutant huGMRα expressed in stoichiometric proportions with
wildtype huGMRα, inhibited GM-CSF function, suggesting the requirement for at least two functional GMRα chains in the huGMR complex (Lia et al., 1996).

The above mentioned experiments and modelling studies suggest a model in which the active receptor complex is formed following the dimerisation of two 1:1:1 high affinity complexes of huGM-CSF:huGMRα:huβc forming an active receptor complex with a ratio of 2:2:2. However, other reports suggest conflicting models (Wheadon et al., 1997) or that higher order complexes may also be active (McClure et al., 2001b). The stoichiometry of the activated huGM-CSF receptor complex is yet to be confirmed.

1.5.3 Signalling events involving the GMRα, IL-3Rα and IL-5Rα

For many years, it was thought that all signalling through the GM-CSF, IL-3 and IL-5 receptors emanated from the βc. However, recently experiments have uncovered a number of proteins which interact with α subunits and have assigned functional importance to some of these interactions. Firstly, a series of truncation mutants were used to demonstrate that the cytoplasmic domain of GMRα plays an important role in the activation of the JAK/STAT pathway (Doyle and Gasson, 1998). Furthermore, JAK2 has been found to constitutively associate with the IL-5Rα subunit and become activated upon ligand stimulation (Ogata et al. 1998). GMRα directly interacted with c-kit, the receptor for stem cell factor (SCF), via the cytoplasmic domains of both receptors. Binding of GMRα inhibited c-kit ligand-induced autophosphorylation (Chen et al., 2006,). GMRα also co-immunoprecipitated with the p85 subunit of PI 3-kinase via the cytoplasmic tail of GMRα. This interaction may play a role in GM-CSF-induced glucose uptake in oocytes and myeloid cell lines (Dhar-Mascareno, et al.,
2003). GMRα co-immunoprecipitates with GM-CSF receptor alpha subunit-associated protein (GRAP), but a functional role for this interaction is yet to be described, (Tu et al., 2000). Finally, the cytoplasmic portion of the IL-5Rα binds syntenin, an interaction which is required for ligand-mediated activation of the transcription factor sox-4 (Geijsen et al., 2001).

1.5.4 Intracellular signalling from the \( \beta_c \)

Upon activation of the receptors for GM-CSF, IL-3 or IL-5, signal transduction is mediated by the \( \beta_c \). As is common for cytokine receptor signalling, the activated hu\( \beta_c \) has no intrinsic enzymatic activity, but activates specific signalling pathways via the recruitment of kinases and other proteins to the complex, resulting in signal transmission via a number of pathways. Three major signal transduction pathways are activated by the \( \beta_c \); the Jak/STAT pathway, the ras/raf/ERK pathway and the PI 3-kinase pathway. Other proteins such as Shc are also activated by receptors containing hu\( \beta_c \). These pathways are described below and summarised in Figure 1.4.

1.5.4.1 The Jak/STAT Pathway

Although GMR, IL-3R and IL-5R exhibit no intrinsic tyrosine kinase activity, activation of these receptors leads to tyrosine phosphorylation of the hu\( \beta_c \) and other cellular proteins (reviewed in Guthridge et al., 1998, Blalock et al., 1999). This phosphorylation is mediated by the activation of receptor-associated tyrosine kinases such as the Jak2 and src family kinases. The tyrosine kinase Jak2 has been shown to be associated with the \( \beta_c \) of the
GMR via the conserved, membrane-proximal, intracellular proline-rich motif of the βc, “Box 1” (Figures 1.2 and 1.3)(Quelle et al., 1994). The basic sequence of events involved in JAK signalling and signal transducer and activator of transcription (STAT) activation is thus:

1. ligand binding to receptor α subunits (GMRα, IL-3Rα or IL-5Rα), resulting in
2. recruitment of huβc and receptor complex assembly, then
3. activation of the kinase activity of JAK proteins bound to βc subunits, transphosphorylation of JAK proteins,
4. JAK mediated phosphorylation of βc tyrosine residues,
5. recruitment of STAT5 proteins to the receptor,
6. activation of STAT5 proteins via phosphorylation by JAKs,
7. dimerisation of STAT5s, and, finally
8. nuclear translocation of STAT5 proteins leading to regulation of gene expression.

This sequence of events is explained in more detail below.

Upon ligand binding to GMRα or IL-3Rα, and recruitment of the βc to the receptor complex, Jak2, associated with the proline-rich region “Box 1”, becomes phosphorylated and activated (Quelle et al., 1994). Alternatively, huβc as part of the IL-5R complex binds JAK1 (Ogata et al., 1998). JAK activation most likely occurs due to transphosphorylation events whereby two JAK proteins phosphorylate each other, potentiated as JAK proteins
Figure 1.4 Summary of the major signalling pathways emanating from the activated huβc (only the cytoplasmic portion of one huβc is pictured). Upon ligand events depicted which follow ligand stimulation include the activation of JAK2, bound to “Box 1”, tyrosine phosphorylation of 6 residues, recruitment of STAT5a and b via phosphorylated tyrosine residues, activation of c-myc, activation of the ras/MAPK pathway through either Shc or shp2 recruitment to tyrosine residues, all which can contribute to cell proliferation. Also depicted are differentiation, survival and effector cell functions, cellular outcomes of signalling of which the regulating signalling pathways are not well characterised.
come into close proximity during receptor complex assembly (Quelle et al., 1994). At least two JAK2 proteins are likely to be brought together into a receptor complex of 2:2:2 (ligand:α subunit:βc), bound to each of the two βc subunits. It is possible that JAK2 may bind to the α subunits and play a role in JAK2 transphosphorylation upon receptor complex assembly, given that each α subunit contains a proline rich motif with homology to Box 1, and this region, at least for IL-5, has been shown to be necessary for JAK2 activation (Kouro et al., 1996; Takaki et al., 1994). Indeed, JAK2 has been shown to constitutively associate with huIL-5Rα and become activated upon IL-5 stimulation (Ogata et al., 1998).

Ligand-induced activation of huβc-associated JAK2 leads to phosphorylation of 8 intracellular βc tyrosine residues (Watanabe et al., 1996b). At least 6 of these tyrosine residues (huβc Y577, Y612, Y695, Y750, Y806 and Y866) are conserved from human to mouse βc. Phosphorylation of these tyrosine residues is an important step in the activation of the βc and leads to the activation of a number of specific signalling pathways including the STAT pathway (described below) and the ras/MAP kinase signalling pathway (described in Section, 1.5.4.2). The STAT family consists of latent transcription factors which move from the cytoplasm into the nucleus and promote transcription of specific genes when activated (reviewed in Paukku and Silvennoinen, 2004). Upon ligand stimulation of the βc, activation of STAT family members STAT5a and STAT5b occurs, which is dependent upon JAK2 activity (Mui et al., 1995; Ihle, 1995). It is probable that STAT5s are brought to the huβc by their interaction with huβc tyrosine residues, via SH2 domains of STAT5s an event which has been shown to occur during muβcIL-3 activation.
When in close proximity to huβc bound Jak2, these STAT5 become phosphorylated and activated by Jak2. Interestingly, studies using Ba/F3 cells expressing mutant huβc proteins containing substitutions of intracellular tyrosine residues, have shown that loss of any one tyrosine does not reduce GM-CSF stimulated STAT5 phosphorylation, but the loss of all 8 intracellular tyrosines leads to the abolishment of STAT5 phosphorylation (Itoh et al., 1998; Okuda et al., 1997). Redundancy therefore exists among the βc intracellular tyrosine residues in terms of their ability to mediate JAK2 phosphorylation of STAT5 proteins.

Once phosphorylated, STAT5s then undergo dimerisation (via an interaction between SH2 domain of one STAT molecule and the phosphorylated tyrosine residue of its partner (reviewed in Paukku and Silvennoinen, (2004)) and this results in translocation from the cytoplasm to the nucleus, where they bind to regulatory sites within the promoters of specific genes, leading to increase in transcription (Stocklin et al., 1996). Examples of genes activated via the STAT5 pathway in response the GM-CSF signalling include pim1, Oncostatin M (OSM), Id-1, Cyclin D3 and bcl-xL (Hara and Miyajima, 1996; Yoshimura et al., 1996; Stoat et al., 2004; Dumon et al., 1999). The functional significance of the expression of these genes in response to βc signalling is mostly unknown, however the actions of Bcl-xL and Pim1 are likely to inhibit apoptosis in response to IL-3 (Bcl-xL) (Dumon et al., 1999) and GM-CSF or IL-5 (Pim1) (Stoat et al., 2004). In addition, STAT5 proteins have been shown to activate expression of CIS and SOCS proteins which are involved in the negative regulation of Jak2 signalling (Helman et al., 1998; Endo et al., 1997; Starr et al., 1997). Furthermore, JAK2 is necessary for the activation of c-myc expression in Ba/F3 cells stimulated with GM-CSF (Watanabe et al., 1996a). However, induction of c-myc expression by IL-3 does not require STAT5 activation, since it is not
disrupted by expression of a dominant negative form of STAT5 (Mui et al., 1996). Indeed, GM-CSF stimulated c-myc expression in leukaemic cell lines was shown instead to require PI 3-kinase activation (Kobayashi et al., 2003), a signalling event described in Section 1.5.4.3.

Experiments in cell lines have shown that Jak2 is required for cell proliferation in response to GM-CSF (Watanabe et al., 1996b). These experiments are described in further detail in Section 1.5.5.1.

1.5.4.2 The Ras/MAP Kinase Pathway

The Ras/MAP kinase (also known as the ras/raf/MEK/ERK cascade) is one of the best studied signal transduction pathways during signalling by cell surface receptors. It is involved in the stimulation of proliferation, regulation of apoptosis and numerous other functions in many cells types, and is activated by many different cytokine receptors.

The huβc forms a ternary complex with either She or SHP2, and Grb2 (growth factor receptor bound protein-2), an adaptor protein able to couple proteins containing SH2 and SH3 domains. This complex is thought to recruit the ras-specific guanine nucleotide exchange-promoting protein, SOS (Lanfrancone et al., 1995). Activation of SOS enables the activation of ras, raf-1, MEK-1 and Erk proteins (Hall et al., 2001; reviewed in Blalock et al., 1999). Ras is a small GDP/GTP-binding protein which is tethered to the membrane via farnesylation (a 15-chain fatty acid attachment). Active SOS exchanges the GDP bound to ras for GTP, enabling translocation and activation of the serine/threonine kinase raf family members (raf-1, A-raf or B-raf), via binding to ras. Active raf phosphorylates MEK1 (mitogen activated extracellular regulated kinase 1, a serine/threonine and tyrosine
kinase) which then phosphorylate the MAPks ERK1 and ERK2. Activated ERKs can translocate to the nucleus where they can phosphorylate and activate specific transcription regulators such as fos and jun, in the case of huβc signalling (reviewed in Blalock et al., 1999).

Mutagenesis studies have implicated the importance of huβc tyrosines 577, 612 and 695 in the activation of the Ras/MAP kinase pathway (Itoh et al., 1998; Okuda et al., 1997). Two proteins have been demonstrated to separately form ternary complexes with huβc and Grb2 to initiate the ras/MAPK pathways. Firstly, Shc, an adaptor protein, is recruited only to tyrosine 577, via Shc’s PTB domain. Interestingly, a huβc mutant in which tyrosine 577 is substituted is not defective in its ability to promote raf-1 and Erk2 activation (Itoh et al., 1998). Therefore it is likely that Shc is not necessary for activation of the ras/MAPK pathway by huβc, and that redundancy exists between the roles of Shc and SHP2 in the control of this signalling pathway.

It is predicted that phosphatase SHP2, like Shc, recruits Grb2 to the huβc, which then leads to activation of SOS and the ras/raf/MEK/Erk cascade. The recruitment of SHP2 to the huβc requires the presence of either tyrosine 577, 612 or 695 and the presence of either of these three tyrosines is sufficient to promote SHP2 phosphorylation upon receptor activation (Itoh et al., 1998; Okuda et al., 1997). Consequently, the presence of either of these three residues was sufficient to promote Erk activation (Itoh et al., 1998).

The activity of Erks 1 and 2 leads to the regulation of transcription factors which control gene expression (reviewed in Blalock et al., 1999). GM-CSF elicited c-fos expression via the ras/raf/MEK/Erk cascade is predicted to promote proliferation of the human factor-
dependent myeloerythroid cell line, TF-1 (Kolonics et al., 2001). c-fos and c-jun
transcription factors bind AP-1 sites in the promoter regions of many genes of which
expression is regulated by IL-3 and GM-CSF (reviewed in Blalock et al., 1999).

1.5.4.3 The PI 3-kinase Pathway

The lipid kinase, PI 3-kinase, is activated during the signal transduction from numerous
receptors and has pleiotropic effects which include cell survival, chemotaxis, cell adhesion,
ROS production and phagocytosis (reviewed in Funaki et al., 2000). PI 3-kinase is
involved in the regulation of effector cell functions which contribute to the combat of
micro-organisms by mature haemopoietic cells, and is therefore of great interest in the
study of huβc signalling, as the regulation of effector cell functions is the most important in
vivo role of the huβc (see Section 1.4.3.5).

PI 3-kinase is a heterodimeric protein consisting of a regulatory subunit, p85, containing
both SH2 and SH3 domains and a catalytic subunit, p110. Upon ligand stimulation, PI 3-
kinase is activated, involving its recruitment to the huβc (reviewed in Funaki et al., 2000).
However, there is no apparent huβc consensus binding site for PI 3-kinase, therefore the
recruitment of PI 3-kinase to the βc is therefore likely to be mediated by an adaptor protein.
Activation of PI 3-kinase by the βc leads to activation of downstream targets AKT (also
known as protein kinase B) and p70S6K. The activation of Akt has been shown to be
important in a great number of cellular processes; for example cell survival, regulation of
glucose uptake and metabolism, protein translation, cell cycle progression, angiogenesis
and cancer metastasis (Plas and Thompson, 2005; Kim and Chung, 2002). In response to
IL-3 stimulation, one effect of AKT activation is the promotion of survival. There are at
least two signalling outcomes of PI 3-kinase and Akt activation that have been shown to promote cell survival. Firstly, serine phosphorylation of the pro-apoptotic BAD by Akt promotes binding to the adapter/chaperone protein 14-3-3 (see 1.5.4.5), sequestering it in the cytoplasm, in a process which inhibits apoptosis (del Peso et al., 1997), discussed in greater detail in Section 1.5.5.2. Secondly, Akt phosphorylates FoxO transcription factors, leading to their sequestration in the cytoplasm, which also inhibits apoptosis (Chong and Maieze, 2007; Cahill et al., 2001; Arimoto-Ishida et al., 2004; reviewed in Arden and Biggs, 2002). Interestingly this process also involves 14-3-3, and is discussed further in Section 1.5.4.5.

However, few studies have addressed the involvement of GM-CSF, IL-3 or IL-5 elicited PI 3-kinase or Akt activation in regulation of glucose uptake, angiogenesis, cell cycle, translation or metastasis. Interestingly, PI 3-kinase activation is important in a number of innate immune processes important for fighting invading pathogens, such as ROS production, phagocytosis and chemotaxis (reviewed in Hannigan et al., 2004). Whether this pathway is involved in GM-CSF-, IL-3- or IL-5-control of these effector cell functions has not been studied. This shortfall needs to be addressed, as these effector cell functions can be considered the most important functions of the βc, given that they are the only ones severely disturbed in the βc knockout (see Section 1.4.3.5).

1.5.4.4 Shc signalling

Shc, an adaptor protein which can be a substrate for tyrosine kinase activity as well as a docking site for proteins containing SH2 domains, is recruited to the huβc upon activation (reviewed in Blalock et al., 1999). Human Shc contains a phosphotyrosine binding domain
(PTB domain) and an SH2 domain, both capable of binding to phosphorylated tyrosine residues, as well as three tyrosine residues (Y239, Y240 and Y317) which can become phosphorylated upon activation and interact with phosphotyrosine-binding proteins (Ravichandran, 2001). Upon ligand stimulation, Shc is recruited to tyrosine 577 (as part of the Shc consensus binding site NXXY (Kavanaugh and Williams, 1994; Blaikie et al., 1994)) of the huβc, via binding of its PTB domain to this phosphorylated residue (Bone and Welham, 2000). huβc tyrosine residue 577 is essential for Shc phosphorylation and association of Shc with the huβc (Itoh et al., 1996; Okuda et al., 1997). Binding of Shc to huβc is important for the binding of the adaptor protein grb2, most probably via Shc tyrosine residue Y317 (Gotoh et al., 1996). As mentioned in Section 1.5.4.2, grb2 recruits the nucleotide exchange factor SOS, which is essential for the activation of ras. IL-3-induced Shc activation (via phosphorylation of shc Y317) has been shown to be important in the activation of the ras/MAP kinase pathway (Gotoh et al., 1996). Activation of ras through Shc leads to activation of the transcription factor c-fos (Gu et al., 2000; Itoh et al., 1996) and downstream gene transcription.

In addition to the activation of the ras/MAP kinase pathway, Shc recruitment to huβc tyrosine 577 can also regulate PI 3-kinase activity (Gu et al., 2000). Shc binding of grb2 can lead to recruitment of Gab2 (Gu et al., 2000). Gab2 contains two proline rich regions able to bind SH3 domains of grb2, and multiple binding sites for SH2-containing proteins (reviewed in Liu and Rohrschneider, 2002). Gab2 was shown to bind and recruit the p85 subunit of PI 3-kinase, leading to PI-3 kinase activation in response to IL-3 stimulation (Gu et al., 2000). In support of a role for Gab2 in huβc-regulated PI 3-kinase activity, a dominant negative Gab2 protein reduced PI 3-kinase activity in response to IL-3 (Gu et al., 2000). However, it has not been demonstrated that huβc tyrosine 577 is necessary for PI 3-
kinase activity. Indeed, Shc activation itself is not essential for survival since a mutant hu\(\beta\)c unable to signal through Shc was still able to promote survival in Ba/F3 lines (Okuda et al., 1997).

Signalling through Shc recruitment can therefore lead to two known pathways: the ras/raf/MEK/Erk pathway and an alternate pathway where Gab2 is recruited leading to PI 3-kinase activation. Despite the activation of these two pathways via Shc binding to hu\(\beta\)c Y577, this residue, as well as Shc activation, is not essential for either proliferation or survival in response to ligand.

1.5.4.5 14-3-3 Signalling

At the outset of this thesis, unpublished data from the Lopez laboratory, Adelaide, South Australia, indicated that the hu\(\beta\)c is capable of binding a phosphoserine-binding protein named 14-3-3 (Figure 3.2). 14-3-3 family members are highly conserved among eukaryotes and are expressed in most, if not all, tissues of humans and mice. 14-3-3 proteins serve a variety of purposes such as adaptors, chaperones, scaffolds and maskers of active sites such as nuclear localisation sequences (reviewed in Tzivion et al., 1998; Bridges and Moorhead, 2005). Multiple isoforms of 14-3-3 have been found ranging from 2 in yeast to 7 in humans (\(\beta, \epsilon, \varphi, \eta, \sigma, \tau\) and \(\zeta\)) to 12 in the flowering plant, *Arabidopsis thaliana*. They bind motifs containing phosphoserine or phosphothreonine residues in a large number of proteins involved in signal transduction. Table 1.2 lists most of the protein interactions known at the outset of this thesis. In the last few years a vast increase in the number of 14-3-3-interacting proteins has been documented and the experiments of (Pozuelo Rubio et al., 2004) indicated that 14-3-3 is able to bind over 200 phosphoproteins
<table>
<thead>
<tr>
<th>Protein</th>
<th>Interaction site(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASK1</td>
<td>RSISLE</td>
<td>(Zhang et al., 1999)</td>
</tr>
<tr>
<td>IGF1 receptor</td>
<td>MNESVP</td>
<td>(Craparo et al., 1997)</td>
</tr>
<tr>
<td>IL9 receptor α</td>
<td>RSWTF</td>
<td>(Sliva et al., 2000)</td>
</tr>
<tr>
<td>Middle T antigen</td>
<td>RSHSYD</td>
<td>(Cullere et al., 1998)</td>
</tr>
<tr>
<td>raf-1</td>
<td>RSTSP</td>
<td>(Pallard et al., 1995), (Quelle et al., 1995),</td>
</tr>
<tr>
<td>PTPH1</td>
<td>RSLSVE</td>
<td>(Zhang et al., 1997)</td>
</tr>
<tr>
<td>p53</td>
<td>QSTSRH</td>
<td>(Falini et al., 1995)</td>
</tr>
<tr>
<td>cdc25b</td>
<td>RSP SMP</td>
<td>(Mils et al., 2000)</td>
</tr>
<tr>
<td>cdc25c</td>
<td>RSP SMP</td>
<td>(Nicholson et al., 1995)</td>
</tr>
<tr>
<td>BAD</td>
<td>RSRHYSYP</td>
<td>(Murata et al., 1992)</td>
</tr>
<tr>
<td>FKHRL1</td>
<td>RCTWP</td>
<td>(Brunet et al., 1999)</td>
</tr>
<tr>
<td>K18</td>
<td>RFVSSASVT</td>
<td>(Lemoli et al., 1995)</td>
</tr>
<tr>
<td>PKCζ</td>
<td>HMDSVMP</td>
<td>(van der Hoeven et al., 2000)</td>
</tr>
<tr>
<td>Gp Ib-IX</td>
<td>RYGSHLS</td>
<td>(Gu et al., 1999)</td>
</tr>
</tbody>
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Table 1.2 Known interactors with 14-3-3 proteins at the outset of this thesis, and their sites of interaction
Putative phosphorylated serine or threonine residues are shown in red.
from proliferating HeLa cells. The 14-3-3 binding site was defined as RSXSXP by Muslin et al., (1996). Using phosphoserine-oriented peptide libraries to probe mammalian and yeast 14-3-3 proteins, (Yaffe et al., 1997) identified two different 14-3-3 binding motifs RSXSXP and RSY\(\xi\)XP. The X-ray crystallographic structure of 14-3-3 demonstrates that it exists as a dimer, with dimerisation mediated by interaction between the N-terminal domain of each subunit (Liu et al., 1995; Xiao et al., 1995). Each subunit of the 14-3-3 dimer contains a groove which is able to bind a peptide containing a phosphoserine residue (Yaffe et al., 1997). These studies suggest that a 14-3-3 dimer can bind phosphoserine residues of two different proteins or two different regions of a single protein. In this way, 14-3-3 proteins can act as scaffolds or adaptors, bringing two phospho-proteins into close proximity. An example of 14-3-3 proteins functioning in this way is the co-localisation of 14-3-3\(\zeta\) with tau and glycogen synthase kinase 3\(\beta\) (GSK-3\(\beta\)), leading to greater phosphorylation of tau by GSK-3\(\beta\) (Agarwal-Mawal et al., 2003).

A number of studies have shown that the 14-3-3 family of proteins play a role in the inhibition of apoptosis. Upon survival signals such as IL-3 stimulation, 14-3-3 interacts with the pro-apoptotic BAD, sequestering BAD into the cytoplasm and promotes survival (del Peso et al., 1997) (described in further detail in Section 1.5.5.2). The interaction of 14-3-3 and BAD is dependent on the phosphorylation of at least one of two serine residues of BAD which lie in 14-3-3 binding motifs (serine 112 and serine 136) (Zha et al., 1996). In the absence of survival signals, BAD remains unphosphorylated and is able to bind Bcl-X\(_L\) at the mitochondrial membrane, promoting apoptosis. Experiments utilising a dominant negative 14-3-3\(\zeta\) mutant \textit{in vitro} and \textit{in vivo} further demonstrated that 14-3-3\(\zeta\) is necessary for the prevention of apoptosis (Xing et al., 2000). In these experiments, a dominant negative 14-3-3\(\zeta\) was created by substitution of conserved arginine residues within the
phosphoserine-binding groove, resulting in inhibition of 14-3-3ζ monomer binding to substrate peptides (Thorson et al., 1998). Mutant 14-3-3ζ monomers would sequester wildtype monomers into dimers unable to bind more than one 14-3-3 binding site. Fibroblasts transfected with this 14-3-3ζ isoform exhibited markedly increased apoptosis in response to UV-C irradiation. In similar experiments, targeted expression of dominant negative 14-3-3η to murine cardiac tissue resulted in massive cardiomyocyte apoptosis and increased mortality, in response to transverse aortic constriction, a treatment which in normal tissue produces only a modest response (Xing et al., 2000). Recently, it has been shown that 14-3-3 proteins play a role in PI 3-kinase-mediated inhibition of apoptosis via their ability to sequester the pro-apoptotic FoxO transcription factors in the cytoplasm (Chong and Maiieze, 2007; Cahill et al., 2001; Arimoto-Ishida et al., 2004). PI 3-kinase activates the serine kinase Akt which phosphorylates FoxO family members in up to 4 places which either masks their nuclear localisation sequences, preventing transport into the nucleus, or unmask their nuclear export sequence, stimulating nuclear export (Arden and Biggs, 2002). 14-3-3 binding to one or more of these phosphorylated FoxO serine and threonine motifs has been demonstrated to lead to cytoplasmic retention (Brunet et al., 1999; Cahill et al., 2001; Biggs et al., 1999; Nakae et al., 2000). One mechanism by which the FoxO family members promote apoptosis is likely to be through transcriptional activation of pro-apoptotic Bim, which has a FoxO3a site in its promoter (Rosas et al., 2005; Gilley et al., 2003).

The 14-3-3 proteins may also play a role in the prevention of cell cycle progression in response to DNA damage. 14-3-3ζ was shown to bind phosphoserine 216 of the cell cycle-regulating protein phosphatase cdc25c (Peng et al., 1997; Sanchez et al., 1997). Serine 216 of cdc25c is phosphorylated by Chk1, which is activated in response to DNA damage. 14-
3-3β binding to this site was shown to be necessary for the inactivation of cdc25c, and subsequent inhibition of entry into mitosis in response to DNA damage (Sanchez et al., 1997; Peng et al., 1997). Studies in yeast suggest that 14-3-3 regulates cdc25c activity by enhancing cdc25c nuclear export in response to DNA damage (Lopez-Girona et al., 1999).

In studies performed as part of this thesis, two possible 14-3-3 binding sites in the cytoplasmic region of the βc were identified. These sites are 582HSRSLP587 (designated motif 1) and 820RSKPSSP826 (designated motif 2) whose putative phosphoserines are underlined, (also detailed in Figure 1.3). In vitro studies have shown that GST-14-3-3ζ can interact with the huβc in fibroblast lysates (Stomski et al., 1999). These studies are described in further detail in Section 3.1, where the potential interactions between 14-3-3 and huβc or muβc were investigated.

1.5.5 Activation of cellular functions by the GM-CSF/IL-3/IL-5 receptors

Deletion and point mutagenesis studies of the huβc have shown that the functional activities mediated by the active huβc are dependent on distinct regions of the intracellular portion of the subunit. Specific regions have been found to be necessary for the ability of huβc to promote proliferation, survival and differentiation (see Figure 1.5), however, specific residues required for these functions have not yet been described.
Figure 1.5 Schematic representation of the cytoplasmic domains of the huβc and their proposed roles in GM-CSF, IL-3 and IL-5 signalling. Studies utilising truncation mutants by a number of groups have predicted the domains of the huβc responsible for growth, survival, differentiation and negative regulation (Sato, 1993; Sakamaki K, 1992; Kinoshita, 1995; Smith, 1997). Amino acid residues at which specific truncations were made are listed to the right. Conserved regions Box 1 and Box 2 are indicated, conserved tyrosines are indicated by upper-case Y, nonconserved by lower case. Boxes indicate the domains important for cell proliferation, differentiation and negative regulation of huβc signal transduction. Figure adapted from Smith (1997).
1.5.5.1 Signalling for regulation of proliferation

A huβ, membrane-proximal region of 31aa (from arginine 456 to phenylalanine 487) has been shown to be necessary and sufficient for the stimulation of a transient proliferative response by GM-CSF in transfected Ba/F3 cells (Sato et al., 1993; Sakamaki K et al., 1992; Itoh et al., 1996). This proliferative response is transient due to the lack of survival signals transduced from this mutant receptor (Inhorn et al., 1995; Kinoshita et al., 1995). The membrane-proximal region contains a conserved motif (in type 1 cytokine receptors) known as “Box 1”, where Jak2 is known to bind the huβ, (Section 1.5.4.1). Receptor dimerisation is predicted to lead to activation of Jak2 molecules bound to Box 1 of the dimerising huβ, subunits via cross-phosphorylation. Experiments utilising deletion mutants of Box 1 or dominant negative Jak2 show that Jak2 is both necessary and sufficient for the mediation of proliferation by huGM-CSF in Ba/F3 cells (Watanabe et al., 1996b). Primary foetal liver myeloid progenitor cells isolated from Jak2-deficient mice failed to survive or proliferate in response to IL-3, but did respond to other cytokines such as G-CSF (Parganas et al., 1998). A second conserved motif in the first 35 aas of the intracellular huβ, “Box 2”, is not necessary for Jak2 binding but does enhance the proliferative signal (Itoh et al., 1996). In summary, there is evidence that cytokine stimulation of huβ, requires the activity of the kinase Jak2 for the promotion of proliferation in cell lines and primary cells.

A number of studies have addressed the question of how Jak2 activation by huβ, ultimately leads to cell proliferation. Expression of the transcription factor c-myc has been shown to be essential for cell proliferation in a variety of biological settings (Watanabe et al., 1996a;
Although Jak2 is required for the induction of c-myc expression, it does not directly induce c-myc expression, (Watanabe et al., 1996a). Jak2 activation of STAT5 proteins via the phosphorylation of huβc intracellular tyrosine residues (Section 1.5.4.1) is also likely important in the promotion of proliferation, since GM-CSF stimulated proliferation of bone marrow-derived macrophages from STAT5a deficient mice was significantly reduced compared to wildtype mice (Feldman et al., 1997).

In transfected Ba/F3 cells expressing huβc mutants where all eight intracellular tyrosines are substituted (huβc\(^{F8}\)), the proliferative response to GM-CSF is reduced (Okuda et al., 1997; Itoh et al., 1998). GM-CSF activation of SHP2, Shc, Grb2, raf-1, ERK2 and JNK was significantly reduced in these cells. Despite the obvious importance for tyrosine residues in the activation of these proteins, residual proliferation is detected in huβc\(^{F8}\) expressing cells. Ba/F3 cells expressing huβc mutants where all but one tyrosine have been substituted (i.e. huβc\(^{F8}\) where one tyrosine has been added back – eight in total were made) have shown that the sole presence of either of tyrosine residues 577, 612 or 695 is sufficient for the full proliferative response (Itoh et al., 1998). Redundancy therefore exists among the role played by individual tyrosine residues in proliferation. These results are consistent with the finding that the ras/raf/MEK/Erk cascade can be activated by cytokine in the presence of any of these three tyrosine residues. The ras/raf/MEK/Erk cascade has been shown to promote proliferation in a variety of biological settings (Maruta and Burgess, 1994; Campbell et al., 1998) and is likely to be important in the promotion of proliferation mediated by huβc tyrosine residues.

In addition to the promotion of proliferation by the huβc, the negative regulation of proliferation can be mediated by a distinct region of the huβc. Studies utilising truncation
mutants of the huβc have determined that the C-terminal domain (aa 767-881) negatively regulates proliferative responses to GM-CSF in CTLL cells, as well as the activation of STAT5 (Mui et al., 1995).

1.5.5.2 Signalling for regulation of survival

GM-CSF, IL-3 and IL-5 transmit signals through the huβc which promote cell survival. Studies utilising cell lines transfected with truncation mutants of the huβc have described distinct intracellular regions which are necessary and sufficient for these survival signals. Cell lines expressing a mutant huβc truncated at residue 544 failed to survive in response to GM-CSF stimulation (Kinoshita et al., 1995). However, GM-CSF stimulated a transient proliferative response in these cells before apoptosis. These experiments showed that constitutive proliferation signals without concomitant survival signals results in transient proliferation followed by cell death and further indicate that the receptor utilises different signalling mechanisms for the independent regulation of survival and proliferation. Further studies utilising truncation mutants described a region which mediates survival (huβc 544-763, Survival domains 1 and 2 combined, Figure 1.5). When cell lines were cultured in the absence of serum, this entire domain was necessary for survival, but in the presence of foetal calf serum, only the huβc region 544-626 (Survival domain 1, Figure 1.5) was necessary and sufficient for the promotion of cell survival (Kinoshita et al., 1995; Inhorn et al., 1995). The C-terminal domain survival domain (only required for signalling in the absence of serum), was designated Survival Domain 2 (Figure 1.5). The experiments described above have indicated that elements within the huβc region 544-626 (Survival domain 1) are absolutely necessary for the promotion of survival in Ba/F3 cells, regardless of addition of serum. Two tyrosine residues lie within survival domain 1, Y577 and Y612.
Both of these residues are involved in the activation of the ras/raf/MEK/ERK cascade, as described in Section 1.5.4.2. Tyrosine 577 also plays a role in the recruitment of Gab2 and activation of PI 3-kinase, a pathway which is involved in promoting survival (Section 1.5.4.4). However, GM-CSF promotion of survival in cells expressing huβc mutants where either of these residues are substituted (huβc<sup>Y577F</sup> and huβc<sup>Y612F</sup>) is not defective (Durstin et al., 1996). GM-CSF activation of Shc was dramatically reduced in cells expressing the mutant huβc<sup>Y577F</sup>, therefore the activity of Shc is dispensable for GM-CSF promotion of survival in this cell line.

Interestingly, experiments in the laboratory of Prof. Angel Lopez (Guthridge et al., 1998), and others (Okuda et al., 1997; Itoh et al., 1998) have shown that cells expressing a mutant huβc where all intracellular tyrosine residues are substituted (huβc<sup>F8</sup>) are able to survive in response to GM-CSF. These experiments show that tyrosine phosphorylation is not essential for the regulation of survival. The huβc must therefore utilise alternate mechanisms for the mediation of survival.

It has been shown that IL-3 mediation of survival requires the activation of PI 3-kinase (Sato et al., 1993; Scheid et al., 1995) (Section 1.5.4.3). Cell survival also depends on the activation of the serine/threonine kinase AKT, a downstream target of PI 3-kinase (Songyang et al., 1997). There are many possible downstream effects which can follow activation of Akt, although few of these have been examined in terms of PI 3-kinase/Akt inhibition of apoptosis in response to GM-CSF or IL-3. It is known that expression of the anti-apoptotic gene mcl-1 is increased upon IL-3 stimulation of Ba/F3 and TF1 cell lines and inhibition of PI 3-kinase reduces this effect (Wang and Wood, 1995). Another role of Akt activation in response to IL-3 stimulation is to initiate the serine phosphorylation of
apoptosis promoting BAD, a promoter of apoptosis (briefly described in Section 1.5.4.5) (del Peso et al., 1997). Serine phosphorylation of BAD by the serine/threonine kinase Akt leads to its binding of the adaptor/chaperone protein 14-3-3, which sequesters the BAD:14-3-3 complex in the cytoplasm, in a process which inhibits apoptosis (del Peso et al., 1997). Consistent with this mode of action for PI 3-kinase promotion of survival, IL-3 stimulated phosphorylation of BAD (del Peso et al., 1997) as well as cell survival (De Groot et al., 1998), is abolished by inhibitors of PI 3-kinase. It is not known exactly how PI 3-kinase is activated by the huβc. It is likely that PI 3-kinase is recruited to the huβc and therefore into the vicinity of the membrane. The experiments of (Gu et al., 2000) highlight the possibility that PI 3-kinase activation is mediated by huβc tyrosine 577. However, the presence of tyrosine 577 was shown to be dispensable for cell survival (Durstin et al., 1996; Guthridge et al., 1998; Okuda et al., 1997; Itoh et al., 1998). PI 3-kinase activity in response to huβc ligand is therefore likely to be mediated by other motifs in the huβc intracellular region. This possibility is examined in the third chapter of this thesis.

1.5.5.3 Signalling for regulation of differentiation

In contrast to the amount known about how signalling through the huβc can control proliferation, the mechanisms by which GM-CSF, IL-3 and IL-5 regulate differentiation is much less understood. Influential among these studies has been the use of the M1 leukaemic cell line as an in vitro model for haemopoietic differentiation. M1 cells engineered to express wildtype or mutant GM-CSF receptors have been examined to determine the mechanisms and signalling pathways that may regulate cellular differentiation in response to GM-CSF (Smith et al., 1997). When stimulated with GM-CSF, M1 cells gain the morphology of macrophages and express surface lineage markers
consistent with their identity as macrophages. Studies utilising a truncation mutant of the huβc showed that a region of the huβc encompassing aas 525 to 610 (the “differentiation domain”, Figure 1.5) was required for the ability of GM-CSF to cell stimulate M1 cell differentiation (Smith et al., 1997). Motifs within this region and the receptor proximal events required for differentiation are unknown. In addition to the above mentioned study, the membrane proximal proline rich region, but not the cytoplasmic tail, of the GMRα was necessary for GM-CSF induced differentiation of FDCP1 cells (Matsuguchi et al., 1998).

1.5.5.4 Signalling for regulation of effector cell functions

The effector cell functions of mature monocytes/macrophages and granulocytes are essential to the immune response. GM-CSF activates the effector cell functions of mature monocytes/macrophages and neutrophils leading to cell adhesion, degranulation and potent priming of ROS production and phagocytic activity (Sections 1.4.4.3 to 1.4.4.5). The priming of ROS production and phagocytic activity by GM-CSF is one of the most potent effects of GM-CSF on mature cells and plays an essential role in the killing of microbial pathogens. IL-5 and IL-3 have been shown to regulate the effector cell functions of eosinophils (Lopez et al., 1986b; Lopez et al., 1988) and IL-3 also plays a role in the activation of basophils (Hirai et al., 1988b; Kurimoto et al., 1989).

The essential in vivo role for the βc during insult to the immune system has been demonstrated in muβc−/− mice. Mice lacking muβc are defective in their ability to fight specific infections (Nishinakamura et al., 1995). Ex vivo experiments have shown that the effector cell functions (adhesion and phagocytosis) of muβc−/− neutrophils and alveolar macrophages are unable to be stimulated by GM-CSF (Scott et al., 1998). It is likely that
these specific cellular defects are the underlying cause of the inability of muβC-/- mice to fight off parasitic infections.

The process by which the huβc signals to regulate effector cell functions has gone unstudied, therefore the huβc regions, motifs and signalling pathways by which these essential in vivo functions are regulated, are unknown. It is likely that these functions have been left unstudied as experiments necessary to characterise the molecular mechanisms involved are difficult. For such experiments it would be necessary to use primary cells which are devoid of wildtype βc and have been reconstituted with mutant βc proteins. These cells need to be purified and functional assays performed. Described in this thesis are a method for the generation and purification of such cells (Chapter 4), and subsequent functional assays (Chapters 5 and 6).

1.5.5.5 The limitations of previous studies on GM-CSF/IL-3/IL-5 regulation of specific cellular functions

GM-CSF, IL-3 and IL-5 regulate pleiotropic cellular responses and these different responses are regulated by multiple signalling pathways. The pathways and residues of the huβc that regulate specific responses are not well understood. For example; how the huβc initiates the signals that lead to survival are unknown. Most studies have utilised huβc mutants (truncation and point mutations) in cell lines to determine how GM-CSF regulates specific responses. This has proven to be successful when examining cell proliferation, for example, and the results indicate that tyrosine residues of the βc, most likely through JAK2 activation, are essential for the regulation of proliferation. However, there is signal redundancy in βc tyrosine requirements with no one specific tyrosine being important in
the regulation of the proliferation response in cell lines. In sharp contrast to proliferation responses, huβc tyrosine residues are NOT required for survival in cell lines. Other mechanisms must therefore be involved, one possibility is phosphorylated serine or threonine residues. The most significant drawback of the preceding studies is that they utilise cell lines and not primary haemopoietic cells. The main function of cytokine signalling through the huβc is the regulation of effector cell functions of myeloid cells, as demonstrated by knockout studies (Sections 1.4.3.5). However, effector cell functions such as phagocytosis and ROS production are highly specialised and cannot be readily measured in cell lines. Thus, despite all the published work on huβc and the mechanisms by which it regulates cellular responses, no studies have undertaken a detailed examination of the truly physiological functions of this receptor. One reason for this shortfall is technical: ROS production, adhesion, phagocytosis and other effector cell functions can only be studied in primary cells where expression of mutant forms of the huβc is very difficult.
1.6 Aims of this thesis

Although both the signal transduction pathways activated by the huβc and the functional outcomes of huβc signalling have been the subject of many investigations, some important aspects are essentially uncharacterised. A key function of huβc signalling is the regulation of effector cell functions, but the huβc signal transduction pathways which regulate these are unknown. Similarly, the importance of phosphotyrosine-independent signal transduction is also undetermined. It is the aim of this thesis to increase the understanding of huβc signal transduction and regulation of cellular functions by addressing these gaps in our knowledge. To this end, the experiments presented here investigate huβc phosphotyrosine-independent signal transduction, as well as the GM-CSF regulation of effector cell functions. The aims of this thesis are thus:

1) To investigate signal transduction emanating from phosphoserine residues in huβc.

2) To develop an approach for the purification of mature primary cells expressing the huGMRα and wildtype or mutant huβc, in which effector cell functions can be analysed.

3) To investigate the roles of specific serine and tyrosine residues of huβc in the GM-CSF priming of reactive oxygen species production and phagocytic activity.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and reagents were of analytical grade or the highest purity available. Chemicals and reagents listed below were obtained from BDH except where indicated.

acetic acid, acrylamide (Biorad), agarose (Promega), APS (Gibco BRL), aprotinin (Sigma), bis-acrylamide(biorad), butanol, blocking- reagent (Boehringer Manneheim), caesium chloride (Gibco BRL), calcium chloride, chloroform, dextran sulphate (Sigma), digitonin (WAKO), disodium hydrogen orthophosphate, EDTA, ethanol, glucose, glycerol, glycine, HEPES (Sigma), iodoacetamide (Sigma), isopropanol, iso-amyl alcohol, leupeptin (Sigma), low melting point agarose (Sigma), magnesium chloride, magnesium sulphate, methanol, Nonidet P40-substitute (Fluka Biochemica), phenol, SDS (Research Organics), sodium acetate, sodium chloride, sodium dihydrogen orthophosphate, sodium hydroxide, sucrose, TEMED (Biorad), tris (Gibco BRL), triton-X100 (Calbiochem), trypsin inhibitor (Sigma), tryptone (Oxoid), tween, urea, yeast extract (Oxoid)

2.1.2 Enzymes for manipulation of DNA

Restriction endonucleases were obtained from New England Biolabs or Promega, T4 DNA ligase from New England Biolabs, RNase A from Sigma, Pfu DNA polymerase from Promega.
2.1.3 Kits

For purification of DNA bands out of agarose gels: Qiagen QIAquick gel purification (Qiagen)

For the large scale isolation and purification of plasmid DNA form bacterial cells: Qiagen MIDI tip-100 kit (Qiagen)

For the detection of western blotting: ECL (enhanced chemiluminescence): (Amersham Pharmacia Biotech)

2.1.4 Antibodies

Antibodies used for the detection of huβc on western blots and during flow cytometry and the immunoprecipitation of huβc:

1C1: monoclonal mouse-anti-huβc IgG1 (raised and purified in the Lopez Laboratory)

8E4: monoclonal mouse-anti-huβc IgG1 (raised and purified in the Lopez Laboratory)

Antibodies used for the detection of muβc on western blots and during flow cytometry:

rabbit-anti-muβc (SC-678,K17), (Santa Cruz)

Antibodies used for the detection of huGMRα chain during flow cytometry and FACS:

4H1: monoclonal mouse-anti-huGMRα chain IgG1 (raised and purified in the Lopez Laboratory)
Antibodies used for the detection of 14-3-3 chain on western blots
rabbit-anti-14-3-3β (SC-629, K19), (Santa Cruz)

Antibodies used for the detection of phosphorylated tyrosine residues chain on western blots
4G10 monoclonal mouse-anti-phosphotyrosyl (Upstate Biotechnology)

Antibodies used for the as controls during flow cytometry and FACS
A14: monoclonal mouse-anti-huIL5Rα chain IgG1 (raised and purified in the Lopez Laboratory)
12CA5 mouse-anti-HA tag (ATCC)

Antibodies used for the detection of phosphorylated huβc serine585 on western blots
anti-huβc phosphoSer585 (Provided by Dr M. Berndt, Baker Institute, Melbourne)

Antibodies used for opsonisation of sheep RBCs in phagocytosis assays
anti Sheep-RBC (AccurateChemical and Scientific Corp., USA)

Antibodies used for lineage marker determination in cultured bone marrow cells
rat-anti-muLy6G (Gr-1), (Pharmingen)
rat-anti-MAC-1 (Provided by Dr. R. D’Andrea, Hanson Centre for Cancer Research, S.A.)
rat-anti-F4/80 (Provided by Dr. R. D’Andrea)
rat-anti-B220 (Provided by Dr. R. D’Andrea)
rat-anti-Thy-1 (Provided by Dr. R. D’Andrea)
Secondary antibodies used in Western-blotting, flow cytometry and FACS

- goat-anti-mouse-HRP (Pierce)
- goat-anti-rabbit-HRP (Pierce)
- sheep-anti-rat-FITC (Silenus)
- sheep-anti-mouse-FITC (Silenus)
- goat-anti-mouse-PE (Southern Biotechnology Associates Inc.)

2.1.4 Bacterial strains and genotypes

strain: JM109

genotype: F'[traD36, proAB, lacF'Z Δ M15], recA1, supE44, endA1, hsdR17, (rK', mK'), gyrA96, (Nalr), relA1, th,i Δ(lac-proAB), mcrA

2.1.5 Factors

Factors used to maintain factor dependant cell lines:
- muGM-CSF (produced by Mrs B. Cambareri, Lopez Laboratory)
- muIL2 (provided by DNAx Research Institute)

Factors used to activate receptors on cell lines and during primary cell assays:
- hGM-CSF (cloned by Dr T Hercus, Lopez Laboratory)
- huIL3 (cloned by Dr S. Barry, Lopez Laboratory)
- huTNFα (Research and Development)
- muGM-CSF (produced by Mrs B. Cambareri, Lopez Laboratory)
Factors used to promote growth and survival of primary cell cultures:

muSCF

2.1.6 Cloning and expression vectors

pCDNA (Invitrogen)
pBluescript SK
pReCMVpuro
pRufneoIRES (provided by Prof. T. Gonda, modified by Dr H. Ramshaw, Lopez Laboratory)
pRufhygro (provided by Prof. T. Gonda, Hanson Centre for Cancer Research, S.A.)
pPGK (provided by Dr R. D’Andrea, Hanson Centre for Cancer Research, S.A.)

2.1.7 Cloned DNA sequences

cDNA constructs for the following genes were provided by colleagues:

huβc (wildtype): provided by Dr. R. D'Andrea (Hanson Centre for Cancer Research, S.A.)

huβcF8: provided by Prof. J. Griffin (Dana-Farber Cancer Institute, Boston, Massachusetts, USA)

muβc: provided by Prof. T Gonda (Hanson Centre for Cancer Research, S.A.)

14-3-3ζ: cloned by Dr F. Stomski, Lopez Laboratory

2.1.8 Molecular weight standards

Prestained protein ladder (Gibco-BRL)

SPP1 DNA digested with EcoRI (Geneworks)
2.1.9 Oligonucleotides

**Primers for site-directed mutagenesis of huβc:**

Primers for changing huβcS585 to G:

- huβcS585G1: 5’ GGCCGCCCCACAGCCGCTCCCTACCTGACATCCTGG 3’
- huβcS585G2: 5’ CCAGGATGTCAGGTAGGCAGCGGCTGTGGGGCGGCC 3’

Primers for changing huβcS585 to D:

- huβcS585D1: 5’ GGCCGCCCCACAGCCGCGACCTACCTGACATCCTGG 3’
- huβcS585D2: 5’ CCAGGATGTCAGGTAGACAGCGGCTGTGGGGCGGCC 3’

Primers for changing huβcS585 to E:

- huβcS585E1: 5’ GGCCGCCCCACAGCCGCGAGCTACCTGACATCCTGG 3’
- huβcS585E2: 5’ CCAGGATGTCAGGTAGCTCAGCGGCTGTGGGGCGGCC 3’

Primers for changing huβcY577 to F:

- huβcY577F1: 5’ GCTTTGACTTCATGGGCCCTACCTGGGGCCGCC 3’
- huβcY577F2: 5’ GGCGGCCCCAGGTCGCGGGCCGATGAGTCAGC 3’

**Primers for site-directed mutagenesis of muβc:**

Primers for changing muβcS583 to G:
muβ,S583G1: CCTACCTGGGGCCTCCCCAATCCCACGGCTGCCAGATCTC CCAGACCAGC 3’
muβ,S583G2: GCTGGTCTGGGAGATCTGGCAGGCCGTGGGATTGGGGAG GCCCCAGGTAGG 3’

Primers for changing muβc581HSL583 to AAA:
muβcAAA1: 5’ GCTGGTCTGGGAGATCTGGCCGCCGCCGGGATTGGGGAGGC CCCAGGTAGG 3’
muβcAAA2: 5’ CCTACCTGGGGCCTCCCCAATCCCGGGCGGCCAGATCTCC CAGCAACGA 3’

Primers for changing muβcY575 to F:
muβcY575F1: 5’ GCTTTGACTTCAATGGGCCTTTCCTGGGGCCTCCC CAATCC 3’
muβcY575F2: 5’ GGATTGGGGAGGCCCCAGGAAAGGCCCATTGAAGTC AAAGC 3’

Primers for introducing a XhoI site into the 5’UTR of mβc:
muβcXhoI5’1: 5’ CCATAGCCTCCAGTGCCAGCCTCGAGAAGTGCCAAAAT GGACC 3’
muβcXhoI5’2: 5’ GGTCATTTTGCACTTCTCGAGGCTGCACTGGAAGCTATGG 3’

Primers for sequencing huβc cDNA constructs:
primer #99: 5’ TTGGCCCAAGAGGAGG 3’ nts 2072 - 2053
primer #97: 5' CCCAACCCCAGCAAGGC 3' nts 1462 - 1482

Primers for sequencing muβc cDNA constructs:

muβc1220seqf: 5' CCTCCAACCCTCAACCTGACC 3' nts 1220 - 1240
muβc2220seqr: 5’ CCTGTTCCTCCATGCTCAGC 3’ nts 2240 - 2221
muβc2070seqf: 5’ GGAGTACATGTGTCTGGCC 3’ nts 2068 - 2086
muβc3070seqr: 5’ CCCATAGAAGGCAGAAAACTGC 3’ nts 3097 - 3072
β1: 5’ CGATTTTCCATCACAAACGCC 3’ nts 497 - 517
β2: 5’ TCAATGAATGAGTAAGCCATC 3’ nts 1302 - 1282
muβcexon14seq: 5’ GGAGGCAGAGGCAGG 3’ nts 3218 - 3233

2.1.10 Mice strains

All mice used in these studies were 129SV strain, acquired from Prof G Begley, (WAIMR, W.A.)

129SV double knockout mice were used with genotype: muβc<sup>−/−</sup>;β<sub>IL3</sub><sup>−/−</sup>

2.1.11 Tissue culture reagents

DMEM, RPMI-1640, IMDM, 10 x PBS, Penicillin, streptomycin, FCS, L-glutamine, Baxter Water (enotoxin free), 2.5% trypsin (all obtained from CSL, Australia)

2.1.12 Cell lines

COS (monkey kidney fibroblasts, Large T-antigen positive)
M1 (myeloid leukemic cell line, with stable transfections of huGMRα chain): provided by Dr. N. Nicola, WEHI. Further stable transfections of huβc performed by Dr F. Stomski, Lopez Laboratory

CTL-EN (subline of mouse IL-2 dependent T-cell line CTLL-2, engineered to express a surface retroviral receptor to allow transduction by retroviral constructs produced by Ψ2 cells): A gift from Dr J. Norton, Paterson Institute for Cancer research, manchester.

Ψ2 (murine fibroblast cell line, derivative of NIH3T3): provided by Prof. T Gonda

2.1.13 Standard solutions and bacterial media

**30% Acrylamide/8% bisacrylamide:** 30%(w/v) acrylamide and 8%(w/v) bisacrylamide were dissolved in MQ by stirring and stored at 4°C in the dark.

**6 x agarose gel loading buffer:**
- **Destain:** 5% methanol, 10% acetic acid
- **Facs Fix:** PBS with 2% D-glucose, 0.2% sodium azide, 1% formaldehyde
- **L-broth:** 1% bacto tryptone, 0.5% bacto yeast extract, 1% NaCl, pH 7.0
- **Lower Gel Buffer:** 182g tris and 4g SDS were dissolved in 1L MQ
- **1 x PBS:** 10L made with 2g KH₂PO₄, 11.5g Na₂HPO₄, 80g NaCl, 2g KCl
- **Tris-saturated phenol:** phenol crystals were melted and mixed with 1M tris-HCl pH8, layers were allowed and aqueous layer was replaced with fresh 1M tris-HCl pH 8, this was repeated twice, then a layer of 0.1M tris-HCl pH 8 was mixed with phenol and 8-hydroxyquinoline added for storage.

**Restriction endonuclease buffers:** as recommended and supplied by manufacturers
1 x SDS running buffer: 2L was made from 60.6g tris, 288G glycine and 20g SDS dissolved in MQ.

1 x TAE: 4L was made from 387.52g tris, 91.5ml acetic acid and 160ml 0.5M EDTA pH 8, dissolved in MQ and made up to 4L.

1 x TE: 10mM tris pH 8, 1mM EDTA

1 x TNT: 1mM tris, 150mM NaCl, 0.005% tween 20, pH adjusted to 8

Transfer Buffer: 3 litres was made from 787g tris, 33.78g glycine, 3g SDS and 600ml methanol dissolved in MQ and made up to 3 litres

Upper Gel Buffer: 30g tris and 2g SDS were dissolved in 500ml MQ and pH adjusted to 6.8

Western Stripping Solution: 62.5mM tris pH6.8, 2%SDS, with 700µl βME freshly to each 100ml at time of use

2.1 Methods

2.2.1 DNA Manipulation

2.2.1.1 Restriction Digests

DNA was placed in an aqueous solution of 1 X the recommended commercial buffer for specific enzymes and 100µg/ml BSA added where recommended by the company. For complete digests, 5 to 20 units of enzyme was per µg of plasmid DNA. Digestions were incubated at 37°C, or recommended temperature for specific enzymes, from 2 hours to overnight.
2.2.1.2 Electrophoresis of DNA on agarose gels

1% molten agarose in 1 x TAE was poured into horizontal gel casters with wells formed by suspended plastic combs. The combs were removed and gels submerged into 1 x TAE in an electrophoresis tank. DNA samples in 1 x loading buffer were loaded into wells and a 100V current was applied until loading dyes had migrated through the gel at an appropriate distance. After staining in dilute EtBr solution for 5 minutes and destaining under running tap water, DNA was visualised under UV light and photographed on polaroid film.

2.2.1.3 Small scale preparation of plasmid DNA

A single colony was inoculated into 2.5ml of L-broth containing 100µg/ml ampicillin and incubated overnight at 37°C whilst shaking. 1.5ml of this culture was used for plasmid DNA preparation and 1ml for making glycerol stocks for long term storage. 1.5ml culture was centrifuged at 13krpm for 30 seconds. The supernatant was removed and the pellet was resuspended in 500µl STE at 4°C and centrifuged at 13krpm. The supernatant was removed and the pellet was resuspended in 100µl GTE. At room temperature, 200µl of freshly made 0.2M NaOH/1% SDS was added and mixed in by gentle inversions. Cells were allowed to lyse at room temperature for 5 minutes, then 150µl of a neutralisation solution of 3M KAc pH5.5 was added and the solutions were mixed by shaking. Tubes were kept on ice for 20 minutes, then centrifuged at 13krpm for 5 minutes. The supernatant was transferred into fresh tubes and 1µl of 10mg/ml RNAse A was added. Solutions were mixed by shaking and incubated at 37°C for 20 minutes. 300µl tris-saturated phenol, pH8 and 300µl chloroform were added and solutions were vortexed to mix. Tubes were centrifuged at 13krpm for 3 minutes and the aqueous (top) layer removed to a fresh tube.
1ml 100% ethanol was added and mixed in by shaking, tubes were then incubated at -20°C for 1 hour, centrifuged at 13krpm, 4°C for 15 minutes, pellets washed in 500µl 70% ethanol, centrifuged at 13krpm, 4°C for 15 minutes. Pellets were dried by 10 minutes spinning in a vacuum drier and resuspended in 20µl of TE.

2.2.1.4 Large scale preparation of plasmid DNA by Qiagen MIDI kit

A sterile scraping of a glycerol stock was inoculated into 5ml of L-broth containing 100µg/ml ampicillin and incubated for 8 hours at 37°C with shaking. This was added to 200ml or 400ml L-broth containing 100µg/ml ampicillin and incubated overnight at 37°C with shaking. Cells were harvested by centrifugation at 5krpm in a Beckman 10.5 rotor for 10 minutes. Pelleted cells were resuspended in 6ml per 200ml culture of 4°C P1 (50mM tris-HCl, pH8.0; 10mM EDTA; 100mg/ml RNaseA). 6ml of room temperature P2 (0.2M NaOH; 1% SDS) was added and mixed in by inverting 5 times. Cells were allowed to lyse at room temperature for 5 minutes then 6ml of a neutralisation solution of 3M KAc pH5.5 was added and the solutions were mixed by shaking and left on ice for 20 minutes. Solutions were centrifuged at 16krpm for 30 minutes and supernatants were then centrifuged at 16krpm for 15 minutes. Supernatants were applied to Qiagen MIDI 100 Tip which had been pre-equilibrated with 5ml of QBT (750mM NaCl; 50mM MOPS pH7; 15% isopropanol; 0.15% triton X-100). The solution was allowed to enter the column by gravity flow. The column was washed twice with 10ml QC (1.0M NaCl; 50mM MOPS pH7; 15% isopropanol). DNA was eluted with addition of 5ml of QF (1.25M NaCl; 50mM tris pH 8.5; 15% isopropanol) Eluted DNA solution was added to 3.5ml isopropanol and incubated at -20°C for 1 hour or overnight. Solution was centrifuged at 12krpm for 15 minutes in a Beckman 13.1 rotor at 4°C. Pellets were washed in 5 ml of 70% ethanol and
centrifuged at 12krpm for 15 minutes in a Beckman 13.1 rotor at 4°C. Pellets were resuspended in 50µl MQ and concentration determined by spectrometer absorbance readings at $\lambda_{260}$.

2.2.1.5 Large scale preparation of plasmid DNA by CsCl purification

500ml of L-Broth containing appropriate antibiotic was inoculated with culture from a glycerol stock and incubated overnight at 37°C whilst shaking. Cells were harvested by centrifugation in a Beckman JA10 rotor at 5krpm for 8 minutes at 4°C. Cells were resuspended in ice cold TES (25mM tris pH 8, 10mM EDTA, 15% w/v sucrose) at 4ml per 250ml culture. 8mg of lysozyme was added and mixed and cells incubated on ice for 30 minutes. 8ml of 0.2M NaOH/1% SDS was added and the solutions were incubated on ice for 10 minutes. 5ml of 3MKAc/2M acetic acid was added and solutions incubated on ice for 40 minutes, then centrifuged at 17krpm for 30 minutes in a Beckman JA20 rotor. To the supernatant was added 10ml phenol and 10ml chloroform/isoamyl-alcohol 24:1. Solution was vortexed and centrifuged at 10krpm for 10 minutes at 10°C in a Beckman JA20 rotor. Plasmid DNA was precipitated from the upper layer by adding 1 volume of isopropanol and incubating at -20°C for 1 hour then centrifugation at 15krpm for 15 minutes in a Beckman JA20 rotor at 4°C. Pellets were resuspended in 2.3ml MQ and this was added to 2.45g CsCl. 60µl 10mg/ml EtBr was added and the solution was heat sealed inside a Beckman Quick-seal tube (13 x 32mm). Tubes were balanced and centrifuged in a TLA 100.3 ultracentrifuge rotor overnight at 80krpm. Tubes were punctured and plasmid DNA bands recovered using a 19 gauge needle and 1ml syringe. EtBr was removed by 6 x extractions in equal volumes of MQ saturated butanol, centrifuged for 5 minutes at room temperature and upper phase discarded. Volumes were adjusted to 2ml and DNA was
precipitated by adding 200µl 3MNaAc pH 5.4 and 5ml ice cold 100% ethanol and
incubating at -20ºC for 1 hour, followed by 30 minutes of centrifugation in a Beckman
JA20 at 15krpm, 4ºC. Pellets were washed in 5ml 70% ethanol and centrifuged at 15krpm
for 15 minutes at 4ºC. Pellets were resuspended in 400ul MQ and transferred to 1.5ml
eppendorf tubes where 40µl 3M NaAc pH5.4 and 1ml ice cold ethanol were added and
DNA was precipitated at -20ºC for 1 hour, followed by centrifugation for 15 minutes at
13krpm in a microfuge at 4ºC. Pellets were washed in 1.5ml 70% ethanol, centrifuged for
10 minutes and then dried for 10 minutes in a vacuum drier and resuspended in 300µl MQ.
Plasmid DNA concentration was determined by spectrometer absorbance readings at λ260.

2.2.1.6 Cycle sequencing of DNA

0.5µg Qiagen or CsCl plasmid, of 1µl (approx. 1µg) of miniprep plasmid DNA was added
to 4µl of “big dye” mix (containing ddNTPs, dyes and DNA polymerase), 3.2pmoles of
appropriate primer and MQ to a volume of 20µl. Reaction solution was subject to 30
cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes in a GeneAmp
PCR system 9700 cycler (PE Applied Biosystems). Reactions were then added to 2µl 3M
NaAc pH5.4 and 50µl 100% ethanol and incubated on ice for 10 minutes. Precipitated
DNA was centrifuged at 13krpm for 30 minutes at 4ºC in a microfuge, pellets were washed
with 500µl 70% ethanol and centrifuged for 10 minutes at 13krpm at 4ºC, vacuum dried
for 10 minutes DNA sequence determined by automated sequencing performed by the
Molecular Pathology Department of the IMVS.
2.2.1.7 Preparation of JM109 *E. coli* electrocompetent cells.

50ml of L-Broth was inoculated with culture from a JM109 glycerol stock and incubated overnight at 37°C with shaking. 400ml L-Broth was then inoculated with 10ml of overnight culture and incubated at 37°C with shaking until OD at 600nm was 0.6 (approx. 2.5 hours). Cells were harvested by centrifugation at 5krpm for 10 minutes in a Beckman J6 rotor at 4°C. Cells were carefully resuspended on ice in 20ml ice cold buffer G (1mM Hepes/10% glycerol pH7). Cells were centrifuged at 3.6krpm for 8 minutes at 4°C in a Beckman J6 rotor and pellets were carefully resuspended in 20ml ice cold buffer G. Cells were recentrifuged and pellets were carefully resuspended in 20ml ice cold 10% glycerol. Cells were recentrifuged and pellets were carefully resuspended in 1500µl ice cold glycerol. 50µl aliquots of electrocompetent cells were frozen in liquid nitrogen then transferred to -80°C for storage.

2.2.1.8 Purification of DNA from agarose gels

For the purpose of subcloning, digested DNA was electrophoresed in agarose gels as described in Section 2.3.1.2 and appropriate bands were purified from the gel using the QIAquick gel extraction kit, following the manufacturers instructions. Bands were stained with EtBr and visualised under low intensity UV light. Bands were excised using a scalpel blade and transferred to eppendorf tubes, where 300µl of QG buffer (formulation not provided by manufacturer) was added and gels were incubated at 55°C for 10 minutes. 100µl of isopropanol was added and the solution was vortexed, then transferred to a QIAquick spin column and centrifuged at 13krpm for 1 min. A further 300µl of QG was centrifuged through the column to remove left-over agarose. 750µl of buffer PE was centrifuged through the column as a wash. Flow-through was removed from the collection
tub and the column centrifuged again for 1 min. 25µl of buffer EB (10mM tris-HCl, pH 8.5) was added to the column and centrifuged through for 1 min. to elute and store DNA.

2.2.1.9 DNA Ligation Reactions

7µl of digested, gel purified insert DNA was added to 2µl of digested, gel purified vector DNA in a solution of 1 x T4 ligase buffer and 200U/10µl of T4 ligase. Ligation reactions were incubated overnight at 16°C.

2.2.1.10 Calf Intestinal Phosphatase (CIP) Digestions

To a 30µl digested solution of vector DNA was added 4ml of 10 x CIP buffer, 1ml of CIP and 5ml of MQ and CIP digestions were incubated for a further hour at 37°C.

2.2.1.11 Transformations

An aliquot of JM109 competent cells were thawed on ice and 2µl of ligation reaction mixed with one 50µl aliquot. Mixes were transferred to a precooled 2mm electroporation cuvette and pulsed at settings of 25µFD, 200Ω and 2.0kV. 1ml of LB was added and cells were incubated at 37C for 20 min. Cells were then centrifuged at 6krpm for 2 min., resuspended in 200µl of LB and spread onto LB plates containing 100µg/ml ampicillin.

2.2.1.12 Site-Directed Mutagenesis

Site-directed mutagenesis was performed by cycling reaction designed to replicate whole small (<6.5kb) plasmids incorporating primers containing the site change. Typically a
reaction mixture of 50µl consisted of 100ng DNA template, 150ng each of two complementary primers, 2.5U/ml *Pfu* DNA polymerase, 1X *Pfu* reaction buffer, 0.2mM dNTPs and MQ. Cycling parameters were as follows: 1 incubation of 95°C for 30 sec., 15 cycles of: 95°C, 30sec., 55°C for 1 min. and 68°C for 15min. (5.4kb plasmids) or 20min. (6.2kb plasmids). 5ml of this reaction was electrophoresed (Section 2.3.1.2) on an agarose gel to determine the success of the reaction. The remaining 45ml was digested (Section 2.3.1.1) for approx 8 hours at 37°C with 10U *Dpn*I then 2 ml was transformed (see 2.3.1.11) into electrocompetent JM109 cells. *Dpn*I digests methylated, non-mutated DNA, leaving only that polymerised in the reaction. Resultant colonies were screened by miniprep (see 2.3.1.3) and sequencing (see 2.3.1.6).

### 2.2.1.13 Site-directed mutagenesis to create huβc cDNAs containing mutations which disrupt intracellular serine and tyrosine motifs

Site-directed mutagenesis was performed based on the Quikchange method described by Stratagene (http://www.stratagene.com/mutagenesis/quikchng.htm), and Section 2.3.1.12. It was necessary to perform the site-directed mutagenesis on huβc cDNA contained in the smaller bluescript vector (pB) as the pRcCMV vector is too large for the cycling reaction to be successful. huβc cDNA was supplied in pB, subcloned via *Eco*RI (5' cloning site) and *Xba*I (3' cloning site). Site-directed mutagenesis was performed on this plasmid to make huβc<sup>S585G</sup> and huβc<sup>584AAA586</sup>. Restriction enzyme digests on DNA isolated from colonies produced by the Quikchange technique confirmed the presence of a mutation by loss of a *Bsr*B1 site which is removed from the DNA sequence in each case. Sequencing was used to confirm each mutation. Sequences of the mutant cDNAs are shown in table 3.2. Both primers #97 (forward) and #99 (reverse) were used to confirm the cDNA mutations.

huβc<sup>Y577F</sup> cDNA was kindly provided to us by Prof. J. Griffin (Dana-Farber Cancer
Institute, Boston, Massachusetts, USA). A mutant cDNA, huβc\(^{Y577F/S585G}\), was made which combines this mutation with the huβc\(^{S585G}\) mutation. To make this construct, huβc\(^{Y577F}\) was subcloned into pBluescript and site directed mutagenesis was performed to introduced the S585G mutation into the huβc\(^{Y577F}\) cDNA (Section 2.3.1.12).

2.2.1.14 Subcloning of wildtype and mutant human βc cDNAs into the pReCMVpuro expression vector

Wildtype and mutant huβc cDNA constructs were subcloned into pReCMVpuro for transfection into cell lines. huβc was excised from pB plasmids by restriction enzyme digestion with EcoRI, XbaI and PvuI. EcoRI and XbaI excised the huβc cDNA, whereas PvuI cleaved the pB vector. This was necessary as the huβc cDNA at 2.8kb is similarly sized to pB (2.9Kb) and gel extraction is made easier if the vector DNA is cleaved and therefore electrophoreses in two smaller pieces, away from the huβc cDNA band. Colonies resulting from the electrophoration of ligations into competent cells were screened for the presence of insert huβc and each mutation. EcoRI/XbaI double digests confirmed the presence of the 2.8kb insert in the 5.1kb pReCMVpuro vector, and BsrBI digests confirmed the presence of a mutation. Sequencing of each construct with both primers #97 and #99 confirmed the presence of the desired mutation in each case.

2.2.1.15 Construction of mutant mouse βc cDNA constructs by site-directed mutagenesis

muβc cDNA was supplied in the pcDNA1 vector from the Lopez laboratory. A restriction digest with EcoRI and XbaI was recommended for excising the cDNA from the vector, with EcoRI cleaving once at the 5’ end of the cDNA and XbaI cleaving once at the 3’ end.
However an \textit{XbaI} digest unexpectedly cleaved the plasmids twice. Sequencing of the 5' and 3' insertion sites revealed the presence an unexpected \textit{XbaI} cleavage site exists within the 3' UTR was also present in the cDNA, following the translation stop codon in the last exon of the cDNA.

It was necessary to subclone the mu\textsubscript{\textbeta} c cDNA into pB, as the cDNA in pcDNA1 was too large for the Quikchange reaction. mu\textsubscript{\textbeta} c was excised from pcDNA using a single digest with \textit{XbaI} which cleaved 5' and 3' of the cDNA. The fragment was gel purified and ligated into \textit{XbaI} digested, phosphatase treated pB. Restriction digests confirmed the presence of the 3.5kb mu$\beta$ c insert in the 2.9kb pB vector.

It was decided that the 5'UTR and extraneous vector sequence in the mu\textsubscript{\textbeta} c cDNA would be too long a sequence to allow optimal protein expression if it was situated between the PGK promoter and the translation start site. Therefore these sequences excluded from the final vector. This was achieved by the introduction of the an \textit{XhoI} site immediately 5' of the translation start site. Site-directed mutagenesis was performed on wildtype mu\textsubscript{\textbeta} c in pB to introduce the \textit{XhoI} site. Restriction digests with \textit{XhoI} and sequencing using the T7 promoter confirmed the presence of the added restriction site. The resulting plasmid was labelled "pBmu$\beta$ c 5'XhoI".

Site-directed mutagenesis was performed on pBmu$\beta$ c 5'\textit{XhoI} to introduce mutations in the putative 14-3-3 binding site homologous to hu$\beta$ c motif 1. The mutations mu$\beta$ c\textsubscript{SSS}G and mu$\beta$ c\textsubscript{SAAAS} were made by the Quikchange method. Resultant colonies were screened for the presence of mutation by restriction digest and sequencing. The loss of the restriction cleavage site \textit{BglII} confirmed the presence of a mutation, as this site is removed from the
DNA sequence in each of the mutant sequences. Sequencing with the primer muβc2220seqr confirmed the mutations in each case.

2.2.1.16 Subcloning of mutant mouse βc cDNAs into the pPGK expression vector

Wildtype and mutant μβc cDNA constructs were subcloned into pPGK for transfection into cell lines. μβc was excised from pB plasmids by restriction enzyme digestion with XhoI and NotI. The cDNA fragment was gel purified and ligated into XhoI and NotI digested, phosphatase treated pPGK. Colonies resulting from the electrophoration of ligations into competent cells were screened for the presence of insert μβc and each mutation. XhoI and NotI restriction digests confirmed the presence of the 3.5kb μβc insert in the 2.9kb pPGK vector, and BglII digests confirmed the presence of a mutation. Sequencing of each construct with the primer μβc2220seqr confirmed the presence of the desired mutation in each case.

2.2.1.17 Creation of pRFneoIRES retroviral vectors which express huGMRα and either wildtype huβc or mutant huβc.

A retroviral vector was created which co-expresses huGMRα and wildtype huβc or mutant huβc from one transcribed message. This vector, pRFneoIRES, was created by Dr. H Ramshaw of the laboratory of Prof. Angel Lopez, by a modification of the pRFneo retroviral construct, described by Rayner, (Rayner and Gonda, 1994).

The pRFneo vector was modified for the co-expression of the huGMRα and huβc by the introduction of an encephalomyocarditis internal ribosome entry site (IRES), which
permits translation of two open reading frames from the one messenger RNA (Jang et al., 1988; Jackson et al., 1990). The huGMRα cDNA was subcloned into this modified vector (pRUFneoIRES), 3’ to the IRES, and wildtype or mutant huβc cDNAs were subcloned into the vector, 5’ to the IRES, by Dr. H. Ramshaw. This vector is described in Fig. 2.1. One mRNA transcript is produced by integrated retrovirus, leading to expression of wildtype or mutant huβc, driven by the LTR promoter, and expression of huGMRα driven by the IRES. The mutant huβc cDNAs subcloned into pRUFneoIRES were huβcS585G, huβcY577F, huβcY577F/S585G, and huβcF8. In addition, a pRUFneoIRES construct was also created which expresses only huGMRα. These constructs are described in Table 4.1.

2.2.2 Tissue Culture Techniques

All cell lines were stored in a Napco Model 4100 E series CO2 incubator at 37°C, 5% CO2. Sterile handling of cell lines was performed in a Biosafety Cabinet Class II (Gelman Sciences Australia). Centrifugation of cells for the purpose of maintaining stocks and experiments was typically performed at 1500rpm for 5 min. in a Beckman Allegra 6R benchtop centrifuge.

2.2.2.1 Maintenance of CTL-EN lines

CTL-EN cell lines were maintained in DMEM containing 2mM glutamine, penicillin (50U/ml) and streptomycin (50µg/ml) and 10% FCS. Cells were typically diluted to a
Figure 2.1 pRUFneoIRES retroviral vector, expressing huβc and huGMRα. The pRUFneoIRES retroviral vector was created by modification of the pRUFneo retroviral construct described by (Rayner and Gonda 1994). This modification was performed by Dr H. Ramshaw of the Laboratory of Prof. Angel Lopez. Transcription of the vector yields one mRNA transcript from which most of the \textit{gag} gene (0.84kb) is spliced out. This mRNA expresses wildtype huβc or mutant huβc (cDNA 2.8kb) from the LTR promoter (593kb), and huGMRα (cDNA 1.2kb) from the encephalomyocarditis internal ribosome entry site (IRES) (0.7kb). Other features of the retroviral construct include a neomycin resistance gene (MC1neoR) (1.09kb) of which the expression is driven by the f9 polyomavirus enhancer and myoproliferative sarcoma virus long terminal repeats (LTR) (593kb), which are known to function well in hemopoietic cells (Stocking, 1985). The total size of this construct is 10.35kb.
concentration of $1 \times 10^5$ cells/ml to maintain lines. muIL2 growth factor was added to the
CTL-EN media at a concentration of 20ng/ml. Where necessary, to select for stably
transfected constructs, puromycin was added at a concentration of 2.5µg/ml or hygromycin
at a concentration of 300µg/ml

2.2.2.2 Maintenance of ψ2 lines

ψ2 cell lines were maintained in DMEM containing 2mM glutamine, penicillin (50U/ml)
and streptomycin (50µg/ml) 2mM glutamine and 10% FCS. To turn over lines: flasks were
washed with 1 x PBS, trypsinised in a 1/5 dilution of 2.5% trypsin solution at room
temperature for 5 min. Cells were removed by a wash in DMEM containing 10% FCS
which also stopped the trypsin reaction. Cells were washed and seeded at 1/10 X the
concentration of a confluent flask to maintain stocks.

2.2.2.3 Maintenance of COS lines

COS cell lines were maintained in RPMI containing 2mM glutamine, penicillin (50U/ml)
and streptomycin (50µg/ml) 2mM glutamine and 10% FCS. To turn over lines: flasks were
washed with 1 X PBS, trypsinised in a 1/5 dilution of 2.5% trypsin solution at room
temperature for 5 min. Cells were removed by a wash in RPMI containing 10% FCS which
also stopped the trypsin reaction. Cells were washed and seeded at 1/10 X the
concentration of a confluent flask to maintain stocks.
2.2.2.4 Maintenance of M1 lines

M1 cells were maintained in DMEM containing 2mM glutamine, penicillin (50U/ml) and streptomycin (50µg/ml) 2mM glutamine 10% FCS, 50µg/ml hygromycin and 5µg/ml puromycin. Selection drugs maintained the stable trasfection of huGMRα and huβ, chain expression constructs. Cells were typically diluted to a concentration of 1 x 10^5 cells/ml to maintain lines.

2.2.2.5 Generation of CTL-EN lines by Stable Transfections

2.2.2.5.1 Purification of DNA  Plasmid DNA was produced by Qiagen MIDI kit for transfections of which 20µg was linearised by digestion overnight with a restriction enzyme which cleaves once in the vector DNA region. The DNA was purified by addition of 50µl of phenol and 50µl chloroform, vortexed, centrifigation for 5 min. at room temperature, 13krpm. The aqueous layer was removed and DNA precipitated by the addition of 10µl 3M NaAc and 250µl 100% ETOH followed by incubation at -20°C for one hour. Precipitations were centrifuged at 13krm, 4°C for 10 min. 1.5ml of 70% ETOH was added to pellets followed by centrifugation at 13krpm for 10 min at 4°C. In a laminar flow hood, the supernatant was aspirated and pellets resuspended in 20µl of sterile MQ.

2.2.2.5.2 Transfection of CTL-EN cells  CTL-EN cell lines containing stable transfections of huIL3Rα chain were produced by Mrs B. Cambareri in the Lopez Laboratory. For each transfection, 4.6 x 10^6 CTL-EN cells were used. Cells were washed in PBS and centrifuged, pellets were then resuspended in 800µl CTL-EN medium with no selection drugs added. 10µg DNA was added to 800µl cells, followed by mixing and incubation at room temperature for 10 min. Mixtures were electroporated at 270V, 960µFD followed by
5 min. incubation at room temperature. 1ml CTL-EN medium with no selection drugs was added and this solution was layered over 1ml FCS in a 15ml polypropylene tube, followed by centrifugation to remove dead cells. The pelleted cells were resuspended in 6ml CTL-EN medium with no selection drugs and incubated as usual for 48 hours. After this time, cells were washed and resuspended in CTL-EN medium with appropriate selection drugs: puromycin at 2.5µg/ml, or hygromycin at 300µg/ml.

### 2.2.2.6 Generation of COS lines by Transient Transfection

One flask containing 182cm² of confluent COS cells was used per transfection. Media was aspirated from the flask and cells were trypsinised by addition of a 1/2 dilution of 2.5% trypsin solution followed by incubation at 37°C for 5 min. 9ml of COS medium was used to resuspend cells from the flask. Cells were centrifuged, washed in PBS and recentrifuged. Pelleted cells were resuspended in 800µl PBS and transferred to sterile tissue culture cuvettes containing CsCl purified (see 2.3.1.5) plasmid DNA (10µg receptor α chain, 25µg of hu or mu β chain). Cells were electroporated at 300V, 500µFD and incubated on ice for 10 min.. 1ml COS medium was added to cells and this solution was layered over 1ml FCS in a 15ml polypropylene tube, followed by centrifugation to remove dead cells. Pelleted cells were resuspended in 5ml COS medium and transferred to a 182cm² flask containing a further 15ml media. Cells were harvested after 48 hours.

### 2.2.2.7 Antibody staining of cell lines for surface expression of specific proteins

1 x 10⁵ to 1 x 10⁶ cells per sample were stained for analysis of surface expression by flow cytometry. Cells were centrifuged and resuspended in 100ml primary Ab solution at
10µg/ml Ab concentration. Typically, control Abs were isotype-matched irrelevant Abs. Cells were incubated on ice for 20 to 30 min. and washed once in 4ml PBS, followed by centrifugation. Pelleted cells were resuspended in PBS and appropriate dilutions of secondary Ab conjugated to either PE or FITC fluorescent compounds. Alternatively biotinylated primary Ab solutions were stained with streptavidin conjugated PE (Rocklands). Cell surface fluorescence was analysed by flow cytometry using a Coulter EPICS-XL-MCL flow cytometer. Between 5000 and 10,000 events were recorded for each sample.

2.2.2.8 Determining cell concentrations using the trypan blue exclusion method

Viable (trypan blue excluding) cells were counted in a 1:1 solution of 0.1% trypan blue using a Neubauer haemocytometer.

2.2.3  Protein Manipulation

2.2.3.1 Preparation of whole cell lysates

1 x 10^5 to 1 x 10^6 cells were used for each lysate preparation, depending on expression levels of the desired protein. Cells were centrifuged and resuspended in 400µl PBS, then transferred to an eppendorf tube, followed by 10 sec. centrifugation at 13krpm. Supernatants were aspirated off and pelleted cells resuspended in 100µl ice cold 1% NP-40 lysis buffer solution containing 5mM EDTA, pH8, 1mM PMSF, 1µg/ml aprotinin, 1µg/ml leupeptin, 1mM sodium vanadate and 1mM sodium fluoride. Lysing cells were incubated on ice for 5 min. followed by 15 min. centrifugation at 13krpm, 4°C. Supernatants were
recovered and an equal volume of 2 X SDS load buffer was added, followed by incubation at 100°C for 2 min. Lysates were analysed by PAG electrophoresis (see 2.3.3.2).

2.2.3.2 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed using Hoefer Mighty Small SE250 mini-gel equipment according to manufacturers instructions. Two spacer plastics were used to separate a ceramic plate and a glass plate between which the gel was poured, and these components were assembled in a gel caster. Lower layers of polyacrylamide gel mixtures consisted of reagents as described in Table 2.1.

<table>
<thead>
<tr>
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<th>7.5% acrylamide</th>
<th>10% acrylamide</th>
<th>12% acrylamide</th>
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<tbody>
<tr>
<td>4 x lower gel buffer</td>
<td>2.6ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
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<tr>
<td>30% acrylamide</td>
<td>2.6ml</td>
<td>3.2ml</td>
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<tr>
<td>MQ water</td>
<td>5.2ml</td>
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<td>APS</td>
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<tr>
<td>TEMED</td>
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Table 2.1 Formula for making polyacrylamide gels

After mixing of reagents, lower gel layers were poured into prepared casters and approx. 1ml of water-saturated butanol was added to the top of the gel. Gels were allowed to stand at room temperature for 20 min. for polymerisation of acrylamide. Upper gel layer mixture consisted of 1.25ml 4 x upper gel buffer, 0.7ml of 30% acrylamide, 3ml of MQ, 50µl of APS and 20µl of TEMED. Butanol was poured off the lower layer and the upper layer
mixture was poured on the gel, a well forming comb was suspended into the upper gel mixture and gel was allowed to stand at room temperature for 20 min. for polymerisation. When the gel was set, the comb was removed and gel clamped into a running tank. The tank was filled with 1 x SDS running buffer and wells flushed before loading. Protein samples were mixed with equal amounts of 2 x SDS loading buffer and incubated at 100°C for 2’. Protein samples were loaded into lanes, including prestained protein markers, a 15mA current was applied until dye fronts had migrated through the upper gel layer. The current was increased to 25mA until the dye front had migrated to the bottom of the lower gel.

2.2.3.3 Protein transfer to nitrocellulose membrane by western transfer

Following PAG electrophoresis, proteins were transferred to 0.45µm pore size nitrocellulose membrane (Advantec MFS Inc, Pleasanton, CA, USA) using a Hoefer TE series transphor electrophoresis. To set up the transfer, gels were placed on to nitrocellulose membrane prewetted in transfer buffer. Gel/nitrocellulose layers were sandwiched between three layers of prewetted whatman paper, between prewetted sponges. These components were clamped into plastic holders and placed into a transfer tank where a current of 250mA was applied for one and a half hours. Nitrocellulose membranes were removed and incubated at 4°C in 100ml 1% blocking reagent overnight, whilst rotating.

2.2.3.4 Protein transfer onto nitrocellulose membrane by dotblotting

A dotblotting apparatus was loaded with dry nitrocellulose filter and vacuum pressure applied. Peptides were pipetted onto the filter without leaving any bubbles of air in the
solution covering the filter. Vacuum was applied for a further 20 min. then filters were incubated in 100ml 1% blocking reagent overnight, whilst rotating.

2.2.3.5 Antibody detection of specific proteins transferred onto nitrocellulose membranes

Following overnight incubation in blocking reagent, nitrocellulose membranes were incubated in a solution of 1 x TNT, 0.5% blocking reagent and primary Ab, in a sealed plastic bag, at room temperature, rotating, for 2 to 4 hours. Nitrocellulose was then washed over 30 minutes with 3 changes of 100ml 1 x TNT, followed by incubation in a solution of 1 x TNT, 0.5% blocking reagent and secondary Ab, in a sealed plastic bag, at room temperature, rotating, for 1 hour. This was followed by washes over 30 minutes with 3 changes of 100ml 1 x TNT.

2.2.3.6 ECL detection of antibody stained proteins on nitrocellulose membranes

Following the final TNT wash, nitrocellulose membranes were drained of excess buffer, an incubated with 1ml each of ECL reagents 1 and 2 with vigorous mixing for 1 minute only. Nitrocellulose was immediately drained of ECL reagents and sandwiched between two clear plastic sheets and exposed to X-ray film (Fujifilm SuperRX) in a light-proof cassette for exposure times ranging from 5 seconds to overnight. X-ray films were developed using a Ilfospeed 2240 (Ilford) X-ray film developer.
2.2.3.7 Immunoprecipitations and co-immunoprecipitations of specific proteins from cell lines

$2 \times 10^7$ to $1 \times 10^8$ cells were used per immunoprecipitation, depending on the expression levels of the desired proteins and the cell lines. Cells were centrifuged and pooled into one 50ml tube, followed by a wash in cold PBS and recentrifugation. Cells were then lysed in 1ml of an ice cold solution of 1% NP-40 lysis buffer, 10mM $\beta$ glycerol phosphate, 1mM PMSF, 5mM sodium fluoride, 10mM sodium vanadate, 5mM EDTA, 1µg/ml aprotinin, 1µg/ml leupeptin, 1mM sodium pyrophosphate, 5mM triethanolamine and 10mg iodoacetamide. Lysates were centrifuged at 13krpm, 4°C for 15 minutes and supernatants were then precleared by 2 hours incubation with 50µl of a 2 x slurry of Protein-A conjugated sepharose at 4°C whilst rotating. Mixtures were then centrifuged for 10 minutes at 13krpm, 4°C and precleared supernatants were transferred to new ependorf tubes. To supernatants were added 50µl 2 x slurry of protein-A sepharose beads (preabsorbed with Ab for one hour at 4°C whilst rotating, then washed 3 times with PBS, with centrifugation at 13krpm, 4°C, for 1 min.). Lysates and Ab conjugated sepharose were incubated for 1 hour at 4C whilst rotating, followed by 5 washes in 1ml PBS, with centrifugation at 13krpm, 4°C, for 1 min. After the final wash, the supernatant was aspirated off the sepharose and 25µl of 2 x SDS reducing load buffer was added to pelleted sepharose, followed by incubation at 100°C for 2 min to remove protein from the sepharose. Recovered proteins were analysed by PAG electrophoresis (Section 2.3.3.2) and western blotting (Sections 2.3.3.3-5).
2.2.2.8 Pull-downs of specific proteins from cell lysates by GST-14-3-3 conjugated sepharose

When pulldowns were performed using transfected (over-expressing) cell lines, 2 x 10^7 cells were used per sample. When pulldowns were performed using untransfected (endogenous expressing) cell lines, 5 x 10^7 cells were used per sample. Cells were centrifuged at 4°C and washed in 25ml ice cold PBS, followed by recentrifugation. Cells were lysed in 1ml of lysis buffer containing 1% digitonin, 1 tablet (per 10ml) of protein inhibitor cocktail (Boehringer Manneheim), 2mM sodium vanadate, 1mM PMSF, 1mM EDTA, 1mM EGTA, 2mg/ml soybean trypsin inhibitor, 10mM sodium pyrophosphate, 0.1% SDS, 0.1% deoxycholate and 50mM sodium fluoride. Lysing cells were rotated at 4°C for 30 minutes, followed by 15 min. centrifugation at 13krpm, 4°C. Supernatants were precleared by incubation with GST conjugated sepharose (50µl of a 50% slurry) for 1 hour at 4°C while rotating. Solutions were centrifuged for 15 min. at 13krpm, 4°C and the supernatants divided over 2 eppendorf tubes. Pulldowns were performed by addition of GST-14-3-3 (50µl of a 50% slurry) to one of the two tubes. As a control for each sample, sepharose conjugated to GST only (50µl of a 50% slurry) was added to the remaining precleared supernatant. Pulldown and control tubes were incubated at 4°C overnight while rotating, then washed 6 times in PBS, with 5 min., 13krpm centrifugation between each wash. After the final wash, the supernatant was aspirated off the sepharose and 25µl of 2 X SDS reducing load buffer was added to pelleted sepharose, followed by incubation at 100°C for 2 min to remove protein from the sepharose. Recovered proteins were analysed by PAG electrophoresis (Section 2.3.3.2) and western blotting (Sections 2.3.3.3-5).
2.2.3.8 Stripping detection reagents from western blots

Western blotted filters were immersed in 100ml of western stripping solution with freshly added 700µl of βME in a sealed plastic container, followed by 30 min. incubation at 55°C in a shaking waterbath. Filters were then washed four times in TNT at room temperature with rotation, followed by overnight incubation in blocking solution at 4°C.

2.2.4 Bone Marrow Cell Manipulation

2.2.4.1 Extraction of bone marrow from mice

Mice were killed by cervical dislocation, tibia and femur removed by dissection. In a sterile laminar flow hood, where necessary, bones were freed of surrounding meat and with a sterile scalpel blade, were cut approx. 1mm from each end. Bone marrow was flushed with IMDM from tibiae using a 26 gauge needle (Teruma) and from femurs using a 21G needle. Recovered bone marrow cells were counted in a solution of 1% crystal violet using a Neubauer hemocytometer.

2.2.4.2 Cytocentrifugation of bone marrow cells

Bone marrow cells were suspended in a 100ml solution of IMDM/10%FCS containing approx. 7 x 10^4 cells per slide. Cells were centrifuged for 5 min. at 500rpm onto glass slides using a Shandon Cytospin 3 cytofuge.
2.2.4.3 Jenner/Giemsa staining of bone marrow cells

Slide of cytofuged bone marrow cells were stained automatically using a Midas II (Merck) cell stainer. The sequence of staining solutions was as follows: 2 min. fixing in 20% methanol, 2 min. staining incubation Jenner’s stain, 2 min. incubation in 10% Giemsa stain, 30 sec. wash in water followed by 2 min. wash in PBS, pH 7. Slides were air-dried and mounted in CV-mount (Leica, Nussloch, Germany) and sealed with cover-slips.

2.2.4.4 Transduction of bone marrow cells with retroviral constructs

Bone marrow cells from muβc−/−βIL3−/− double knockout 129SV mice were transduced with retrovirus produced by ψ2 cell lines with stable transfections of retroviral constructs (pRufneoIRES) engineered to produced huGMRα and huβc proteins. ψ2 cells were drained of media and washed with PBS, followed trypsinisation in 2.5% trypin solution diluted 1/5 in PBS. ψ2 cells were incubated at room temperature for 5 minutes, followed by recovery of cells by washing with IMDM/10% FCS. Recovered ψ2 cells were centrifuged and pelleted cells resuspended in 10ml of IMDM/10%FCS, followed by irradiation at 30Gy in a CIS Biointernational IBL437 Blood Cell Irradiator. Irradiated ψ2 cells were cultured at 9.6 x 10⁶ cells per 182cm² flask in 22.5ml transduction medium (IMDM, 15% FCS, penicilin, streptomycin, 100ng/ml muSCF and 5µg/ml polybrene). ψ2 cells were cultured at 9.6 x 10⁶ cells per 182cm² flask. Freshly isolated muβc−/−βIL3−/− bone marrow cells were co-cultured with ψ2 cells at 4 - 5 x 10⁶ bone marrow cells per 182cm² flask. ψ2 and bone marrow cells were co-cultured for 48 hours, followed by harvesting of cultured bone marrow cells away from the adhered ψ2 cells. Harvesting was carried out by 3 “bashes” per flask, followed by 5 washes in the transduction media present. Cultured bone
marrow cells were transferred and pooled in a fresh flask and cultured for 24 hours longer to allow any contaminating ψ2 cells to adhere. Recovered bone marrow cells were stained for surface expression of huGMRα and βc proteins (Section 2.3.2.7) and receptor positive cells purified by FACS (Section 2.3.4.5).

2.2.4.5 Purification of receptor positive cells from mouse bone marrow transduced with ψ2 cell retrovirus

Cultures of mouse bone marrow cells from Section 2.3.4.4 were purified of dead cells by Ficoll gradient centrifugation. Cultured bone marrow cells were centrifuged and resuspended in 35ml of IMDM/10% FCS, followed by underlay of 15ml Ficoll (Lymphoprep, Nycomed Pharma AS, Norway, Oslo). Cells were centrifuged for 20 min. at 1500rpm, room temperature with no braking and the cell layer at the media/ficoll interface was removed with a transfer pipette. Cells were washed with 2 changes of 50ml PBS. Recovered cells were stained with 4H1 (anti-huGMRα) at a concentration of 10µg/ml with a 1/10 dilution of normal rabbit serum for 30 min. on ice. Negative control staining was performed with A14 (anti-huIL5Rα) under the same conditions. Cells were washed with PBS and centrifuged. Secondary antibody staining was performed in a 1/50 dilution of anti-mouse-IgG conjugated PE with a 1/10 dilution of normal rabbit serum for 30 min. on ice. Positively staining cells were purified by FACS using a Becton Dickinson FACStarPLUS cell sorter. The sorting profile was set up using uncultured bone marrow cells to determine a gating profile which was optimized for purification of monocytes and macrophages.
2.2.5 Assays for Specific Cellular Functions

2.2.5.1 Assay for production of Reactive Oxygen Species (ROS)

Production of ROS in response to priming by factors and stimulation by fMLP, was measured in both cultured and freshly isolated bone marrow cells. The compound 5(6)-carboxyfluorescein diacetate succinimidylester (5(6)-CFDA,SE) binds to ROS produced by a cell and fluoresces in the FL1 scale (measured at 525nm) once bound, allowing measurement by flow cytometry. 1 x 10^5 cells were used in each of duplicate samples. Cells were placed in 4ml 4DT tubes and were stimulated by addition of factor for 45 minutes or less, in a 37°C shaking waterbath. Factors for stimulation included muGM-CSF, huGM-CSF or huTNFα (positive controls) at varying concentrations. 2nM 5(6)-CFDA,SE and 10^-7M fMLP was added for a further 15 minutes incubation. Cells were washed in ice cold PBS, followed by centrifugation. Pelleted cells were resuspended in 500ml PBS and kept on ice. Measurement of fluorescence in the FL1 scale (extent of ROS production) by flow cytometry was performed immediately, using a Coulter EPICS-XL-MCL. Between 2000 and 5000 events were recorded for each sample.

2.2.5.2 Assay for phagocytosis of opsonised sheep erythrocytes

Cultured bone marrow receptor positive monocyte/macrophages were assayed for their ability to phagocytose opsonised sheep erythrocytes in response to priming by factor. Sheep erythrocytes (IMVS Veterinary services) were opsonised prior to each experiment by 40 min. incubation with 1/200 dilution of anti Sheep-RBC, rotating at room temperature, followed by a wash in PBS and centrifugation at 2500rpm. 10^4 purified receptor positive bone marrow monocyte/macrophages per duplicate sample were placed in
4ml tubes (Falcon catalogue #2063) in IMDM/10% FCS and incubated at 37°C for 60 min. Cells were primed by addition of factor during incubation for time courses up to 60 mins, in a 37°C shaking waterbath. Factors for stimulation included, 100ng/ml huGMCSF and 50ng/ml huTNFα (positive control). 5 x 10⁴ Opsonised sheep erythrocytes were added and cells centrifuged at 100g (Beckman J6 rotor, 750rpm) for 20 sec. Followed by incubation at 37°C for 10 min. with no shaking, to allow phagocytosis to occur. Reactions were stopped by addition of 4ml ice cold PBS and centrifugation. Unphagocytosed erythrocytes were lysed by resuspending pelleted cells in 2mM tris-HCl/0.83% ammonium chloride (made up in MQ) and incubation for 10min. on ice. Lysis was stopped by addition of 4 ml cold PBS and centrifugation. Pelleted cells were resuspended in IMDM/10% FCS and cytofuged (Section 2.3.4.2) then stained with Jenner/Giemsa. Extent of phagocytosis was measured by counting the numbers of monocytes/macrophages with internalised erythrocytes and the numbers of internalised erythrocytes/cell. Erythrocytes which were attached to monocytes/macrophages but not internalised were not counted. At least 500 cells were analysed for each sample.

2.2.5.3 MTS metabloic activity assay

CTL-EN cells incubated in DMEM with 0.1% FCS at a concentration of 5 x 10⁶ cells/ml were stimulated with either 10ng/ml huIL-3 or 10ng/ml mouse IL-2. Metabolic activity was measured using the CellTiter 96 Aqueous one solution cell proliferation assay (Promega) according to the manufacturers instructions.
2.2.5.4 Measurement of apoptosis by flow cytometry

To measure apoptosis by flow cytometry, 1 x 10^5 to 1 x 10^6 cells were incubated for 15 min on ice in the dark in 1ml buffer (10mM HEPES/NaOH pH7.4;140mM NaCl;5mM CaCl2) with 20µl of FITC-labelled annexin V (Boehringer-Mannheim) and 20µl of 50µg/ml propidium iodide. Cells were analysed for fluorescence in the FL1 and FL3 scales using a Coulter EPICS-XL-MCL flow cytometer, with double positive cells considered late apoptotic.

2.2.6 Data analysis

Data were graphed and presented using Deltagraph version 2 (Rockware, Inc, Golden Colorado). Mean and standard deviation are shown on figures. Statistical analyses were performed using Graphpad Prism version 2 software (GraphPad Software Inc., San Diego, CA). One-way analyses of variance (ANOVA) were performed using the Tukey’s multiple comparisons test for differences between samples.
Chapter 3: Site-Specific Serine Phosphorylation of $\beta_c$ leads to 14-3-3 Association with a motif required for survival of CTL-EN cells

3.1 Introduction

The aim of Chapter 3 was to examine the regulation and functional significance of an interaction between hu$\beta_c$ and the signalling molecule 14-3-3.

The recruitment of proteins containing SH2 and PTB domains to phosphotyrosine residues on receptors has become recognised as a central mechanism in signal transduction. These phosphotyrosine binding proteins are either direct mediators of signalling (e.g. PLC-$\gamma$ (Zhu et al., 2001)), or act as adapters (e.g. SHC (Ravichandran et al., 1995)) or SHP2 (Li et al., 1994)) to recruit further signalling components. Ligand activation of the GM-CSF, IL-3 or IL-5 receptors results in recruitment of SH2 and PTB domain containing proteins to the hu$\beta_c$ which leads to signal transduction. However, experiments performed in our laboratory (Guthridge et al., 1998) and others (Okuda et al., 1997; Itoh et al., 1998) have shown that a mutant hu$\beta_c$ in which all eight cytoplasmic tyrosines are substituted for phenylalanine (hu$\beta_c^{F8}$) is able to support cell survival in haemopoietic cell lines in response to GM-CSF (data not shown) and IL-3 (Figure 3.1). The myeloid cell line BA/F3, stably transfected with receptors for huIL-3 (huIL-3R$\alpha$ and wildtype hu$\beta_c$ or hu$\beta_c^{F8}$), was cultured in the presence of huIL-3. The data in Figure 3.1 show that cells expressing hu$\beta_c^{F8}$ were able to survive in response to IL-3, albeit with a reduced efficiency compared to cells expressing wildtype hu$\beta_c$. However, these BA/F3 cells also expressed the mouse $\beta_c$ and therefore it is possible that IL-3 was able to signal through a complex of huIL-3R$\alpha$ and mouse $\beta_c$, as demonstrated in the experiments of McClure et al., (2001a). Indeed, later
Figure 3.1 BA/F3 cultured with IL-3 lines expressing huβc F8 exhibit survival deficiencies compared to lines expressing wildtype huβc. BA/F3 cells stably transfected to express huIL-3Rα and wildtype huβc or huβc F8 mutant were cultured at 1 x 10^4 cells/ml in triplicate, in the presence or absence of huIL-3, over a timecourse of 4 days. On each day the number of live cells per ml were counted using the trypan blue exclusion method (Section 2.3.2.7).
Chapter 3: Site-Specific Serine Phosphorylation of \( \beta_c \) leads to 14-3-3 Association with a motif required for survival of CTL-EN cells

Table 3.1 Comparison of potential 14-3-3 binding sites in the hu\( \beta_c \), mu\( \beta_c \) and mu\( \beta_{IL-3} \). Putative phosphoserines are underlined.

<table>
<thead>
<tr>
<th></th>
<th>motif 1</th>
<th>motif 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>hu( \beta_c )</td>
<td>582<strong>HSR</strong>S_LP587</td>
<td>820<strong>RSK</strong>PSSp826</td>
</tr>
<tr>
<td>mu( \beta_c )</td>
<td>580<strong>QSH</strong>S_LP585</td>
<td>804<strong>HSK</strong>PPSp810</td>
</tr>
<tr>
<td>mu( \beta_{IL-3} )</td>
<td>580<strong>QSH</strong>S_LP585</td>
<td>804<strong>HSK</strong>PPSp810</td>
</tr>
</tbody>
</table>

Experiments have shown that CTL-EN cells, with no endogenous mouse \( \beta_c \), are incapable of transmitting proliferating signals via a hu\( \beta_c \)\(^ {18} \) mutant receptor (Guthridge, unpublished data). Regardless, the results of Figure 3.1 indicate the interesting possibility that \( \beta_c \) utilises phosphotyrosine-independent mechanisms for the regulation of at least some biological responses such as survival. We therefore examined whether there were any recognisable phosphotyrosine-independent motifs that may be activated during signalling through the \( \beta_c \). 14-3-3 proteins, predicted to act as adaptors, chaperones or scaffolds (described in Section 1.5.4.5 and Table 1.2), bind to phosphoserine and phosphothreonine motifs. We identified two putative 14-3-3 binding sites in the cytoplasmic region of the hu\( \beta_c \). These sites are 582**HSR**S\_LP587 (designated motif 1) and 820**RSK**PSSp826 (designated motif 2) where putative phosphoserines are underlined, (Table 3.1). These motifs were identified on the basis of their similarity to the 14-3-3 binding consensus described in Section 1.5.4.5 and Table 1.2. Conserved motifs were also identified in the mu\( \beta_c \) and mu\( \beta_{IL-3} \), (Table 3.1), although the sequence similarity of the mouse motif 2 was poor, given that the spacing between serine residues is larger than any previously described 14-3-3 binding site (Table 1.2).
To investigate the possibility of phosphorserine dependent $\beta_c$ signalling through
recruitment of 14-3-3, we performed pulldown experiments to ask if 14-3-3$\zeta$ could interact
with hu$\beta_c$ in vitro. For these experiments, HEK 293 cells were transiently transfected with
constructs expressing either the wildtype hu$\beta_c$ or hu$\beta_c$ mutants with substitutions of
putative 14-3-3 binding motifs (motif 1: hu$\beta_c^{582}$HSRSLP$^{587}$, mutated to EFAAAA; motif 2:
hu$\beta_c^{820}$RSKPSSP$^{826}$, mutated to EFAAAAA). GST-14-3-3$\zeta$-sepharose is able to pull-down
wildtype hu$\beta_c$ and hu$\beta_c^{820}$EFAAAAA$^{826}$ (motif 2 mutant), but not hu$\beta_c^{582}$EFAAAA$^{587}$ (motif 1
mutant) (Figure 3.2). These results indicate that GST-14-3-3$\zeta$ is able to bind hu$\beta_c$ and that
motif 1 is likely to be the primary site of interaction.

While these experiments demonstrate an in vitro interaction in lysates derived from
fibroblasts, it was not known if this interaction also occurred in haemopoietic cells.
Furthermore, it was not known whether ligand stimulation regulated the phosphorylation of
serine 585 or recruitment of 14-3-3 to hu$\beta_c$. We were also interested in whether the
interaction between 14-3-3 and the $\beta_c$ was a general event in signalling, e.g. conserved
between species. Most importantly, a role for this interaction in the functioning of
haemopoietic cells was investigated. These specific issues and questions are addressed in
the following aims; 1) To determine if 14-3-3$\zeta$ interacts with hu$\beta_c$ in the context of
haemopoietic cells, 2) To determine if 14-3-3$\zeta$ interaction with hu$\beta_c$ is regulated by
cytokine, 3) To determine if this interaction occurs in vivo, by co-immunoprecipitation
experiments, 4) To determine the generality of this interaction by asking if it is conserved
in evolution, and 5) To determine a role in haemopoietic cell function for this interaction.
Figure 3.2 14-3-3 and huβc interact exclusively through huβc motif 1 in vitro. Pulldowns were performed using transiently transfected HEK293 cell lysates to show huβc interaction with GST-14-3-3ζ. Upper panel: GST-14-3-3ζ-sepharose was used to pulldown interacting proteins from lysates of untransfected (UT) HEK293 cells (control, lane 1) or transiently transfected HEK293 cells expressing wildtype huβc (lane 2), huβc S581EFAAAA (lane 3) or huβc S820EFAAAAS26 (lane 4). ‘Captured’ proteins were subject to PAGE and immunoblotting analysis with mouse-anti-huβc, followed by goat-anti-mouse-HRP secondary antibodies. Lower panel: anti-huβc Western blot of the lysates used in this experiment, showing expression of the huβc from transiently transfected constructs.
3.2 Production of CTL-EN cell lines expressing wildtype huβc, huβc<sub>F8</sub> or huβc containing substitution mutations of the 14-3-3 binding site.

3.2.1 Construction of mutant huβc cDNA expression constructs

While the results of Figure 3.2 would indicate that the HSRSLP motif in huβc was important in the binding of 14-3-3ζ, the mutant used for these experiments (huβ<sub>c</sub><sup>581EFAAAA587</sup>) encompassed the entire 14-3-3 binding site. To better characterise the binding site for 14-3-3 a point mutant huβ<sub>c</sub><sup>S585A</sup> was generated, in which the putative phosphoserine residue was substituted. This construct was cloned into the expression vector pReCMVpuro (Figure 2.1) and was transiently transfected into HEK 293 cells. Western blot analysis indicated that the huβ<sub>c</sub><sup>S585A</sup> protein was expressed in cells, however cell surface expression was not detected by flow cytometry (data not shown). For this reason, site-directed mutagenesis and subcloning were used to make additional point mutations of the 14-3-3 binding site in huβc. huβ<sub>c</sub><sup>S585G</sup> and huβ<sub>c</sub><sup>584AAA586</sup> were constructed to study the importance of the serine 585 for the 14-3-3ζ/huβc interaction, phosphorylation on serine 585 and the role of serine 585 in GM-CSF regulation of cellular functions. To make these mutant constructs, huβc cDNA in the pBluescript vector was obtained. Site-directed mutagenesis was performed, based on the Quikchange™ method (Sections 2.2.1.12 and 2.2.1.13) to create huβ<sub>c</sub><sup>S585G</sup> and huβ<sub>c</sub><sup>584AAA586</sup>. Mutations introduced into the huβc cDNA are described in Table 4.1. Wildtype and mutant huβc cDNAs were subcloned into pReCMVpuro for transfection into cell lines (Section 2.2.1.14).
3.2.2 Production of cell lines expressing wildtype huβc, huβcF8 or huβc containing substitution mutations of the 14-3-3 binding site

The pull-down experiment described in Figure 3.2 indicates that 14-3-3ζ can associate with the huβc and this association occurs through the \(^{582}\text{HSRSLP}^{587}\) motif (motif 1). However, while we were able to observe this interaction in a fibroblastic cell line, it was important to demonstrate the presence of this interaction in haemopoietic cell lines. For these studies we used CTL-EN subline of the mouse IL-2-dependent, CTLL-2 T-cell line to study the proliferative and survival response to GM-CSF, and the M1 murine leukemic cell line, which proliferates upon IL-3 stimulation but differentiates into macrophages upon GM-CSF stimulation (Smith et al., 1997). In addition to being able to test the biochemical interaction of 14-3-3ζ with the huβc in a haemopoietic context, these cell lines would also allow us to examine a number of biological responses to wildtype and mutant βc, which could include cell proliferation and survival.

CTL-EN cells are dependent on muIL-2 for growth and survival. Previously it has been shown that these cells are able to proliferate and survive in the presence of huGM-CSF or huIL-3 following expressing of either the huGMR or huIL-3R respectively (data not shown). CTL-EN cells expressing huIL-3α were transfected with pRcCMVpuro-huβc constructs and transfected cells selected for by growth in puromycin. CTL-EN lines were constructed which stably express huIL-3α, in combination with: wildtype huβc, huβcSS8SG, huβcS84AAA586, huβcS82EFAAA587 or huβcF8. Expression of huIL-3Rα and each huβc were confirmed by Western blotting (data not shown) and analysis by flow cytometry (Figure 3.3).
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Figure 3.3 Surface expression of huIL3Rα and huβc (wildtype or mutants), on CTL-EN cell lines. Cells were incubated with antibodies to detect surface expression of; huIL-3Rα with the monoclonal antibody 9F5 (purple), huβc with the monoclonal antibody 1C1 (red) or as a negative control, the monoclonal antibody 12CA5, (anti-HA-tag, black). Cells were then incubated with the secondary antibody sheep-anti-mouse-FITC. Flow cytometry was used to demonstrate cell surface expression of huIL-3Rα and wildtype or mutant huβc. A; wildtype huβc, B; huβc S585G, C; huβc 584AAA586, D; huβc F8, E; huβc S585G.
3.3 Development and characterisation of a polyclonal antibody which recognises phosphorylated serine 585 of huβc

The previous section describes the production of CTL-EN lines which express the wildtype huβc, huβc F8 and huβc mutants where the 14-3-3 binding motif is substituted. These lines can be used to examine the regulation of phosphorylation of 14-3-3 binding site residues and the association of the huβc with 14-3-3. Firstly we wished to study the regulation of phosphorylation of huβc serine 585, as 14-3-3 has been shown to bind phosphorylated serine residues. For this purpose, we have generated an antibody that recognises the 14-3-3 binding site of huβc, S82HSRSLP ε 587, when serine 585 is phosphorylated. This polyclonal antibody was raised against a huβc peptide in the laboratory of our collaborators Dr. M. Berndt, by Dr. R. Andrews (Monash University, Victoria, Australia). Presented here are experiments which characterise this antibody in terms of its ability to recognise the 14-3-3 binding motif when serine 585 is phosphorylated.

3.3.1 Production of a polyclonal antibody raised against a human βc phosphoserine 585 peptide

New Zealand white rabbits were immunised with a KLH-coupled peptide encompassing serine 585 and the 14-3-3 binding motif in the huβc. This 15 aa peptide (named phosphoSer585), is phosphorylated on serine 585 and its sequence is shown in Table 3.2. After immunisation and two booster injections, rabbits were bled and antibodies specific for phosphoserine 585 were purified from serum using a two step process. Firstly, serum was passed over a phospho-Ser585 peptide column. The bound fraction was eluted and subsequently passed over a non-phospho-Ser585 peptide column (see Table 3.2) column and the
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### Table 3.2 Peptides utilised in the production, purification and specificity testing of the affinity purified antibodies

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Motif</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho-Ser585</td>
<td>motif 1</td>
<td>H-CLGPPHSRSLPDLG-OH</td>
</tr>
<tr>
<td>non-phospho-Ser585</td>
<td>motif 1</td>
<td>H-CLGPPHSRSLPDLG-OH</td>
</tr>
<tr>
<td>phospho-Ser824</td>
<td>motif 2</td>
<td>H-CPLSLRSKSPSGPGP-OH</td>
</tr>
</tbody>
</table>

1 Peptides encompass motifs 1 or 2 of the hu$\beta_c$, plus surrounding amino-acids.
2 Phosphorylated serine residue is underlined, where present.

Flow-through fraction was recovered. Antibody recovered in this flow-through fraction was then tested for its ability to specifically recognise phospho-Ser585. These experiments were performed by our collaborators Dr. M. Berndt and Dr. R. Andrews.

#### 3.3.2 Demonstration of the specificity of the affinity-purified antibodies

##### 3.3.2.1 The affinity-purified antibodies recognise only the phospho-Ser585 peptide on dot-blots

It was necessary to determine if the affinity-purified antibodies were able to specifically recognise the 14-3-3 binding motif in hu$\beta_c$ when serine 585 was phosphorylated. We firstly performed a Western dot-blot experiment using the peptides phospho-Ser-585, non-phospho-Ser585 and phospho-Ser824 (Table 3.2). As shown in Figure 3.4A, the affinity-purified antibodies specifically recognised phospho-Ser-585, but not non-phospho-Ser585 or phospho-Ser824 (Figure 3.4A) peptide. In further experiments, the affinity-purified antibodies failed to recognise a peptide consisting of a scrambled 14-3-3 binding motif or a non-phosphorylated peptide encompassing motif 2 (data not shown). These experiments
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Fig 3.4 anti-phospho-S585βc is specific for phospho-S585huβc. A The peptides phospho-Ser585, non-phospho-Ser585 and phospho-Ser824 (Table 3.2) were dot-blotted onto nitrocellulose filters. Filters were probed with rabbit-anti-phospho-S585βc followed by the secondary antibody goat-anti-rabbit-HRP, then signals were detected by ECL. B Wildtype huβc was immunoprecipitated from transiently transfected HEK cell lysates. One quarter of the total immunoprecipitated protein was subject to electrophoresis on each of four lanes on a PAG. Western transfers were probed with anti-phospho-S585βc in the presence of 100× excess of the indicated peptide, to antibody molecule. This was followed by probing with the secondary antibody goat-anti-rabbit-HRP and signals detected by ECL. C Wildtype huβc or huβcS585A was immunoprecipitated from transiently transfected HEK cell and electrophoresed on a PAG, followed by transfer onto filters. Filters were probed with anti-phospho-S585βc, followed by the secondary antibody goat-anti-rabbit-HRP and signals detected by ECL. Filters were then stripped and then reprobed with the monoclonal antibody 1C1 (anti-huβc), followed by the secondary antibody goat-anti-mouse-HR and signal detection by ECL.
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3.3.2.2 The affinity-purified antibodies recognise hu$\beta_c$ on Western blots, and this interaction is competed out by excess phospho-Ser585 peptide

It was next important to determine if the affinity-purified antibodies recognise the 14-3-3 binding motif within the context of full length hu$\beta_c$ expressed by transfected cells. A wildtype hu$\beta_c$ expression construct was transiently transfected into HEK 293 fibroblasts. hu$\beta_c$ was immunoprecipitated from these cells and transferred onto filters. As shown in Figure 3.4B, lane 1, the affinity-purified antibodies recognised hu$\beta_c$ protein. This recognition is competed out by 100 times molar excess of phospho-Ser585 peptide (Figure 3.4B lane 2), but not non-phospho-Ser585 or phospho-Ser824 peptides (Figure 3.4B, lanes 3 and 4). These results suggest that the affinity-purified antibodies recognise the hu$\beta_c$ 14-3-3 binding motif, only when serine 585 is phosphorylated.

3.3.2.3 The affinity-purified antibodies recognise wildtype hu$\beta_c$ but not hu$\beta_c^{S585A}$ on Western blots

As a further demonstration of the specificity of the affinity purified antibodies, the absolute requirement of serine 585 for the recognition of hu$\beta_c$ was determined. HEK-293 cells were transiently transfected to express wildtype hu$\beta_c$ or hu$\beta_c^{S585A}$. Immunoprecipitated hu$\beta_c$ from these cells was transferred onto filters. Figure 3.4C shows that the affinity purified antibodies recognise only wildtype hu$\beta_c$, not hu$\beta_c^{S585A}$ (of which
the protein expression is detected by Western blotting but expression is not detected on the
surface of the cells by flow cytometry).

Together, these results have characterised the specificity of this novel tool for the detection
of serine 585 phosphorylated huβc. This affinity purified antibody, hereafter termed anti-
phospho-S585 βc, recognises only phosphorylated serine 585 of the huβc.

3.4 Phosphorylation on huβc serine 585 is upregulated by GM-CSF or IL-3 stimulation in cell lines

The specificity of the anti-phospho-S585 βc antibody puts us in a unique position to
determine the regulation of phosphorylation of a single serine residue of the huβc, in
haemopoietic cells in response to cytokine stimulation. We therefore examined the
regulation of serine 585 phosphorylation in response to IL-3 or GM-CSF signalling. For
these studies, we utilised CTL-EN lines which express wildtype huβc and mutant huβc
(Section 3.2) in combination with the huIL-3Rα, and the murine leukaemic cell line M1,
transfected with huGMRα and huβc.

3.4.1 huIL-3 upregulates huβc serine 585 phosphorylation in CTL-EN cells

CTL-EN cells expressing huIL-3Rα and wildtype or huβcS82EΦAAAA587 were stimulated with
huIL-3 over a short timecourse. huβc was immunoprecipitated from these cells and
transferred onto filters. The phosphorylation state of serine 585 was determined by probing
the filters with anti-phospho-S585 βc. huIL-3 stimulation resulted in a transient upregulation
of phosphorylation on serine 585 of huβc (Figure 3.5A, top left panel). However, serine
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585 phosphorylation could not be detected in cells expressing hu\( \beta \)c\textsuperscript{582FEAAAA587}, the mutant in which the 14-3-3 binding site is substituted (Figure 3.5A, top middle panel). In each of these experiments, Western blots were stripped and reprobed for total hu\( \beta \)c to show equal loading each lane (Figure 3.5, lower panels).

3.4.2 hu\( \beta \)c tyrosine phosphorylation is not required for IL-3 stimulated increase in hu\( \beta \)c serine 585 phosphorylation

To ask if hu\( \beta \)c tyrosine phosphorylation is required for serine 585 phosphorylation to occur in response to ligand stimulation, hu\( \beta \)c was immunoprecipitated from CTL-EN cells expressing IL-3\( \alpha \) and hu\( \beta \)c\textsuperscript{F8}, which is not tyrosine phosphorylated by IL-3 stimulation. Western blotting of these immunoprecipitates with anti-phospho\( \beta \)c\textsuperscript{585} showed that hu\( \beta \)c serine 585 phosphorylation was not disrupted by substitution of all intracellular tyrosine residues (Figure 3.5A, top right panel). The absence of IL-3 stimulated hu\( \beta \)c tyrosine phosphorylation was confirmed by immunoblotting with an anti-phosphotyrosine antibody (Figure 3.5B, right panel). These results demonstrate that hu\( \beta \)c tyrosine phosphorylation is not required for serine 585 phosphorylation. However, hu\( \beta \)c tyrosine phosphorylation may be important in the down regulation of serine 585 phosphorylation as the signal persists for a longer time.
Fig 3.5 Phosphorylation on serine 585 of the huβc is upregulated by huIL-3 stimulation

A. CTL-EN cells expressing huIL-3Rα and huβc (either wildtype, huβc<sup>582EFAAAA587</sup> or huβc<sup>F8</sup>) were starved of factor in 0.1% FCS for 4 hours, followed by stimulation with 50ng/ml huIL-3 for the indicated times. huβc was immunoprecipitated with anti-huβc monoclonal antibodies (1C1), from each sample. Immunoprecipitates were subject to electrophoresis and immunoblotting analysis with rabbit-anti-phospho-S585βc antibodies, followed by goat-anti-rabbit-HRP secondary antibodies, and detection by ECL. Filters were then stripped and reprobed with anti-huβc (1C1) antibodies, followed by goat-anti-mouse-HRP, and detection by ECL. This result is representative of at least three similar experiments.

B. Separate samples of the same cell lines were starved and stimulated as described in A. huβc was immunoprecipitated with the anti-huβc monoclonal antibody 1C1, from each sample. Immunoprecipitates were subject to electrophoresis and immunoblotting analysis with monoclonal anti-phosphotyrosine antibodies (4G10), followed by goat-anti-mouse-HRP, and detection by ECL. This is representative of at least three similar experiments.
3.4.3 huβc serine phosphorylation is not required for huβc tyrosine phosphorylation in response to IL-3

To ask if huβc serine 585 phosphorylation is required for huβc tyrosine phosphorylation in response to ligand stimulation, huβc was immunoprecipitated from CTL-EN cells expressing IL-3Rα and wildtype or huβc 582EFAAAA587. Western blotting of these immunoprecipitates with an anti-phosphotyrosine antibody showed no detectable difference in the phosphotyrosine response of the huβc 582EFAAAA587 compared to wildtype huβc (Figure 3.5B, panel 2 compared to panel 1). These results demonstrate that huβc serine 585 phosphorylation is not required for tyrosine phosphorylation.

3.4.4 huGM-CSF upregulates serine 585 phosphorylation in M1 cells

In the experiments described in this section, we examine huGM-CSF regulation of huβc serine 585 phosphorylation in the M1 murine leukemic cell line, with stable transfections that express the huGMRα and huβc. M1 cells can be used to study some functional outcomes of signalling, as they differentiate in the presence of huGM-CSF (Smith et al., 1997).

M1 cells were stimulated with huGM-CSF over a timecourse from 1 to 60 min. huβc was immunoprecipitated from these cells and transferred onto filters. The phosphorylation state of serine 585 was determined by probing the filters with anti-phospho-585 βc. huGM-CSF stimulation results in a transient upregulation of phosphorylation on serine 585 of huβc.
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(Figure 3.6, upper panel). Equal loading of hu\( \beta_\text{c} \) was demonstrated by stripping and reprobing the same membrane to show total hu\( \beta_\text{c} \) protein (Figure 3.6, lower panel).

Our data has therefore demonstrated, for the first time, that cytokine stimulation can lead to a transient upregulation of phosphorylation of a specific \( \beta_\text{c} \) serine residue. Stimulation of hu\( \beta_\text{c} \) with either huIL-3 or huGM-CSF leads to the transient phosphorylation of serine 585, which lies in a motif necessary for 14-3-3 association in our pulldown experiments (Figure 3.2). This phosphorylation event occurs in transfected CTL-EN (Figure 3.5) or M1 (Figure 3.6) cell lines, indicating that it is a general hu\( \beta_\text{c} \) signalling event and therefore may have functional importance. However, 14-3-3\( \zeta \) association has only been demonstrated \textit{in vitro} (pull-down experiments) in the non-haemopoietic cell line HEK 293. To determine if this regulated phosphorylation on serine 585 indeed leads to regulated 14-3-3 association in haemopoietic cells, we needed to perform co-immunoprecipitation experiments.

3.5 Phosphoserine 585 is required for 14-3-3 co-immunoprecipitation with the hu\( \beta_\text{c} \) in response to IL-3 stimulation

We have shown that stimulation of the hu\( \beta_\text{c} \) with huIL-3 or huGM-CSF leads to an upregulation of serine 585 phosphorylation in the CTL-EN and M1 cell lines. In this section we ask two important questions: Does 14-3-3 co-immunoprecipitate with the hu\( \beta_\text{c} \) via serine 585, in factor-dependent haemopoietic cells?; and, is this association regulated by cytokine stimulation?
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Fig 3.6 Phosphorylation on serine 585 of the hu$\beta_c$ is upregulated by huGM-CSF stimulation. M1 myeloid leukemic cells expressing huGMR$\alpha$ and hu$\beta_c$ were starved of factor in 0.1% FCS for 4 hours, followed by stimulation with 10ng/ml huGM-CSF for the indicated times. hu$\beta_c$ was immunoprecipitated with the anti-hu$\beta_c$ monoclonal antibodies (1C1), from each lysate. Immunoprecipitates were subject to electrophoresis and immunoblotting analysis with rabbit-anti-phospho-$^{S585}\beta_c$ antibodies, followed by goat-anti-rabbit-HRP secondary antibodies, and detection by ECL. Filters were then stripped and reprobed with anti-hu$\beta_c$ antibodies, followed by goat-anti-mouse-HRP, and detection by ECL. hu$\beta_c$ appears as two bands in Western blots of M1 cells which represent differently glycosylated forms of the hu$\beta_c$ (Dr. T. Hercus, unpublished data). This result is representative of two similar experiments.
The association of 14-3-3 with the huβc was examined in CTL-EN cells (expressing huIL-3Rα and wildtype huβc, huβc\textsuperscript{582EFAAAA587} or huβc\textsuperscript{F8}, in response to huIL-3 stimulation. For these experiments, cells were starved of factor in 0.1%FCS for 4 hours, then stimulated with huIL-3 over a short timecourse, huβc was immunoprecipitated from lysates. Immunoprecipitates were the subject to PAGE and transfer onto membranes, followed by sequentially immunoblotting with anti-14-3-3 and anti-huβc antibodies. It is clear from Figure 3.7 (left panels) that 14-3-3 co-immunoprecipitates with the huβc and that this association is up-regulated by huIL-3 stimulation. 14-3-3 did not co-immunoprecipitate with huβc\textsuperscript{582EFAAAA587} (Figure 3.7 middle panels), demonstrating that the interaction between 14-3-3 and huβc is mediated by the \textsuperscript{582}HSRSLP\textsuperscript{587} motif. It was also demonstrated that 14-3-3 is recruited to the huβc\textsuperscript{F8} mutant upon IL-3 stimulation (Figure 3.7 right panels), demonstrating that intracellular huβc tyrosine residues of the huβc are not necessary for this interaction. Interestingly 14-3-3 recruitment to huβc\textsuperscript{F8} was delayed compared to recruitment to wildtype huβc.

We have now shown that cytokine stimulation leads to the upregulation of phosphorylation of huβc serine 585, and the adapter protein 14-3-3 is recruited to this site upon cytokine stimulation. Upregulation of serine 585 phosphorylation as well as 14-3-3 recruitment to this site occurred in the absence of tyrosine phosphorylation. This novel βc signalling event is therefore a candidate for the phosphotyrosine-independent βc regulation of cellular functions such as promotion of survival, since it lies in a huβc region required for survival (residues between 544 and 626 (Section 1.5.5.2 and Figure 1.5)). To further characterise this signalling pathway, we asked if it was conserved from human to
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Figure 3.7 14-3-3 and hu\( \beta_c \) interact in vivo. CTL-EN cells stably transfected with huIL-3R\( \alpha \) and either wildtype hu\( \beta_c \), hu\( \beta_c^{582EFAAAA587} \) or hu\( \beta_c^{F8} \) were starved of factor in 0.1% FCS/DMEM for 4 hours. 8 x 10^7 cells were stimulated with 50ng/ml huIL-3 for the indicated times. Cells were then lysed, hu\( \beta_c \) immunoprecipitated (IP) and the immunoprecipitates subject to PAGE and immunoblotting analysis. The lower molecular weight half of the filter was probed with anti-14-3-3\( \beta \) antibodies (Santa Cruz, SC629, K19), followed by goat-anti-rabbit-HRP secondary antibodies and detection by ECL. The higher molecular weight half of the filter was probed with biotin-conjugated anti-hu\( \beta_c \) antibodies (1C1) followed by streptavidin conjugated HRP and detection by ECL. The result is representative of at least three similar experiments.
mouse. Conservation of the $\beta_c/14\text{-}3\text{-}3$ interaction between species would indicate a general mechanism in $\beta_c$ signalling.

### 3.6 14-3-3 is recruited to the mouse $\beta_c$ at serine 583

As described in Section 3.1, there exists an equivalent of the hu$\beta_c$ motif $^{582}\text{HSRSLP}^{587}$ in both the mu$\beta_c$ and mu$\beta_{IL-3}$ receptor subunits (see Table 3.1). In the mu$\beta_c$, serine 583, in the motif $^{580}\text{QSHSLP}^{585}$, is the equivalent of serine 585 of the hu$\beta_c$. In this section we examined the potential for an in vitro interaction between 14-3-3 and the mu$\beta_c$, to determine the generality of the $\beta_c/14\text{-}3\text{-}3$ interaction.

#### 3.6.1 Expression of wildtype and mutant mu$\beta_c$ in COS cells

To ask if 14-3-3 interact with mu$\beta_c$ in COS cells, constructs were made for the expression of wildtype mu$\beta_c$, or a mutation of mu$\beta_c$ where the potential 14-3-3 binding site is substituted. mu$\beta_c$ was subcloned into pBluescript (Section 2.2.1.15) and site directed mutagenesis was performed by the Quikchange method (Sections 2.2.1.12 and 2.2.1.15), to create mu$\beta_c^{S583G}$ and mu$\beta_c^{582\text{AAA}}$. The first mutation substitutes serine 583 of the mu$\beta_c$, the equivalent of hu$\beta_c$ serine 585, and the second mutation, mu$\beta_c^{582\text{AAA}}$, substitutes three amino acids, centred around mu$\beta_c$ serine 583. Mutations introduced into the mu$\beta_c$ cDNA are described in Table 4.1. Wildtype and mutant mu$\beta_c$ cDNAs were subcloned into the expression vector pPGK (Section 2.2.1.16). Transcription of mu$\beta_c$ cDNA from this construct was driven by the mouse phosphoglycerate kinase (PGK) promoter (Takagi et al., 1995). COS fibroblasts were transiently transfected with pPGK-mu$\beta_c$ constructs to express wildtype mu$\beta_c$, mu$\beta_c^{S583G}$ or mu$\beta_c^{582\text{AAA}}$. Expression of these receptors in COS cells was monitored by Western blotting.
confirmed by Western blotting (Figure 3.8) and flow cytometry (Figure 3.9). Although little surface expression of these muβc constructs was detected on COS cells, protein levels were easily detected by Western blotting. The lack of surface staining did not impact on the pull-down experiments described in Section 3.6.2 as these protein interactions occur after cell lysis, and do not require surface expression.

3.6.2 14-3-3 interacts with the mouse βc at serine 583 in COS cell lysates

To determine if 14-3-3 and muβc interact in COS cell lysates, pull-down experiments were performed using GST-14-3-3ζ-Sepharose (Section 2.2.3.8). GST-14-3-3ζ binds wildtype muβc, but not muβcS583G or muβcS82AAA584 from COS cell lysates (Figure 3.10, top panel). These results indicate that GST-14-3-3ζ is able to bind muβc via serine 583.

To determine whether this interaction could occur in haemopoietic cells with endogenous expression of muβc, the same experiments were performed using an untransfected cell line, FDCP1. FDCP1 are a cytokine-dependent myeloid cell line which express endogenous muGMR and muIL-3R and proliferate in the presence of either GM-CSF or IL-3. GST-14-3-3ζ pulled down endogenous muβc, expressed by FDCP1 cells (Figure 3.10, lower panel).

Unfortunately the anti-phospho-S585βc antibody did not recognise serine 583 of the muβc (data not shown), therefore analysis of the phosphorylation state of muβc serine 583 was not possible.

By demonstrating that overexpressed and endogenously expressed muβc from cell lysates can interact with 14-3-3ζ, these experiments indicate that 14-3-3 recruitment to serine 585
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Fig 3.8 Expression of wildtype μβc and mutant μβc in COS cells and FDCP1 cells, demonstrated by Western blotting. Lane 1: Lysates were made of FDCP1 (FD) cells expressing endogenous μβc and were subject to PAGE and immunoblotting analysis with rabbit-anti-μβc, followed by secondary staining with goat-anti-rabbit-HRP. Secondary antibody was detected by ECL. Lanes 2 to 5: COS cells transiently transfected to express wildtype μβc, μβc582AAA584 or μβcS583G were lysed. These lysates were analysed as for lane 1. lane 2: wildtype μβc, lane 3: μβc582AAA584, lane 4: μβc583G by immunoblotting as described above. Lane 5 contains untransfected COS cell lysates as a negative control.
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Fig. 3.9 Surface expression of wildtype and mutant mu\( \beta \) on transfected COS cells

COS cells were incubated with antibodies to detect surface expression of mu\( \beta \) with the rat-anti-mu\( \beta \) monoclonal antibodies (Santa Cruz, SC-678, K17). Cells were then incubated with sheep-anti-rat-FITC secondary antibodies. Flow cytometry was used to demonstrate cell surface expression of wildtype or mutant mu\( \beta \). The black negative control line is rat-anti-mu\( \beta \) staining of untransfected COS cells and the purple line mu\( \beta \) (wildtype or mutant) staining in transfected lines. A; wildtype mu\( \beta \), B; mu\( \beta \)^{582AAA584}, C; hu\( \beta \)_{c}^{S583G}
Fig. 3.10 14-3-3 and mυβc interact in vitro. GST-14-3-3ζ pull-downs were performed on huβc from FD cell lysates (with endogenous mυβc) and transiently transfected COS lysates to show mυβc interaction with GST-14-3-3ζ. **Upper panel:** COS cells were transiently transfected to express wildtype mυβc, mυβc\(^{S583G}\) or mυβc\(^{582AAA584}\). Pull-down experiments were performed using GST-14-3-3ζ-sepharose or GST-sepharose (negative control). ‘Captured’ proteins were subject to PAGE and immunoblotting analysis with rat-anti-mυβc (Santa Cruz, SC-678), followed by goat-anti-rabbit-HRP secondary antibodies and ECL detection. **Lower panel:** Proteins were pulled-down from untransfected COS cells and FDCP1, cells as described above.
is conserved from human to mouse βc. The possible conservation of 14-3-3ζ/βc interaction in two different organisms indicates that recruitment of 14-3-3 may be a general event in βc signalling.

3.7 The 14-3-3 binding motif of huβc is required for IL-3 supported survival of transfected CTL-EN cell lines by suppressing apoptosis

The experiments presented in this Section ask if the 14-3-3 binding motif of huβc is necessary for IL-3-supported cell survival. There were three reasons to believe that this might be so: 1) The 14-3-3 binding motif of huβc lies in a region required for survival, from huβc 544 to 626 (Kinoshita et al., 1995; Inhorn et al., 1995) and described in Section 1.5.5.2 and Figures 1.5, 2). The signalling event/s responsible for survival of BA/F3 cells are independent of huβc intracellular tyrosine residues (Figure 3.1, (Guthridge et al., 1998; Okuda et al., 1997; Itoh et al., 1998), and 3) Serine 585 phosphorylation and 14-3-3ζ recruitment to the βc occur independently of tyrosine phosphorylation and therefore are candidates for signalling events required for cell survival.

3.7.1 The 14-3-3 binding motif of huβc is required for cellular viability in trypan blue assay of transfected CTL-EN cells in response to IL-3

CTL-EN cells expressing huIL-3Rα and either wildtype huβc or huβc^{582EFAAAA587} were cultured in the presence of the control cytokine IL-2, which promotes survival through endogenous IL-2 receptors, or IL-3, signalling through transfected receptor. IL-3 was able to maintain viability of cells expressing wildtype huβc, as measured by counting of trypan blue negative cells over 3 days of culture in media containing 0.1% FCS. Cells expressing huβc^{582EFAAAA587} were deficient in viability in response to IL-3 (Figure 3.11A). Survival of
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**Figure 3.11** The 14-3-3 binding motif of the hu\( \beta \) is required for cell viability and metabolic activity in CTL-EN cell lines. **A** CTL-EN cells expressing huIL-3R\( \alpha \) and either wildtype hu\( \beta \) or \( \text{hu}\beta_582EFAAAA587 \) were incubated at a concentration of 5 x 10^5 cells/ml in DMEM containing 0.1% FCS and either 10ng/ml IL-3 or 10ng/ml IL-2. Viable cells were counted using the trypan blue exclusion method over 3 days to indicate cellular viability (Section 2.2.2.7). **B** CTL-EN cells expressing huIL-3R\( \alpha \) and either wildtype hu\( \beta \) or \( \text{hu}\beta_582EFAAAA587 \) were plated in DMEM containing 0.1% FCS and either no IL-3 or 10ng/ml IL-3. Metabolic activity was measured (absorbance at 490nm) each day using MTS reduction method (Section 2.2.5.3). This result is representative of two similar experiments.
cells expressing huβc\(^{582EFAAAA587}\) in the presence of IL-2 demonstrated that no intrinsic defect existed in these cells ability to survive in response to cytokine. From this data it can be concluded that the 14-3-3 binding motif of huβc is necessary for cellular viability of transfected CTL-EN cells in response to IL-3. Interestingly, initial experiments in the presence of 10% FCS showed no defect in the ability of cells expressing huβc\(^{582EFAAAA587}\) to survive in response to IL-3 (data not shown), indicating that as yet unknown components of the serum were able to compensate for the loss of the 14-3-3 binding motif.

3.7.2 The 14-3-3 binding motif of huβc is required for cell viability in an assay for metabolic activity of transfected CTL-EN cells in response to IL-3

The measurement of cell viability using an assay for metabolic activity (2.2.5.3) of CTL-EN cells expressing huIL-3Rα and either wildtype huβc or huβc\(^{582EFAAAA587}\) in the presence or absence of IL-3 is shown in Figure 3.11B. These data demonstrate that CTL-EN cells expressing huβc\(^{582EFAAAA587}\) suffered a loss in metabolic activity in response to IL-3, compared to cells expressing wildtype huβc. In fact, the metabolic activity of cells expressing huβc\(^{582EFAAAA587}\) in the presence of IL-3 was equivalent to cells cultured in the absence of factor. These data show that the 14-3-3 binding motif of huβc is necessary for the metabolic activity of transfected CTL-EN cells in response to IL-3.

3.7.3 The 14-3-3 binding motif of huβc is required for suppression of apoptosis in transfected CTL-EN lines in response to IL-3

To ask if the 14-3-3-binding motif is necessary for IL-3 mediated suppression of apoptosis, an apoptosis assay was used to analyse transfected CTL-EN cells in the presence or absence of growth factors. Apoptosis of CTL-EN cells was measured after 30 hours of

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culture in media containing 0.1% FCS, using annexin V and propidium iodide staining (Section 2.2.5.4). For these experiments, CTL-EN cells transfected with huIL-3Rα and either wildtype huβc, huβc\(^{S585G}\), huβc\(^{S84AAA586}\) or huβc\(^{S82EFAAA587}\) were cultured in the presence of IL-2, IL-3 or no factor. Figure 3.12 details the percentage of live (annexin V and propidium iodide negative) and late apoptotic (annexin V and propidium iodide positive) CTL-EN cells after 30 hours of culture. Each cell line underwent apoptosis when cultured for 30 hours in the absence of factor. IL-3 was able to inhibit apoptosis of CTL-EN cells expressing wildtype huβc, but not those expressing huβc\(^{S585G}\), huβc\(^{S84AAA586}\) or huβc\(^{S82EFAAA587}\). IL-2 was able to suppress apoptosis of CTL-EN cells expressing each of these 14-3-3 binding site substitution mutants, indicating no intrinsic detect in the ability of these cells to suppress apoptosis. These experiments demonstrate that the 14-3-3 binding motif of the huβc is necessary for the inhibition of apoptosis in transfected CTL-EN cells.

3.8 Discussion

To study phosphorylation of serine 585 in the putative 14-3-3 binding site of huβc, a unique phospho-specific antibody was raised against phosphorylated huβc serine 585 and its specificity verified. Experiments utilising this resource have demonstrated that phosphorylation on huβc serine 585, which lies in the 14-3-3 binding motif \(^{S582}HSRSLP^{S587}\), is transiently upregulated upon ligand stimulation. This phosphorylation event was stimulated by either huIL-3 or huGM-CSF, in two different haemopoietic cell lines, CTL-EN and M1. Serine 585 phosphorylation was independent of tyrosine residues, as it
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Figure 3.12 The 14-3-3 binding motif of the huβc is required for cellular survival by suppressing apoptosis in CTL-EN cell lines. CTL-EN cells expressing huIL-3Rα and either wildtype huβc, huβc<sup>S585G</sup>, huβc<sup>S58AAA585</sup> or huβc<sup>S8EFAAAA587</sup> were plated in either 20ng/ml IL-2, no factor or 50ng/ml IL-3 for 30 hours. Cells were stained with annexin V and propidium iodide and subjected to flow cytometry. The percentage of viable cells (annexin V and propidium iodide negative) and apoptotic cells (annexin V and propidium iodide positive) is indicated next to each sub-population. This result is representative of two similar experiments.
occurred in response to cytokine stimulation of cells expressing hu\(\beta_c\)\(^{F8}\). In addition, it was demonstrated that tyrosine phosphorylation occurred in the absence of serine phosphorylation, by cytokine stimulation of cells expressing hu\(\beta_c\)\(^{582EFAAAA587}\), indicating that these phosphorylation events are separately regulated.

Experiments performed in the Lopez laboratory after the completion of the studies in this thesis describe a concentration range of 0.1 to 30pM GM-CSF leading to hu\(\beta_c\) serine 585 phosphorylation in factor-dependent TF-1 cells (Guthridge et al., 2006). The concentration of GM-CSF used in Figure 3.6 to stimulate M1 myeloid leukemic cells is higher, at 400pM (10ng/ml). Although we did not measure hu\(\beta_c\) serine 585 phosphorylation at lower concentrations, these results neither-the-less indicate that different concentrations of GM-CSF can have different effects on the phosphorylation state of hu\(\beta_c\) in different cell types.

Co-immunoprecipitation experiments demonstrated that the phosphoserine binding protein 14-3-3\(\zeta\) is recruited to the hu\(\beta_c\) motif \(^{582}HSRSLP^{587}\) in response to cytokine stimulation. In addition, we examined the possibility that this signalling event occurs in the mouse. Pull-down experiments showed that 14-3-3\(\zeta\) associated with the mouse \(\beta_c\) and this association was dependent on the presence of the mouse equivalent of the hu\(\beta_c\) 14-3-3\(\zeta\) binding motif. This interaction occurred in two cells lines, COS cells which overexpress mu\(\beta_c\), and FDCP1 cells which endogenously express mu\(\beta_c\). Given its conservation from human to mouse systems, this association would appear to be a general event in hu\(\beta_c\) signalling.

The functional importance of the conserved 14-3-3/\(\beta_c\) interaction was demonstrated when CTL-EN cells transfected with substitution mutants of the hu\(\beta_c\) 14-3-3 binding motif were
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shown to be deficient in their viability and metabolic activity in the presence of IL-3. Furthermore, apoptosis assays showed that a greater proportion of cells expressing these hu\( \beta_c \) mutants underwent apoptosis when cultured with huIL-3 than cells expressing wildtype hu\( \beta_c \). These experiments have demonstrated a functional significance for the \( \beta_c/14-3-3\zeta \) interaction, indicating that 14-3-3 recruitment to the \( \beta_c \) is necessary for the suppression of apoptosis.

Based on the data in this thesis, we propose a model whereby ligand stimulation leads to dimerisation and activation of the hu\( \beta_c \), resulting in \( \beta_c \) tyrosine phosphorylation (as previously characterised) and serine 585 phosphorylation (Figure 3.13). There is no known serine kinase domain of the \( \beta_c \), therefore serine 585 phosphorylation is likely to occur once a serine kinase is recruited to the active \( \beta_c \). Serine 585 phosphorylation of the \( \beta_c \) leads to the recruitment of 14-3-3 to this site. Since 14-3-3 exists as a dimer, capable of binding two separate phosphoserine/threonine motifs, recruitment of 14-3-3 to \( \beta_c \) is likely to lead to recruitment of other 14-3-3 binding partners. Ultimately this recruitment of 14-3-3 (and potential binding partners) to the \( \beta_c \) culminates in the ability of hu\( \beta_c \) signalling to suppress apoptosis in the CTL-EN cell line. In the absence of hu\( \beta_c \) serine 585, 14-3-3 would not be recruited to the \( \beta_c \) upon ligand stimulation and an essential anti-apoptotic pathway would not be activated by ligand binding to the receptor, leading to greater numbers of cells undergoing apoptosis.

Further experiments in the Lopez laboratory demonstrated that a CTL-EN cells expressing a hu\( \beta_c^{S585G} \) mutant were defective in Akt phosphorylation, nuclear factor kappa B (NF-\( \kappa B \)) activation, anti-apoptotic bcl-2 induction, and cell survival (Guthridge et al., 2004). These
data support the above model and provide insight into the mechanism by which huβc is able to support survival through the recruitment of 14-3-3.

Serine phosphorylation of cytokine receptors upon ligand stimulation is known to occur in a small number of instances. For example, the thrombopoietin (TPO) receptor c-Mpl contains eight intracellular serine residues, four of which undergo serine phosphorylation upon TPO stimulation (Miyakawa et al., 2000). These serine residues are likely to contribute to receptor signalling, as substitution of the four that undergo phosphorylation leads to a 50% reduction in the ability of TPO to support growth of BA/F3 cells. The transforming growth factor β−1 receptor, (TGFβ1R), also undergoes phosphorylation of a number of serine and threonine residues upon TGFβ1 stimulation (Souchelnytskyi et al., 1996). When one of these residues, serine 165, is substituted, the mutant TGFβ1R increases TGFβ1 mediated growth inhibition and extracellular matrix formation, but decreases TGFβ1 induced apoptosis. Phosphorylation of serine 165 is therefore likely to be necessary for specific functions of the TGFβ1R. phosphorylation of a number of serine and threonine residues upon TGFβ1 stimulation (Souchelnytskyi et al., 1996). When one of these residues, serine 165, is substituted, the mutant TGFβ1R increases TGFβ1 mediated growth inhibition and extracellular matrix formation, but decreases TGFβ1 induced apoptosis. Phosphorylation of serine 165 is therefore likely to be necessary for specific functions of the TGFβ1R.
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Figure 3.13 Model describing the huβc/14-3-3 interaction and its role in mediating cell survival. Binding of IL-3 to the huIL-3Rα and huβc activates the receptor, leading to phosphorylation on tyrosine residues. Serine 585 of the huβc is also phosphorylated upon receptor activation by an as yet unknown serine kinase. phosphorylation of serine 585 recruits a 14-3-3ζ dimer. This dimer is able to bind other proteins containing phosphoserine or phosphothreonine 14-3-3 binding motifs, and therefore able to recruit further proteins to the huβc. It is likely that as yet unknown binding partners of 14-3-3 will be recruited to the huβc and play a role in the huIL-3-stimulated suppression of apoptosis, which requires serine 585 of the huβc, by CTL-EN cells expressing huIL-3R.
In a similar manner, we have shown that the huβc undergoes a specific serine phosphorylation event upon ligand binding, and importantly that this signalling event is essential for IL-3-supported survival of transfected CTL-EN cells. Similar to both these examples, the experiments of this Chapter have shown that the huβc undergoes a specific serine phosphorylation event upon ligand binding, and importantly that this signalling event is essential for survival of transfected CTL-EN cells. The concept of 14-3-3 being recruited by ligand-induced serine phosphorylation of a receptor is relatively new and only a few examples are known. 14-3-3 has been shown to associate with the IL-9 receptor α subunit in a yeast 2-hybrid and transfected HEK 293 cells. Although the requirement of IL-9 stimulation for this interaction has not been investigated, it does depend on phosphorylation of serine 519 or threonine 521 in the IL-9Rα motif 254RSWTF259 (Sliva et al., 2000). However, a function for this interaction is yet be described. 14-3-3 has also been shown to interact with the insulin-like growth factor I receptor (IGFIR) via two motifs, 1269MNESVP1274 and 1280SSSSLP1285, in a phosphoserine-dependent manner (Craparo et al., 1997). Among many roles for this receptor, it has been shown to efficiently protect transformed and tumour cells from apoptosis, via multiple pathways (Romano, 2003). In the murine haemopoietic cell line, 32D, the IGFIR ligand, IGF, activates an anti-apoptotic pathway which leads to mitochondrial translocation of c-raf and Nedd4. It is dependent on serine residues in IGFIR, known to interact with 14-3-3 (Peruzzi et al., 1999). Peruzzi (Peruzzi et al., 2001) showed that a substitution mutant of one of these 14-3-3 binding motifs, 1280AAALP1285, fails to protect 32D cells from apoptosis through disruption of the anti-apoptotic pathway which normally would lead to translocation of Nedd4 and raf-1 to mitochondria. This interaction of 14-3-3 and IGFIR can be initiated by serine autophosphorylation induced by dimerisation, likely due to the serine/tyrosine kinase
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domain of IGFIR (Parvaresh et al., 2002), indicating that for this receptor, no separate kinase is necessary for the 14-3-3 interaction. A third receptor for which an interaction with 14-3-3\( \zeta \) has been investigated is the platelet glycoprotein GPIb\( \alpha \) (Gu et al., 1999). GPIb\( \alpha \) is one of three subunits of the GPIb-IX complex, which is the platelet receptor for von Willebrand Factor (vWF), regulating platelet adhesion upon vWF stimulation. GPIb\( \alpha \), the subunit which contacts vWF, binds 14-3-3 via the motif \( ^{604} \text{RYSGHSL}^{610} \) in its cytoplasmic domain, in a phosphoserine 609 dependent manner (Bodnar et al., 1999).

Interestingly, the interaction of 14-3-3\( \zeta \) with GPIb\( \alpha \) was found to be necessary for the ability of GPIB-IX to bind vWF (Dai et al., 2005), indicating a role for 14-3-3 recruitment in regulating ligand binding of a receptor. In addition to these examples of known 14-3-3 interactions with receptors, other cell surface receptors exist which contain putative 14-3-3 binding sites, including the TPO receptor c-Mpl \( ^{475} \text{SSWSDP}^{480} \), c-kit \( ^{974} \text{ASSSQP}^{979} \), trk3 receptor \( ^{453} \text{VSLSLP}^{458} \), VEGF receptor \( ^{1206} \text{ESISAP}^{1211} \) and the 5\( \alpha \) acetylcholine receptor \( ^{382} \text{ESGSGP}^{386} \). Although 14-3-3 interactions with these receptors are untested, the existence of putative 14-3-3 binding motifs on numerous receptors indicates that ligand dependent 14-3-3 recruitment to cell surface receptors may be a widespread signalling mechanism, which has only recently come to light.

Not surprisingly, the promiscuous nature of 14-3-3 as a sequestering protein able to bind many different proteins (up to 170, (Jin et al., 2004)), leads to its involvement in numerous different cellular functions, which are only recently being characterised. As described in Section 1.5.4.5, 14-3-3 has reported roles which include preventing cell cycle progression in response to DNA damage (Sanchez et al., 1997) and inhibiting apoptosis in response to UV-C irradiation (Xing et al., 2000). There are some known instances of ligand-induced 14-3-3 binding to cell-surface receptors to phosphoserine residues (described above), and
one of these interactions, with IGFIR, is known to be necessary for anti-apoptotic pathways in a cell line. Consistent with these studies, we have shown that 14-3-3ζ recruitment to the huβc is necessary for suppression of apoptosis in transfected CTL-EN cells. It is possible that similarities exist between the mechanism by which 14-3-3 recruitment acts to inhibit apoptosis through its interactions with huβc and through IGFR1.

The requirement of a tyrosine independent βc serine phosphorylation event for survival is consistent with the finding that IL-3 stimulation of BA/F3 cells expressing huβcF8 leads to survival of these cells despite the loss of all signalling through intracellular tyrosine residues (Figure 3.1) (Guthridge et al., 1998; Okuda et al., 1997). The presence of a survival motif at aa residue 585 of the huβc is also consistent with the requirement of a region between aa residues 544 and 626 for cellular survival (Kinoshita et al., 1995; Inhorn et al., 1995). Interestingly, 14-3-3 recruitment was delayed by about 10 minutes when all huβc intracellular tyrosines were mutated (Figure 3.7). This result introduces the interesting possibility that huβc tyrosine residues are at least somewhat involved in the recruitment of 14-3-3 to huβc. However, this involvement is very unlikely to be during the induction of serine 585 phosphorylation, as it is not delayed in the huβcF8 mutant (Figure 3.5). Indeed, the influence of intracellular tyrosines must not be great or essential, since the delay in 14-3-3 recruitment is only short (10 minutes) before recruitment occurs and there seems to be no difference in the amount of 14-3-3 recruited to huβcF8 compared to wildtype huβc after 15 minutes stimulation.

Our findings have demonstrated the existence of a novel huβc signalling event whereby a phosphoserine residue of the huβc recruits 14-3-3, leading to the promotion of survival in
transfected CTL-EN lines. This signalling event is part of a newly emerging paradigm of receptor signalling and has the potential to be widespread and play an important role in the process by which receptors such as the $\beta_c$ are able to convert ligand stimulation into a variety of functional outcomes for a cell. Our experiments, however, have been limited to factor-dependent cell lines where we can measure proliferation, survival and in some cases differentiation. Perhaps the most important functions of cytokines such as GM-CSF, IL-3 and IL-5 lie in their ability to stimulated an array of effector functions of mature myeloid cells such as macrophages, designed to kill invading micro-organisms (Sections 1.4.4.3 to 1.4.4.5), since it is these functions which are lost in $\beta_c$ knockout mice (Scott et al., 1998). The following chapters in this thesis describe the investigation of a potential for role for $\beta_c$ serine 585 in the GM-CSF control of mature cell functions: The priming of ROS production and phagocytic activity.
Chapter 4: Development of a Novel Approach to Allow Measurement of the Effector Cell Functions of Primary Haemopoietic Cells

4.1 Introduction

The aim of Chapter 4 was to develop a novel approach for the isolation of primary mouse myeloid cells expressing wildtype or mutant huβc in which the regulation of mature cell functions by huGM-CSF can be investigated.

4.1.1 A novel approach to isolating primary myeloid cells where the measure of huβc regulation of effector cell functions is possible

Many studies have examined the role of the cytoplasmic domains of the βc as well as specific intracellular βc tyrosine residues in conversion of ligand (GM-CSF, IL-3 or IL-5) binding into intracellular signalling pathways which regulate some cellular functions, such as proliferation. However, previous studies using cells expressing mutant huβc to examine specific residues have predominantly been performed in immortalised cell lines (reviewed in Guthridge et al., 1998). While these studies have identified specific βc cytoplasmic domains or tyrosine residues which are important in regulating signalling events or pathways, ascribing specific βc tyrosine residues to biological outcomes has proven difficult. These preceding studies have mainly focused on the regulation of proliferation (Sakamaki K et al., 1992; Watanabe et al., 1996a; Okuda et al., 1999; Itoh et al., 1998; Guthridge et al., 1998), with very few on survival (Kinoshita et al., 1995; Inhorn et al., 1995) and differentiation (Smith et al., 1997) of cell lines. These studies therefore have not addressed an important function of the βc: its ability to regulate (prime) myeloid cell
activation, that function which is disrupted in βc knockout mice (Nishinakamura et al., 1995; Scott et al., 1998).

Cell lines that allow the analysis of βc mutants in terms of their regulation of priming are not available. The macrophage cell line RAW 264 can be primed to produce superoxide (Murakami et al., 2000), however, the presence of endogenous GM-CSF receptors in the cell line make it difficult for us to analyse huβc mutants due to cross-talk between endogenous wildtype and transfected mutant receptors (mouse and human βc subunits are able to dimerise across species creating a receptor containing muβc which could be activated by huGM-CSF, (McClure et al., 2001a)). The most appropriate system to examine GM-CSF priming of effector functions, such as ROS production and phagocytosis, is primary myeloid cells. Unfortunately, such studies in these cells are technically challenging as (1) they are not readily available for genetic manipulation, (2) purification of primary cell populations from rodents usually yields low cell numbers, and (3) primary myeloid cells obtained from mouse bone marrow or foetal livers express endogenous muβc, which would produce receptor cross-talk, as explained above.

This chapter describes the development of an approach which overcomes each of these problems and therefore allows biologically relevant activities of GM-CSF, such as priming of effector cell responses, to be examined in primary myeloid cells.

4.1.2 Attributes of primary haemopoietic cells required for their use in assays for the regulation of effector cell functions by huGM-CSF

To determine which huβc signalling pathways are necessary for regulation of effector cell functions, it was necessary to introduce the co-expression of huGMRα chain and wildtype
or mutant huβc, into mature primary cells. For these primary cells to be useful in assays for the regulation of mature functions by huGM-CSF, a number of criteria are required:

Firstly, the primary cells must be devoid of endogenous mouse β subunits (both muβc and muβIL-3), to avoid cross-talk between the human and mouse receptors (McClure et al., 2001a). Mutant huβc needs to be introduced and activated with huGM-CSF, without activation of endogenous mouse β subunits which would mask the effects of mutations in the huβc. This was achieved by deriving primary cells from muβc−/−;muβIL-3−/− double knockout mice.

Secondly, it was necessary to develop a method for inducing the co-expression of human GM-CSF alpha and wildtype or mutant huβc receptor subunits in primary haemopoietic cells. It has been demonstrated that primary haemopoietic cells can only be transfected with plasmid expression constructs at low efficiency, whereas retroviral transduction is a much more efficient method of transfer of genes into these cells (Magli et al., 1987). Therefore we transduced mouse bone marrow and foetal liver cells with a retroviral construct which co-expresses both huGMRα and huβc.

Thirdly, it was necessary to purify mature myeloid cells co-expressing receptor subunits from non-expressing cells and confirm the identity of these cells. FACS was used to purify a population of cells which express huGM-CSF receptors. The lineage of these receptor expressing cells was then determined by differential counts, surface lineage marker expression and Giemsa staining for morphology.
This chapter therefore describes a novel approach established for the derivation of primary cells expressing wildtype huβc and a range of huβc mutants, in which the regulation of mature cell functions by huGM-CSF could be investigated without interference by muβc signalling.

4.2 Assembly of retroviral constructs for the co-expression of huGMRα and wildtype huβc or mutant huβc in primary haemopoietic cells

To reconstitute functional huGMR in mouse cells, it was necessary to develop an approach for the co-expression of huGMRα and wildtype or mutant huβc, in mouse primary haemopoietic cells. To this end, we have used a retroviral transduction method, based on that described by Rayner and Gonda (Rayner and Gonda, 1994). Described in this section is the creation of these retroviral constructs, the first step in the assembly was to make mutant huβc cDNAs in which specific serine and tyrosine residues are substituted.

To determine if huGM-CSF priming mature cell functions is disrupted in the absence of serine 585 or tyrosine 577, we utilised the huβcS585G and huβcY577F cDNAs previously described (Section 3.3). A mutant cDNA, huβcY577F/S585G, was constructed which combines the huβcY577F mutation with huβcS585G. Site directed mutagenesis was performed as described in the Methods Chapter (Sections 2.2.1.12 and 2.2.1.13). In addition, mature cell functions were examined in cells expressing a huβc mutant lacking all tyrosine residues, huβcF8. These constructs as well as appropriate controls are compared in Table 4.1.
<table>
<thead>
<tr>
<th>Construct name</th>
<th>GMR subunits expressed</th>
<th>Residue/s of huβc mutated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRUFneoIRES-huGMRα</td>
<td>huGMRα only</td>
<td>none (huβc not expressed)</td>
</tr>
<tr>
<td>pRUFneoIRES-huβc</td>
<td>huGMRα and huβc</td>
<td>none</td>
</tr>
<tr>
<td>pRUFneoIRES-huβcS585G</td>
<td>huGMRα and huβcS585G</td>
<td>Serine 585</td>
</tr>
<tr>
<td>pRUFneoIRES-huβcY577F</td>
<td>huGMRα and huβcY577F</td>
<td>Tyrosine 577</td>
</tr>
<tr>
<td>pRUFneoIRES-huβcS585G/Y577F</td>
<td>huGMRα and huβcS585G/Y577F</td>
<td>Serine 585 and Tyrosine 577</td>
</tr>
<tr>
<td>pRUFneoIRES-huβcF8</td>
<td>huGMRα and huβcF8</td>
<td>All intracellular tyrosine residues</td>
</tr>
</tbody>
</table>

**Table 4.1 Features of pRUFneoIRES retroviral constructs**

The pRUFneoIRES retroviral vector was created by modification of the pRUFneo construct (Rayner and Gonda, 1994), to co-expresses huGMRα and wildtype huβc or mutant huβc from one transcribed message, by addition of an internal ribosome entry site (IRES), as described in Section 2.2.1.17 and Figure 2.1. This modification was performed by Dr. H Ramshaw of the laboratory of Prof. Angel Lopez.
4.3 Transduction of foetal liver and bone marrow cells from muβc⁻/⁻;βIL3⁻/⁻ mice with pRUFneoIRES constructs, by co-culture with Ψ2 cell lines

This Section describes a series of experiments which established an optimal protocol for retroviral transduction of primary haemopoietic cells. It was necessary to determine which primary cell population to infect and the length of time necessary in culture to produce viable cells co-expressing huGM-CSF receptors. Before transduction of primary cells was possible, it was first necessary to transfect the Ψ2 ecotrophic packaging cell line (described in Mann et al., 1983) with the pRUFneoIRES constructs. Ψ2 cells would then produce and release virus containing this construct.

4.3.1 Stable transfection of Ψ2 lines with retroviral constructs

The Ψ2 ecotrophic packaging cell line was transfected with pRUFneoIRES constructs expressing both the huGMRα and wildtype or mutant huβc described in Section 4.2 and Table 4.1. These transfections were performed by Dr. H. Ramshaw. Ψ2 cells with stable integration of pRUFneoIRES constructs were selected via culture in media containing G418 as well as FACS on surface expression of huGMRα in Ψ2 cell lines. Expression of huGMRα and wildtype or mutant huβc was verified by flow cytometry (Figure 4.1). These cell lines produce and release pRUFneoIRES retrovirus, and therefore are likely to mediate transduction of primary cells with this retrovirus.
Figure 4.1 Surface expression of huGMRα and wildtype huβc or mutant huβc in Ψ2 cell lines by integrated pRUFneoIRES retrovirus. Ψ2 cells were transfected with pRUFneoIRES retroviral constructs co-expressing huGMRα and wildtype huβc or mutant huβc by Dr. H. Ramshaw. Cells were incubated with antibodies to detect surface expression of; huβc with the monoclonal antibody mouse-anti-huβc (1C1) (blue); huGMRα with the monoclonal antibody mouse-anti-huGMRα, (4H1) (black), or as a negative control, the monoclonal antibody mouse anti-huIL-5Rα(A14), (red). Cells were then incubated with the secondary antibody sheep-anti-mouse-FITC. Flow cytometry was used to demonstrate cell surface expression. All cells expressed huGMRα. A: huGMRα only, B: wildtype huβc, C: huβc585G, D: huβc577F, E: huβc585G577F, F: huβc8.
4.3.2 Infection of muβ<sup>−/−</sup>;muβ<sub>IL3</sub><sup>−/−</sup> mouse bone marrow and foetal liver cells with pRUFneoIRES retroviral constructs by co-culture with Ψ2 cells

To determine optimal transduction conditions, both foetal liver cells and bone marrow cells from muβ<sup>−/−</sup>;muβ<sub>IL3</sub><sup>−/−</sup> double knockout mice were transfected with pRUFneoIRES constructs expressing huGMRα and wildtype huβ<sub>c</sub>, according to the protocol described in Figure 4.2. Foetal liver cells were removed from foetuses of 13.5 day pregnant muβ<sup>−/−</sup>;muβ<sub>IL3</sub><sup>−/−</sup> double knockout mice. Bone marrow cells were flushed from femurs and tibias of adult muβ<sup>−/−</sup>;muβ<sub>IL3</sub><sup>−/−</sup> double knockout mice. Retrovirus-producing Ψ2 cells were irradiated for five minutes at 30Gy to inhibit proliferation and overpopulation when in culture. Primary and Ψ2 cells were cultured together (co-cultured), for 48 hours, resulting in a monolayer of Ψ2 cells adhered to the flask in combination with unattached primary cells. Culture conditions included the presence of 100ng/ml muSCF to promote primary cell survival and 5µg/ml polybrene (Section 2.2.4.4), a charge-neutralising agent which enhances retrovirus infection (Andreadis and Palsson, 1997). After 48 hr co-culture, primary cells were harvested and cultured in the absence of Ψ2 cells for a further 24 to 48hr. At this point in the protocol, the resultant cell populations were analysed for surface expression of huGMR subunits, viability and morphology in order to determine optimal transduction conditions. These data are presented in Section 4.3.3 which describes the characterisation of the unpurified population of transduced primary cells. On the lower half of Figure 4.2 is described the purification process whereby transduced primary cells
Chapter 4: Development of a Novel Approach to Allow Measurement of the Effector Cell Functions of Primary Haemopoietic Cells
Figure 4.2 Outline of the optimised experimental protocol for the isolation of \( \text{mu}\beta_c^{-/};\text{mu}\beta_{\text{IL-3}}^{-/-} \) derived primary cells expressing huGMR (with wildtype or mutant hu\( \beta_c \)). This protocol is described in detail in Sections 4.3.2 and 4.3.3, as well as 2.2.4.1, 2.2.4.4 and 2.2.4.5. The analyses of the resultant populations for ROS (reactive oxygen species) production and phagocytic activity are presented in Chapters 5 and 6 respectively.
expressing huGMR were harvested and sorted for GMRα expression. The sorting protocol is described in Sections 2.2.4.5 and 4.3.4. This purified cell population was analysed for morphology and maturity, via cell specific surface marker expression and this data presented in Section 4.3.4.

4.3.3 Characterisation of the unpurified population of pRUFneoIRES transduced cells

A protocol was developed and optimised which produces primary cells with optimised huGMR expression, viability and percentage of mature cells which would be most useful in assays for effector cell functions. To this end, we investigated three properties of cell populations during the transduction process; (1) the ability of two different populations of primary haemopoietic cells (foetal liver and bone marrow) to express GMR subunits after incorporation of the pRUFneoIRES retrovirus, (2) the percentage of viable cells in the final cell population, and (3) and the morphology of the transduced cell population (Figure 4.2). For all of the data presented in Sections 4.3.3.1 to 4.3.3.2, primary cells were harvested after 48 hr co-culture with Ψ2 cells and further incubated for 24 to 48 hr, as described in Figure 4.2.

4.3.3.1 Surface expression of GMRα by pRUFneoIRES-transduced foetal liver and bone marrow cells.

Transduced foetal liver and bone marrow cells were analysed for surface expression of huGMR by flow cytometry (Figure 4.3). By day three, huGMRα was expressed on the surface of 12.2% of cultured foetal liver cells and 10.0% of cultured bone marrow cells by retroviral transduction. On day four, the expression was only a little higher than on day...
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Figure 4.3 Expression of huGMRα on cultured muβc−/−;muβIL-3−/− bone marrow and foetal liver cells. Bone marrow and foetal liver cells were co-cultured with Ψ2 cells which produce and release pRUFneoIRES retrovirus. Samples of primary cells were removed from the adherent Ψ2 cells at days 1 to 4 of culture. Surface expression of huGMRα and wildtype huβc or mutant huβc were verified by flow cytometry. Cells were incubated with antibodies to detect surface expression of; huGMRα with the monoclonal antibody mouse-anti-huGMRα, (4H1) (purple), or as a negative control, the monoclonal antibody mouse anti-huIL-5Rα (A14), (black). Cells were then incubated with the secondary antibody goat-anti-mouse-PE. Surface expression of receptor subunits was detected by flow cytometry. The estimated percentage of cells expressing huGMRα is indicated for each population.
three, indicating that three to four days of culture produced essentially equivalent surface expression levels. Surface expression of huβc was confirmed, as shown in Figure 4.4.

4.3.3.2 Viability of pRUFneoIRES-transduced foetal liver and bone marrow cells.

Viability of the transduced foetal liver and bone marrow cell populations was measured initially, then after each day of culture by the trypan blue exclusion method (Section 2.2.2.7). These data show that cell viability was reduced with each day of culture (Table 4.2). By the third day of culture, 37.5% of bone marrow derived primary cells are viable and 45% of foetal liver derived cells are viable. By the fourth day, both primary cell populations had slightly lower cell viability.

<table>
<thead>
<tr>
<th></th>
<th>Bone marrow</th>
<th>Foetal liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>94.7%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Day 1</td>
<td>87.0%</td>
<td>90.0%</td>
</tr>
<tr>
<td>Day 2</td>
<td>62.5%</td>
<td>83.3%</td>
</tr>
<tr>
<td>Day 3</td>
<td>37.5%</td>
<td>45.0%</td>
</tr>
<tr>
<td>Day 4</td>
<td>36.0%</td>
<td>41.0%</td>
</tr>
</tbody>
</table>

Table 4.2 Percentage of viable cells recovered from co-culture of primary muβc⁻/-;μβIL-3⁻/-;haemopoietic cells with pRUFneoIRES producing Ψ2 cells.

4.3.3.3 Morphology of pRUFneoIRES-transduced foetal liver and bone marrow cells.

To determine the morphology of transduced foetal liver and bone marrow cells, differential counts on cytocentrifuged, Giemsa-stained cells were performed (Sections...
Figure 4.4 Expression of huGMRα and huβc by cultured mouse bone marrow cells. μβc^-/-,μβc^IL-3^-/- mouse bone marrow cells were co-cultured with Ψ2 cells producing and releasing pRUFneoIRES retrovirus for 2 days, then in media containing retrovirus for 1 day. Cultured bone marrow cells were incubated in primary antibody: mouse-anti-huGMRα, (4H1) (red), mouse-anti-huβc (1C1) (purple), or negative control, mouse anti-huIL-5Rα(A14) (black). Cells were then incubated in the secondary antibody, goat-anti-mouse-PE. Surface expression of receptor subunits was detected by flow cytometry.
2.2.4.2 and 2.2.4.3). Of primary interest in optimisation of the transduction procedure were mature cells, eg monocytes/macrophages and neutrophils, which would be of most use in assays for effector cell functions. Figure 4.5 shows that bone marrow cells cultured for 3 or 4 days yielded a population which was approximately 50% monocytes/macrophages and 10% neutrophils as determined by differential counts based on the morphology of Giemsa-stained cells. After 3 to 4 days, cultured foetal liver cells yielded 30 to 40% monocytes/macrophages and only 2% neutrophils. Bone marrow cultures were therefore more effective in the production of mature cells than foetal liver cells.

4.3.3.4 Optimal culture conditions for generation of pRUFneoIRES-transduced primary cells

Optimal culture conditions for the generation of pRUFneoIRES-transduced primary cells (first half of the procedure described in Figure 4.2) were chosen on the basis of the above experiments. A protocol was chosen which included three days of primary cells culture (two days of co-culture with Ψ2 cells and one day in the absence of Ψ2 cells). Three days of culture allowed maximal surface expression of huGMRα but minimised loss of cell viability which increased with each day. Bone marrow cells were chosen due to a greater number of mature cells (monocytes /macrophages and neutrophils) present after 3 to 4 days of culture compared to foetal liver cells. This optimised protocol was then used in the complete procedure which culminated in the purification of mature cells expressing huGMR, as described below.
Figure 4.5 Differential counts of cultured μβc−/−;μβIL-3−/− bone marrow and foetal liver cells. Bone marrow and foetal liver cells were co-cultured with Ψ2 cells which produce and release pRUFneoIRES retrovirus. Samples of primary cells were removed from the adherent Ψ2 cells after 1 to 4 days of co-culture. These cells were cytocentrifuged and Giemsa stained. Differential counts were performed on Giemsa stained cells to indicate which cell lineages were present in the culture. Results are presented for each lineage as the percentage of the total number of viable cells. A: Cultured bone marrow cells, B: cultured foetal liver cells. It should be noted that the lack of eosinophils was expected in these μβc−/−;μβIL-3−/− mice due to their defective IL-5 signalling which leads to very low numbers of eosinophils (Nishinakamura et al., 1995).
4.3.4 Characterisation of pRUFneoIRES-transduced bone marrow cells purified by FACS on the basis of huGMR expression

The second half of the transduction protocol described in Figure 4.2 involves the purification of primary cells on the basis of huGMR\(\alpha\) surface expression. Approximately 10% of the cultured bone marrow population expressed huGMR\(\alpha\) on their cell surface. Prior to sorting, these cells were purified on a Ficoll gradient to remove some of the dead cells (Section 2.2.4.5). Ficoll-purified cells were stained for huGMR\(\alpha\) expression using specific antibodies and fluorescent detection then sorted to enrich for huGMR\(\alpha\) expressing cells by FACS (Section 2.2.4.5, Figure 4.6). During the sorting process, the cells were analysed for forward scatter (a measurement of cell size) and side scatter (a measurement of granularity). A ‘gate’ was constructed using the FACS software to select for a cell population based on these properties, as shown in Figure 4.6A. This gate selected cells of relatively high granularity and size, where macrophages were most likely to lie, based on previous experience with sorting this cell population. The cells which fell into this gated area and were positive for huGMR\(\alpha\) constituted the purified population of bone marrow cells expressing huGMR.

4.3.4.1 Morphology of huGMR positive FACS-purified pRUFneoIRES-transduced bone marrow cells

The FACS-purified pRUFneoIRES-transduced bone marrow population was analysed for cell morphology to determine which lineages of cells were present. Giemsa staining of cytocentrifuged cells showed that the gating described in Figure 4.6A lead to enrichment of cells with monocyte/macrophage-like morphology in the purified population (Figure
Figure 4.6 Purification of huGMR expressing monocyte/macrophages from cultured bone marrow cells. muβc^{−/−}β_{IL-3}^{−/−} mouse bone marrow cells were co-cultured with Ψ2 cells producing and releasing pRUFneoIRES retrovirus for 2 days, then cultured in media containing retrovirus for 1 day. Cultured bone marrow cells were purified by Ficoll gradient and antibodies were used to detect monocytes/macrophages expressing huGMRα. Cells were incubated with the monoclonal antibody biotin-conjugated mouse-anti-huGMRα (4H1) (purple), or negative control, biotin-conjugated mouse-anti-HA tag (12CA5). Cells were then incubated with streptavidin conjugated to PE. A: Forward scatter (X-axis) and side scatter (Y axis) profile of the cell population. Cells were sorted which lay within the polygonal gate R1. B: Detection of huGMRα expressing cells in the gate described in A. Positive cells were isolated by sorting (approximately 10% of the gated population).
4.7). Differential counts of this stained cells are presented in Table 4.3 and indicate that purified populations consisted of approximately 90% monocytes/macrophages.

<table>
<thead>
<tr>
<th>Cell morphology</th>
<th>Percent of population</th>
</tr>
</thead>
<tbody>
<tr>
<td>blasts</td>
<td>1.7%</td>
</tr>
<tr>
<td>promyelocytes/myelocytes</td>
<td>2.1%</td>
</tr>
<tr>
<td>myelocytes/neutrophils</td>
<td>2.8%</td>
</tr>
<tr>
<td>lymphocytes</td>
<td>1.4%</td>
</tr>
<tr>
<td>monocytes/macrophages</td>
<td>90.4%</td>
</tr>
<tr>
<td>eosinophils</td>
<td>0%</td>
</tr>
<tr>
<td>nucleated erythroid cells</td>
<td>1.4%</td>
</tr>
</tbody>
</table>

Table 4.3 Differential count on the purified population of huGMR expressing cultured muβc−/−;muβcIL-3−/− bone marrow cells (>250 cells counted)

4.3.4.2 Cell surface marker expression by FACS-purified pRUFINES-transduced bone marrow cells.

To confirm that the sorted population consisted of a high percentage of monocytes/macrophages, as indicated by differential counts in Table 4.3, surface expression of lineage markers was analysed (Figure 4.8). The sorted population of cells expressed F4/80, a marker found only on mature mouse macrophages and dendritic cells (Leenan et al., 1994; Agger et al., 1990). This population also expressed CD11b, an integrin and complement receptor found on the surface of phagocytes such as macrophages and neutrophils (Ehlers, 2000). A proportion of the sorted population expressed Gr1, found on neutrophils and immature monocytes (Fleming et al., 1993). Minimal Thy1 (expressed on T-lymphocytes) and B220 (expressed on B-lymphocytes) expression was detected. The
Figure 4.7 Photograph of purified bone marrow cells expressing huGMR. pRUFneoIRES transduced bone marrow cells were purified by FACS for huGMRα expression as described in Figure 4.6. These cells were cyto-centrifuged then Giemsa stained and photographed. Differential counts indicated that this population consisted of 90.4% monocyte/macrophages (see Table 4.3). Surface expression of lineage markers confirmed that most of the cells were monocytes/macrophages (Figure 4.8).
Figure 4.8 Surface expression of lineage markers on the FACS-purified population of huGMRα expressing cultured nuβc-/-;nuβIL-3-/- bone marrow cells. After transduction, (Figure 4.2), and purification by FACS, (Figure 4.6), bone marrow cells were incubated with antibodies to detect the surface expression of lineage markers: rat-anti-F4/80, rat-anti-CD11b, rat-anti-Ly6G (Gr-1), rat-anti-Thy1, rat-anti-B220 or rat IgG (negative control). Cells were then incubated in sheep-anti-rat-FITC and surface expression detected by flow cytometry. Detection of lineage marker antibodies are shown in black, and negative control (rat IgG) in red.
expression pattern of lineage markers on the sorted cell population therefore confirms their identity as monocytes/macrophages.

Western blotting experiments performed by Ms E Barry in the Lopez laboratory showed that huGM-CSF stimulation of monocytes/macrophages expressing huGMRα with wildtype huβc, huβcS585G, huβcY577F and huβcY577F/S585G lead to phosphorylation of JAK2 and of STAT5 proteins, indicating that the huGM-CSF receptors were able to be stimulated by ligand. This alleviates the possibility that any of these huβc mutants may be falsely found to be defective in function because of a problem such as protein mis-folding.

Our aim was to produce primary cells co-expressing huGM-CSF receptors for use in assays for effector cell functions. To this end, we have established a protocol for the retroviral transduction of muβc-/-muβc-/-IL-3 bone marrow cells which yields approximately 10% of unpurified cells expressing huGMRα (Figure 4.4), in a population which consists of approximately 50% of cells exhibited monocyte/macrophage like morphology (Figure 4.5). Viable cells expressing surface huGMRα were purified and exhibited approximately 90% monocyte/macrophage morphology (Table 4.3). Surface marker expression of the purified huGMR expressing population was consistent with the monocyte/macrophage phenotype (Figures 4.7 and 4.8).
4.4 Attempts to produce neutrophils co-expressing huGMRα and huβc by transduction of muβc-/-;muβIL-3-/- mouse primary haemopoietic cells with pRUFneoIRES constructs in the presence of huG-CSF

One possible method for the production of neutrophils in a culture of immature haemopoietic cells would be to stimulate them with GM-CSF (Metcalf, 1986). Unfortunately this is not desirable during our experiments for two reasons: firstly, these cells are derived from muβc-/-;βIL-3-/- double knockout mice and do not express muGMR through which GM-CSF would signal, and secondly, stimulation with huGMR would activate the cells, rendering them useless in later assays for the regulation of mature cell functions by huGM-CSF. However, another cytokine which can stimulate the production of neutrophils from immature haemopoietic cells is granulocyte-macrophage colony stimulating factor (G-CSF), (Metcalf and Nicola, 1983).

In an attempt to produce neutrophils co-expressing huGM-CSF receptors, we co-cultured muβc-/-;muβIL-3-/- bone marrow and foetal liver cells with Ψ2 cells producing pRUFneoIRES retrovirus, in the presence of 10ng/ml huG-CSF, 100ng/ml muSCF and 5µg/ml polybrene. After 3 to 4 days of culture, approximately 9% of bone marrow cells and 13% of foetal liver cells expressed surface huGMRα (Figure 4.9). Similar to primary cells cultured in the absence of huG-CSF, the viability of huG-CSF-cultured cells fell with each day of culture (day 4: 30.8% for bone marrow and 32% for foetal liver cells) (Table 4.4). By day three, 33% of cultured bone marrow cells, but less than 2% of cultured foetal liver cells showed neutrophil-like morphology (Figure 4.10). Of primary
## Chapter 4: Development of a Novel Approach to Allow Measurement of the Effector Cell Functions of Primary Haemopoietic Cells

Table 4.1: Bone Marrow and Foetal Liver Cell Cultures

<table>
<thead>
<tr>
<th>Day</th>
<th>Bone Marrow + G-CSF</th>
<th>Foetal Liver + G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>7.9</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>8.2</td>
<td></td>
<td>13.4</td>
</tr>
<tr>
<td>9.0</td>
<td></td>
<td>12.7</td>
</tr>
</tbody>
</table>

**Figure 4.9 Surface expression of huGMRα on muβc−/−,muβcIL-3−/− bone marrow and foetal liver cells cultured in the presence of huG-CSF.** Bone marrow and foetal liver cells were co-cultured with Ψ2 cells which produce and release pRUFneoIRES retrovirus in the presence of 10ng/ml huG-CSF. Samples of primary cells were removed from the adherent Ψ2 cells at days 1 to 4 of co-culture. Surface expression of huGMRα and wildtype huβc or mutant huβc were verified by flow cytometry. Cells were incubated with antibodies to detect surface expression of huGMRα with the monoclonal antibody mouse-anti-huGMRα, (4H1) (purple), or as a negative control, the monoclonal antibody mouse anti-huIL-5Rα(A14), (black). Cells were then incubated with the secondary antibody goat-anti-mouse-PE. Surface expression of receptor subunits was detected by flow cytometry. The estimated percentage of cells expressing huGMRα is indicated for each population.

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Table 4.4 Percentage of viable cells recovered from co-culture of primary muβc--;muβIL-3− haemopoietic cells with Ψ2 cells which produce and release pRUFneoIRES retrovirus. Cultures contained 10ng/ml huG-CSF to promote neutrophil proliferation and survival.

<table>
<thead>
<tr>
<th>Day</th>
<th>Bone marrow (+huG-CSF)</th>
<th>Foetal liver (+huG-CSF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.7%</td>
<td>91.7%</td>
</tr>
<tr>
<td>1</td>
<td>85.7%</td>
<td>87.5%</td>
</tr>
<tr>
<td>2</td>
<td>62.5%</td>
<td>83.3%</td>
</tr>
<tr>
<td>3</td>
<td>38.0%</td>
<td>45.0%</td>
</tr>
<tr>
<td>4</td>
<td>30.8%</td>
<td>32.0%</td>
</tr>
</tbody>
</table>

importance to this experiment was the number of viable neutrophils expressing huGMR which could be purified from the population. Unfortunately, even though more neutrophils were produced by cultured bone marrow, experiments which sorted the huGMRα expressing population by FACS showed that only a very low proportion of these neutrophils expressed huGMRα (data not shown). It was not feasible to scale up the size of the experiment to produce enough huGM-CSF receptor expressing neutrophils for use in assays. Therefore we have not used neutrophils in our assays for huGM-CSF regulation of effector cell functions. The experiments in future chapters were therefore limited to measurement of effector cell functions of monocytes/macrophages, isolated as described in Sections 4.3.3 and 4.3.4.
Figure 4.10 Differential counts of cultured μβc−/−;μβIL-3−/− bone marrow and foetal liver cells in the presence of huG-CSF. Bone marrow and foetal liver cells were co-cultured with Ψ2 cells which produce and release pRUFlneoIRES retrovirus in the presence of 10ng/ml huG-CSF, in order to enhance production of neutrophils. Samples of primary cells were removed from the adherent Ψ2 cells at days 1 to 4 of co-culture. These cells were cytocentrifuged and Giemsa stained. Differential counts were performed on Giemsa stained cells to indicate which cell lineages were present in the culture. Results are presented for each lineage as the percentage of the total number of viable cells. A: Cultured bone marrow cells, B: cultured foetal liver cells.
4.5 Discussion

This chapter describes the development of an *ex vivo* biological system which allows the study of the molecular mechanisms behind GM-CSF regulation of the cellular functions of mature monocytes/macrophages. This system has a number of important features; (1) primary mouse haemopoietic cells are transduced for co-expression of huGMRα and wildtype or mutant huβc, (2) these transduced cells have no endogenous expression of muβc or muβIL-3 so huGM-CSF signalling through huβc can be studied without interference from these receptors, (3) the transduced cell population is enriched for huGMR expressing cells by FACS, and (4) the enriched population consists of a high percentage of viable, mature monocytes/macrophages.

Retroviral constructs were made which expressed the huGMRα and wildtype or mutant huβc from the one viral construct, enabling co-expression of the subunits necessary for production of functional huGMRs. The ecotropic packaging cell line Ψ2 was stably transfected with these constructs (Figure 4.1), to allow production and released of the retrovirus, which infects primary cells during co-culture.

The protocol for transduction of mouse bone marrow or foetal liver cells is described in Figure 4.2. The first half of the protocol (prior to sorting of huGMRα expressing primary cells) was optimised by a series of experiments comparing two different primary cell populations (mouse foetal liver and bone marrow cells) and different culture times (Sections 4.3.1 to 4.3.3). Once the first half was optimised, the second half of the protocol was performed where the transduced cell population was purified and characterised (Section 4.3.4).
A series of experiments determined optimal conditions for the primary cell transduction procedure. A three day co-culture of retrovirus-producing Ψ2 cells with μβc−/−;μβIL-3−/− bone marrow cells was the optimal protocol for transduction. The three day culture time allows a relatively high level of huGMRα surface expression but minimises loss of cell viability seen with longer cultures (Figure 4.3 and Table 4.2). Bone marrow cells were chosen above foetal liver cells due to a higher percentage of mature cells present after three days of culture (Figure 4.5). In addition, the presence of both huGMRα and huβc on the surface of transduced cells were confirmed (Figure 4.4).

Using this optimised transduction protocol to infect μβc−/−;μβIL-3−/− bone marrow cells with the retrovirus expressing huGMR subunits, a population of viable huGMR expressing primary cells was purified by FACS (Figure 4.6) and characterised. This population consisted of cells with the morphology of monocytes/macrophages (Figure 4.7 and Table 4.3), which expressed cell surface markers consistent with their identity as monocytes/macrophages (Figure 4.8). We have therefore established a protocol which produces a population of mature, viable, mouse bone-marrow-derived monocytes/macrophages expressing huGMRα and wildtype or mutant huβc, but not μβc or μβIL-3.

Attempts to produce foetal liver or bone marrow derived neutrophils using the neutrophil growth and differentiation factor huG-CSF during the transduction culture time failed to produce a population sufficiently enriched for cells with neutrophil-like morphology (Figure 4.10).
The development of this *ex vivo* biological system places us in a unique position to examine the regulation of GM-CSF priming of mature cells functions such as ROS production and phagocytic activity.