Inhibition of activation induced CD154 expression on human CD4+ CD25- cells accurately reports suppressor function by human nTreg, iTreg and regulatory T cell lines

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Abstract

Natural Regulatory T cells (Treg) are defined by stable expression of the cell surface proteins CD4 and CD25, low surface expression of CD127 and expression of the transcription factor FOXP3. The contribution of Treg to the prevention of autoimmunity and the maintenance of immune homeostasis is the subject of ongoing interest, as alterations in Treg numbers and function are implicated in a wide range of diseases. The *in vitro* benchmark for determining Treg function is suppression of proliferation of unmatched effector T cells in a mixed leukocyte reaction (MLR) over a 3-6 day time period. As an alternative to this assay we show that a 7 hr CD154 expression assay is rapid, simple, and provides a reliable readout of suppressor function. Using multiple Treg-like cell types including nTreg, iTreg and Treg cell lines we show that suppression of CD154 expression is a surrogate for suppression of proliferation. We propose this as a suitable alternative to the MLR assay, as it is rapid and may be more amenable to high throughput screening, analysing large cohorts of clinical samples, or assaying transiently suppressive populations.

Introduction

A healthy immune system depends on highly reactive cells that are able to distinguish foreign antigens from self antigens [1-4]. Dominant self-tolerance in the periphery is an active process mediated by cells collectively described as having regulatory function. FOXP3 expressing T regulatory cells (Tregs) are critical mediators of dominant self-tolerance. The essential role for these cells in preventing autoimmunity and maintaining immune homeostasis is demonstrated by loss of Treg cells as a result of FOXP3 mutation, which is lethal in both humans and mice [5]. Breakdown of the balance of reactivity and self tolerance in the immune system is believed to lead to a wide variety of autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis, many of which cause progressive and irreversible multi-organ damage [6-9].

Tregs are phenotypically defined by their expression of cell surface markers CD4 and CD25, low expression of the IL-7 receptor (CD127) [10], and expression of the transcription factor FOXP3 [11]. At least two different FOXP3-expressing Treg populations exist in the periphery, and these develop from different sources. Natural Tregs (nTreg) develop in the thymus and are considered a separate CD4 lineage. Inducible Treg (iTreg) are formed from naive CD4 cells in the periphery upon TCR engagement in the presence of the cytokine transforming growth factor-beta (TGF^{-} β) [12-14]. Although FOXP3 is crucial for the development and function of both mouse and human Tregs [15-23], FOXP3 can also be expressed transiently by activated human CD4 T cells without conferring suppressive function [24-25], complicating the use of FOXP3 as a conclusive marker for regulatory function in human cells. Irrespective of the origin of Tregs, measurement of reduced effector cell proliferation *in vitro* is accepted as conclusive functional evidence of a T regulatory cell phenotype. Cell proliferation and Treg function *in vitro* is conventionally measured either through the incorporation of [^{3}H]-thymidine during DNA synthesis or the reduction of 5,6-carboxyfluorescein diacetate

succunimidyl ester (CFSE) label with cell division in a mixed lymphocyte reaction (MLR). Both [^{3}H]-thymidine and CFSE MLRs demonstrate Treg suppression by enumerating a reduction in effector cell proliferation in the presence of Tregs [26].

Although currently the benchmark for assay of *in vitro* suppressor function, the MLR has some drawbacks. Three cell populations are required for the MLR assay: effector cells, irradiated 'feeder' cells, and T regulatory cells. This introduces assay variability due to donor specific differences in effector cell proliferation and response to stimulation, as well as variation in feeder cell function post-irradiation. These factors make MLR assays difficult to standardise, which is necessary for investigating Treg function in clinical settings. Additionally, small variations in assay parameters can be amplified by the 3-6 day culture period.

In order to improve the robustness and application of suppressor assays we have investigated the use of CD154 as a reporter of suppressor function. CD154 (CD40L) is a member of the tumour necrosis factor family [27]. This transmembrane protein is one of the first surface proteins up-regulated on T cells in response to stimulus [28-30], providing co-stimulatory signalling to B cells and antigen presenting cells through CD40 [31]. Importantly, CD154 is not expressed by resting T cells [28], and is only expressed transiently following activation, with expression peaking 6-8 hours after stimulation. Unlike the CFSE and [^{3}H]-thymidine assays, which measure proliferation, the CD154 functional assay measures an early signal preceding proliferation because CD154 is a marker of activation. This has been used to measure T cell activation [28] and recently by Ruitenberg [32] to measure Treg activity.

We have investigated the use of this assay to measure the expression of CD154 on the surface of purified $CD4^{+}$ $CD25^{-}$ effector cells 7 hours after activation. We demonstrate here that suppression of CD154 expression measures comparable suppressor function of freshly isolated and expanded Tregs purified from cord and peripheral blood to that seen when

proliferation is used as a readout of suppression, either by thymidine incorporation or CFSE dilution. Given the growing interest in transient Treg populations such as iTreg and Tr1, which may not be robustly CD25+ve, we have demonstrated that $CD4^+$ $CD25^-$ effector cells can be easily distinguished from suppressive cells by labeling the $CD4^+$ $CD25^-$ effector cells with membrane dyes such as $DIOC_{18}(3)$ prior to input into the assay. We report strong suppressive function by *in vitro* generated iTreg with $DIOC_{18}(3)$ stained Teffector cells. We also report suppression in the CD154 assay by Treg-like cell line Karpas-299. The CD154 assay provides a simple and easily standardised method for analysing the functional potential of freshly isolated or *ex vivo* expanded Treg cells. The rapid nature of the assay and its response to many cells with Treg like characteristics suggest its potential utility for high throughput screens for agents to modulate Treg function and rapid screening of *ex vivo* derived Tregs from clinical cohorts.

Methods

Isolation of Teffector cells from adult blood buffy coat

Buffy Coats from young male donors were collected (Australian Red Cross and Women's and Children's Hospital (WCH) human ethics approved) and processed within 24 hours of collection. $CD4^+$ T cells were enriched using the RosetteSep $CD4^+$ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and ficoll density gradient centrifugation. To isolate $CD4^+$ $CD25^-$ Teffector cells, enriched $CD4^+$ T cell populations were depleted of $CD25^+$ cells by labelling with anti-CD25 microbeads and separation on an Automacs Pro Separator (Miltenyi Biotech, Bergisch Gladbach, Germany). When required, cells were cryopreseved by resuspension in 90% FCS 10% DMSO followed by rate freezing in

isopropanol (Nalgene, Rochester, NY) for 4 hours at - 80°C, then transferred to liquid nitrogen for long term storage.

Isolation of adult peripheral blood Treg cells

Adult peripheral blood $CD4^+$ T cells were enriched using the RosetteSep $CD4^+$ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada) and ficoll density gradient centrifugation as above. Adult peripheral blood Treg cells were then isolated from the enriched $CD4^+$ T cells by labelling with CD4 and CD25 antibodies and collecting the top 2% of $CD4^+$ $CD25^{Bright}$ cells using a FACS Aria II cell sorter (BD). Tregs were cultured at $1x10^6$/mL in RPMI supplemented with 10% foetal bovine serum and 100 U/mL human recombinant IL-2 (eBioscience, San Diego, CA) for 48 hours. Prior to use in suppression assays, Tregs were washed twice in 50mL complete RPMI to remove IL-2, and viable cells were determined by trypan blue counting.

Isolation and expansion of Tregs from cord blood.

Umbilical cord blood samples were collected from the Adelaide Women's and Children's Hospital (WCH) Birthing Suite with approved ethics from the Women's and Children's Health Network Human Subjects Committee. Regulatory T cells were purified from the mononuclear cell fraction (CBMCs) isolated from fresh cord blood by ficoll density gradients as described previously [26]. Regulatory T cells ($CD4^+$ $CD25^+$) were purified using the Invitrogen Dynal Bead Regulatory T cell isolation kit (Invitrogen, Carlsbad, CA, USA). Isolated regulatory T cells were expanded for 7 days with Dynal Bead Human T Expander CD3/CD28 beads at a 3:1 bead:cell ratio (Invitrogen) and 500U/mL IL- 2 (eBioscience) in X-VIVO 15 (Lonza, Basel, Switzerland) supplemented with 5% human serum, 2mM L-glutamine and 20mM Hepes (complete X- VIVO 15). At day 7 of expansion, CD3/CD28

beads were removed and cells were allowed to rest for a further 7 days in complete X-VIVO 15 media with 100U/mL IL-2.

Flow cytometry of expanded nTregs and iTregs

Before use in suppressor assays the phenotype of expanded Treg cells was confirmed by flow cytometry. Expression of CD4 (CD4-APC-H7, BD Biosciences clone SK3) and CD25 (CD25-PECy7, BD Biosciences clone M-A251) were determined by staining cells for 30mins on ice, followed by 2 washes in PBS/BSA. Intracellular FOXP3 staining was subsequently performed using the FOXP3 fix/perm kit and FOXP3-Alexa Fluor 488 and 647 antibodies (BD Biosciences clone 259D/C7). Cells were analysed on a FACSAriaII flow cytometer (BD Biosciences).

CD154 suppression assay

Freshly collected or previously cryopreserved MACS or FACS isolated $CD4^+$ $CD25^-$ cells were used as effector cells in this assay. Freshly collected $CD4^+$ $CD25^-$ effector cells were used within 12 hours. Cryopreserved $CD4^+$ $CD25^-$ effector cells were rested for 12-20 hours at 37^oC in complete RPMI after thawing. The CD154 assay was carried out in 96 well CellStar U-bottomed plates containing a constant number of effector cells ($5x10^4$/well) and serial dilutions of Tregs, giving rise to Teffector:Treg ratios of 1:1, 2:1, 4:1, 8:1, 16:1. To induce CD154 expression stimulated wells received anti-CD3/CD28 T cell expander beads (Invitrogen) at a 0.25:1 bead:cell ratio, and anti-CD154-APC (BD; clone 89-76). Effector cells stimulated in the absence of Tregs and unstimulated effector cells from wells which received anti-CD154-APC alone were used as positive and negative controls respectively, and set up in triplicate. The cultures were incubated at $37^oC/CO_2$ in the dark for 7-8 hours. Following the incubation period, cells were stored at 4^oC for a maximum of 15 hours prior to

staining and analysis on a BD FACSAriaII flow cytometer. Cells were stained for surface expression of CD4 and CD25 prior to FACS analysis by three-colour flow cytometry on a BD FACSAriaII flow cytometer. During analysis the Treg ($CD4^+$ $CD25^{Bright}$) cells were excluded by gating on the $CD4^+$ $CD25^-$ cells. Unstimulated, stained effector cells were used to set the gate for analysis of CD154 expression. Suppression of T cell activation was assessed by comparing CD154 expression in stimulated $CD4^+$ $CD25^-$ effectors alone with CD154 expression in stimulated $CD4^+$ $CD25^-$ effectors co-cultured with Tregs.

Mixed lymphocyte assay: Thymidine incorporation

$CD4^+$ $CD25^-$ effector cells ($2x10^4$ per well), were cultured in U-bottom 96 well plates in the presence of $1x10^5$ irradiated (30 Gy) unmatched PBMCs. Treg cells were washed twice in RPMI media to remove IL-2 before addition to the assays. Treg test cells were added to achieve Teffector:Treg ratios of 1:1, 2:1, 4:1, 8:1, 16:1. Cells were stimulated with 100ng/mL soluble anti-CD3 (OKT3) monoclonal antibody (eBioscience). On day 4 [^{3}H]-thymidine was added to the wells for the last 16 hours of culture. All Teffector:Treg ratios were performed in triplicate and measured using a Microbeta beta counter (Perkin Elmer). Results were expressed in scintillation counts per minute.

Mixed lymphocyte assay: 5,6-carboxy fluorescein diacetate succinimidyl ester (CFSE)

Cell division of effector cells in a mixed lymphocyte reaction was also analysed by CFSE dilution [33]. Effector $CD4^+$ $CD25^-$ cells were isolated as described above, and labelled with 1μM CFSE (Invitrogen, Carlsbad, CA) in PBS for 10 minutes at 37°C. The reaction was quenched by addition of an equal volume of heat inactivated FBS. CFSE labelled effector cells ($2x10^4$ per well) were incubated with $1x10^5$ irradiated (30 Gy) unmatched PBMCs. Treg cells were added at Teffector:Treg ratios of 1:1, 2:1, 4:1, 8:1, 16:1, in the presence of

100ng/mL anti-CD3 (OKT3) monoclonal antibody (eBioscience) in a final volume of 200µl RPMI per well in a 96-well U-bottom plate. Co-cultures were harvested after 5 days of incubation and the proliferation of the effector population was visualised by the dilution of CFSE fluorescence with cell division on a BD FACSAriaII flow cytometer.

Karpas-299 cell line

The Karpas-299 cell line, an anaplastic large T cell lymphoma cell line with regulatory T cell- like properties [34] was used as a substitute regulatory T cell in suppressor assays. This cell line was purchased from HPA Culture collections (Porton Down, UK) and cultured in RPMI supplemented with 2mM Glutamine and 10% FCS. When used in suppressor assays, Teffector:Karpas-299 dilutions from 1:1 to 16:1 were tested.

Generation of iTregs from naive cord blood $CD4^+$ $CD25^-$ T effector cells

To generate iTreg in vitro, $CD4^+$ $CD25^-$ T cells were isolated from cord blood CBMCs using the Invitrogen Dynal Bead Regulatory T cell isolation kit (Invitrogen) and cultured for 7 days in complete X-VIVO 15 supplemented with CD3/CD28 beads (3:1 bead to cell ratio), 500U/mL IL-2, 5ng/mL TGF-β and 10nM all-trans-retinoic acid (ATRA) (Sigma). At day 7, CD3/CD28 beads were removed and cells were allowed to rest for a further 7 days in complete X-VIVO 15 media with 100U/mL IL-2, followed by cryopreservation. Phenotyping of iTregs showed robust expression of FOXP3 and heterogeneous CD25 expression. Induced Treg populations were thawed and rested overnight in complete RPMI before use in assays.

$DIOC_{18}(3)$ labelling of effector cell populations

3,3'- dioctadecyloxacarbocyanine perchlorate ($DIOC_{18}(3)$) labelling was performed when resolution of Treg and Teffector populations could not be achieved with CD25 gating (e.g.

with iTreg). Adult $CD4^+$ $CD25^-$ Teffector cells, isolated by MACS as described above, were labelled with 2µg/mL $DIOC_{18}(3)$ (Molecular Probes) in PBS containing 3% FBS for 45 min at 37°C in a 5% CO_2 incubator. Cells were washed 3 times in PBS supplemented with 3% FCS prior to use in the assay. The $DIOC_{18}(3)$ stained effectors were set up as per standard CD154 suppression assay, however no surface CD4 and CD25 staining was performed prior to FACS analysis.

Calculation of % Suppression:

Percentage suppression was calculated for each assay using the formulae shown below. Thymidine Incorporation: 100%- [(cpm stimulation + treg/cpm stimulation effectors only)*100], CFSE Dilution: 100%- [(% $CFSE^+$ stimulation + treg /% $CFSE^+$ stimulation effectors only)*100] CD154: 100%- [(%$CD154^+$ + Treg/%$CD154^+$ stimulation effectors only)*100].

Results

Ex vivo CB Tregs express CD25 and FOXP3 following expansion.

Cord blood Tregs expanded for 7 days with CD3/CD28 beads, followed by a further 7 day rest period in the absence of expansion beads, expressed high levels of CD25 and FOXP3 (figure 1a,b)(n=8). The addition of a 7 day 'rest' period post-expansion resulted in a reduction in Treg proliferation, rendering these cells suitable to be included in thymidine incorporation assays (figure 1c).

Expanded CB Tregs can suppress induction of CD154 on stimulated $CD4^+$ $CD25^-$ cells

We first demonstrated that cell surface staining with CD4 and CD25 antibodies allowed for resolution of effector cells from Treg cells in co-culture on the basis of CD25 expression.

CD4+CD25- effector cells were analysed alone in the presence and absence of stimulation (fig 2a). A gating strategy that was tightly confined to the CD25- region was selected, as although the mean fluorescence intensity on Treg is a log brighter than CD25- cells (see fig1), we wished to minimise the unintentional inclusion of CD25+ cells in the CD154 analysis to maximise assay signal to noise. Over the seven hour stimulation there was no up-regulation of CD25 on effector cells (figure 2a lower panel ii). This confirmed that the CD25- gating strategy restricted visualisation of CD154 suppression events to the effector cells. When CD25- effector cells alone were assayed in the absence of stimulation, no detectable CD154 expression was observed in the purified $CD4^+$ $CD25^-$ cells, confirming minimal background signal (figure 2a upper panel iii), and confirming that the induction of CD154 was stimulation-dependant (figure 2a lower panel iii). The level of CD154 expression seen with stimulated purified $CD4^+CD25^-$ cells was within the range reported using PBMCs, indicating that $CD4^+$ $CD25^-$ cells do not require bystander cells for optimal CD154 induction by CD3/CD28 beads. When expanded CB Tregs were titrated into this culture, they suppressed CD154 levels in unmatched $CD4^+$ $CD25^-$ effectors in a titratable manner (figure 2b). At maximal suppression (Tregs to effector cell ratio 1:1) CD154 expression ranged from 5 to 26% with a median of 11.4%. The mean fluorescence intensity (MFI) of CD154 expression also decreased upon addition of Treg cells, with a 2 to 3 fold reduction in MFI observed. By serially diluting Tregs to give a range of Teffectors:Tregs ratios from 1:1 to 16:1, measurements of potency of suppression can be determined for Treg populations (figure 2c). For $CD4^+$ $CD25^-$ cells we observed some donor variability in stimulation-induced CD154 expression, and expression of CD154 in the presence of 1:1 Tregs ranged from 5 to 26.2%, with a median of 11.2% (figure 2d). However the percentage suppression was more consistent, ranging from 49 to 78%, with a median of 61% for different donors at maximal Treg suppressor function.

Comparing CD154 assay measurement of suppressive function with benchmark MLR assays

The ability of the CD154 assay to detect Treg suppressive function was then confirmed by comparison with the CFSE and $[^3H]^-$ thymidine assays, two assays routinely used to measure Treg suppressive function *in vitro.* Representative assay readouts for CFSE and H^3-thymidine assays are shown in Supplementary figures 1 and 2 respectively. In order to make an accurate comparison between suppressor assay readouts, paired donor matched assays were performed. Measuring the maximal response to stimulation and suppression in Treg co-cultures and expressing each as percentage suppression, allowed direct comparison of the three assays (figure 3). Equivalent suppressive function was observed in the CD154 and CFSE assays. Measurements of suppressive capacity were higher in $[^3H]^-$ thymidine incorporation assays than both CD154 and CFSE assays. CFSE assays were prepared with the same cells as the $[^3H]^-$ thymidine assay, with the exception of CFSE staining of the effector cells. Therefore the increased suppression observed by $[^3H]^-$ thymidine assays may reflect the short window where DNA synthesis is measured, rather than increased sensitivity of the assay readout. These data indicate that the CD154 assay is a suitable surrogate assay to the traditional MLR assay.

The CD154 assay can be used to detect suppressor function in adult blood Treg populations

We next determined if the CD154 assay could be used to detect suppression by Tregs isolated from adult peripheral blood. Tregs (top 2% of $CD4^+$ $CD25^{Bright}$ cells) were isolated from peripheral blood PBMCs by FACS. Sorted Tregs were rested in 100U/mL IL-2 for 48hours post sorting prior to use in the CD154 assay. This approach has been used to improve viability and suppressive function in sorted Treg populations [35]. A short incubation in IL-2 is sufficient to promote suppressive function in adult Treg cells without need for stimulation or

expansion. Pre-incubation with IL-2 was required for suppressor function in CD154 assays and increased suppressive function in CFSE assays (data not shown).. Using isolated PB Tregs we were able to robustly detect suppression of unmatched $CD4^+$ $CD25^-$ Teffector CD154 induction at various Teffector:Treg ratios using just $5x10^4$ effector cells/well (figure 4). These data suggests that the CD154 assay is sufficiently sensitive to be used with rare Treg populations from peripheral blood, enabling its application to clinical cohort studies.

CD154 assay enables measurement of suppressive function in T cell lines

Large numbers of Treg cells are required to enable high-throughput screens of compounds or genes which modulate Treg function. The use of immortalised cell lines and a cheap and efficient suppression assay would greatly facilitate this. The Karpas-299 cell line has been reported to display Treg characteristics, including expression of Treg-associated genes, production of anti-inflammatory cytokines and suppressive function in classical MLR assays [34]. Karpas-299 cells express high levels of CD4 and CD25, and expression of FOXP3 was confirmed by intracellular flow cytometry (figure 5a,b). The addition of Karpas-299 cells at various Teffector:Karpas-299 cell ratios in the CD154 assay, resulted in suppression of CD154 induction on $CD4^+$ $CD25^-$ effector cell (figure 5c), with an average of 45% suppression of CD154 expression observed at a 1:1 ratio (figure 5d).

Membrane labelling of $CD4^+$ $CD25^-$ Teffector cells enables analysis of suppressive potential of induced Treg populations

High expression of CD25 in Treg populations is necessary to allow for their resolution from the $CD25^-$ effector cell population. However, some suppressor cell populations can have low to intermediate expression of CD25, such as $CD25^-$ Tr1 cells or *in vitro* generated induced Tregs (iTregs), hence an alternative to CD25 based gating is required. We therefore

investigated whether non-antibody based labelling of effector cells could be used. To test this, we generated iTregs from $CD4^+$ $CD25^-$ cord blood cells in the presence of IL-2, TGF-β and all-trans retinoid acid (ATRA). Culturing $CD4^+$ $CD25^-$ cells under these conditions generated iTreg cells that express FOXP3 (figure 6b) and are suppressive in CFSE and [^{3}H]-Thymidine assays (figure 6d). However, as the levels of CD25 expression on iTregs are highly heterogeneous, iTreg cells cannot be easily distinguished from $CD25^-$ Teffector cells in the CD154 assay. We initially labelled the $CD25^-$ cells with CFSE, however, CFSE labelling has been reported to decrease the viability of labelled cells and modulate the expression of activation molecules [36]. We observed that CFSE staining abolished induction of CD154 in response to stimulation (data not shown). An alternative cell labelling dye, $DIOC_{18}(3)$ has been shown to stably integrate into the cell membrane where it remains integrated for up to 48 hours [37]. $DIOC_{18}(3)$ staining has been reported to have little effect on cell viability, and in our hands it robustly labelled $CD4^+$ $CD25^-$ effector cell populations without affecting CD154 expression following stimulation (figure 6b). Using $DIOC_{18}(3)$ labelled effector cells, and assaying CD154 expression in the $DIOC_{18}(3)$- positive gate, we show that iTregs are able to suppress CD154 expression levels in Teffector cells. Two representative assays are shown in fig 6c, and a comparison of iTreg in all three functional assays is shown in fig 6d. Therefore, $DIOC_{18}(3)$ labelling appears to be an effective approach to resolve effector and suppressor populations in the CD154 assay. Using $DIOC_{18}(3)$ stained Teffectors has the added advantage of removing the need for antibody staining with CD4 and CD25 at the end of the assay, simplifying the assay set up and substantially decreasing the cost of the assay.

Discussion

Although widely accepted as the benchmark for human Treg function, the 5 day MLR suppressor assay is relatively complex and is sensitive to the degree of alloreactivity between donors. Generation of high quality and statistically robust data requires multiple assays to minimise the effects of donor to donor variation in both Treg and effector cell function. The cell labelling techniques required for each of these assays introduces additional variability, as wide differences in intensity and distribution of CFSE staining or thymidine scintillation counts are regularly observed between experiments. This creates additional difficulties for comparisons between experiments or for utilising these assays in a longitudinal clinical setting.

Using the [^{3}H]-thymidine incorporation method, DNA synthesis is measured in the last 16 hours of culture and function is therefore not measured across the whole 3-6 day co-culture. This means that any cells that have been suppressed or have died prior to the [^{3}H]-thymidine pulse will not be assayed. Additionally, the MLR using [^{3}H]-thymidine measures total cell proliferation in a mixed population of cells, which may not restrict the readout to the enumeration of division of the effector population in question, and iTreg, Treg like cell lines or Tr1 cells may not be as anergic as nTregs. Although regulatory T cells are generally anergic *in vitro* [38], Tregs will proliferate upon TCR stimulation in the presence of IL2, for example when cultured under expansion conditions or in the MLR itself [39]. CFSE pre labeling of effector cells in a mixed lymphocyte reaction is able to circumvent some of these issues, as it specifically tracks the proliferation of only the effector cell population in co-culture. However, staining procedures can be difficult to control, producing variation in cell viability, uniformity of labeling and mean fluorescence intensity, all of which are critical elements to enumerating proliferation. A further confounder for both MLR assays is in any long co-culture assay, stimulated effector cells may die from activation induced cell death or

IL-2 depletion. This can contribute to assay background, or cause cells which have not responded to stimulation to appear to be suppressed.

This suggests the need for a surrogate for these assays that is simple, rapid, scalable and can be read out on a flow cytometer. The observation that activated $CD4^+$ cells transiently up regulate CD154 on the cell surface following stimulation, with surface expression rapidly lost by internalisation provides such an assay readout. This phenomenon occurs within 4-8hr of stimulation well before activation-induced up regulation of CD25 on the cell surface. Although Tregs can influence proliferation of activated non-$CD25^-$ T cells in some immune settings, measuring suppression of proliferation *in vitro* with a short assay window is not possible. However, observing suppression of early activation marker expression in recently activated naive CD25- effector cells enables rapid identification of suppressive function. The assay utilises the addition of a fluorochrome-conjugated anti-CD154 antibody to the culture media, and although the CD154-antibody complex may be internalised upon surface protein turnover, by keeping the assay window short (7-8 hours), there is little or no degradation of the internalised antibody and hence no need to use agents such as monensin to block intracellular protein degradation machinery [32].

We have confirmed that Treg mediated suppression of CD154 expression correlates with suppression of proliferative activity by comparing the assay against CFSE and thymidine based MLR assays using donor matched CB Tregs. In this comparison, the suppressive functional readout by CD154 assay was equivalent to the degree of suppression observed with the MLR assays, indicating that the short assay correlates with the longer term proliferation results.

Many different markers have been used to sub divide Treg populations, including CD127, CD45RA and ICOS [35, 40-41], however these approaches have so far failed to capture all $FOXP3^+$ or suppressive cells present in peripheral blood. The $CD4^+$ $CD25^{Bright}$ gating strategy

has long been utilised to identify a population of Treg cells with strong FOXP3 expression, TSDR demethylation and suppressive function [41-45]. Utilising this strategy may yield a heterogenous population of Tregs with a spectrum of Ag experiences and phenotypes. We observed that the CD154 assay can measure the suppressive potency of Tregs isolated from peripheral blood by a $CD4^+$ $CD25^{Bright}$ gating strategy and this did not require the 5 day expansion prior to co-culture reported by Ruitenberg. A short incubation in IL-2 has been utilised to rest Treg cells post sorting [35], and when using this treatment we observed increased suppressive function and cell viability in the CD154 assay. We also investigated whether the CD154 assay could be utilised to test a cell line with reported suppressive function, Karpas-299. Although it is not yet clear whether Karpas-299 can be a useful surrogate for primary Tregs, the CD154 assay is able to report suppressive function by this cell line.

Lastly, we show that a simple non-toxic Teffector cell membrane labelling step with long-chain dialkylcarbocyanines such as $DIOC_{18}(3)$ allows for the easy resolution of Teffector cells in the assay co culture. This extends the application of this assay to include populations of Tregs with diverse CD25 expression, such as *in vitro* generated iTreg cells. Furthermore, labelling Teffector cells in this manner eliminates the need for surface staining for CD4 and CD25, further simplifying assay set up and analysis, rendering the assay more time and cost effective. Hence the CD154 assay is amenable to use with multiple Treg sub-populations.

Unlike other MLR suppression assays, only two cell populations are required in the CD154 assay, significantly reducing complexity of set up and reducing assay variability. This assay was initially developed using total PBMC as the effector cell pool [32], however we have now evaluated the use of purified $CD4^+$ $CD25^-$ cells as the effector population. Both PBMC and $CD25^-$ effector cell populations give sensitive responses to stimulation and can report induction of CD154 and its repression by Treg cells. A disadvantage of utilising PBMC is that not all of the cells are CD4+CD25- effector cells. This will introduce inaccuracies in

Teffector:Treg ratios and a high degree of donor variability. Purifying the effector cells allows for more accurate calculation of Teffector to Treg ratios enabling clearer visualisation of the suppressive effects by the Treg populations. In addition, using purified $CD4^+$ $CD25^-$ cells enables a 10-fold reduction in effector cells required and allows for banking of a homogeneous effector cell population. As donor variation in response to stimulation is commonly observed with primary human samples, using banked purified effector cells allows for storage from the same donor for multiple assays, which minimises this donor variable. Using purified effector cells also enables measurement of suppressive effects on the effector cell population directly and excludes any influence of the non- T cell compartments of peripheral blood.

Using the CD154 assay protocol with CD25- effector cells, 5- fold fewer Tregs are required than where PBMCs were used as the effector population [32]. As Tregs are often the limiting cell population for functional studies, lowering the number of cells required increases the possible applications of this assay, including the characterisation of different Treg subpopulations. In addition, a combined reduction of input cell numbers results in a significant reduction in the amount of antibody required, which would provide a major cost advantage when assaying large cohorts of samples.

A rapid and simple assay of suppressor function would be invaluable for applications such as high content screening for new drugs or antibodies to inhibit Treg function. In addition, an assay of acquisition or ablation of Treg function is essential for establishing importance of genes involved in regulating suppressive function. However, the existing 5 day MLR assays are not suitable for transfected populations, as expression of the gene of interest is often lost well before the assay endpoint is reached. The rapid activation readout, sensitivity to Treg suppression and robust staining with anti-CD154 antibody results in an assay amenable to

using transiently transfected cells. The ability to use a Treg cell line in the assay further increases its utility.

In summary, we find that the CD154 assay is a suitable alternative assay of *in vitro* Treg function, can report suppression by many Treg and Treg-like cells, and is fast, robust and reproducible. The CD154 assay could allow for direct high throughput screening of modulators of function in Treg cells for developing new therapeutics to either reduce Treg function (for infectious diseases or cancer therapy), to enhance Treg function (for organ transplantation and autoimmune disease therapy), or direct clinical applications, such as diagnostic screening or post-therapy monitoring of Treg cell function.

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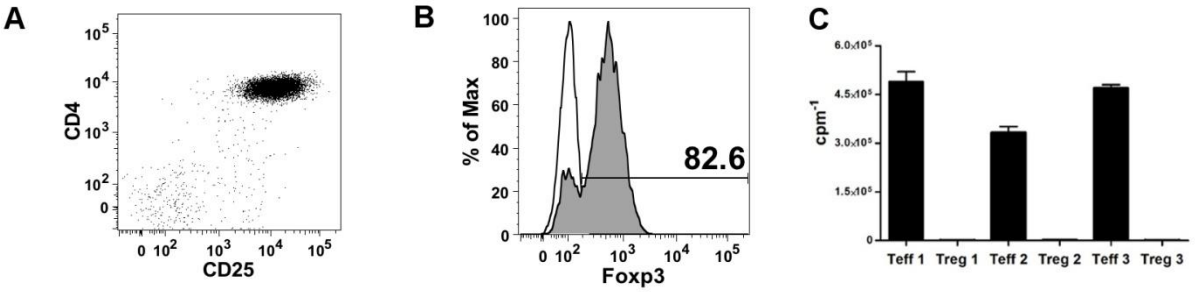

Figure 1: Expanded cord blood Tregs maintain Treg phenotypes. (A) After the 14 day culture period, CB Tregs express CD4 and CD25. (B) These cells robustly express FOXP3. (C) These cells are anergic by [^{3}H]-thymidine assay in the presence of irradiated PBMCs and OKT3 (anti CD3).

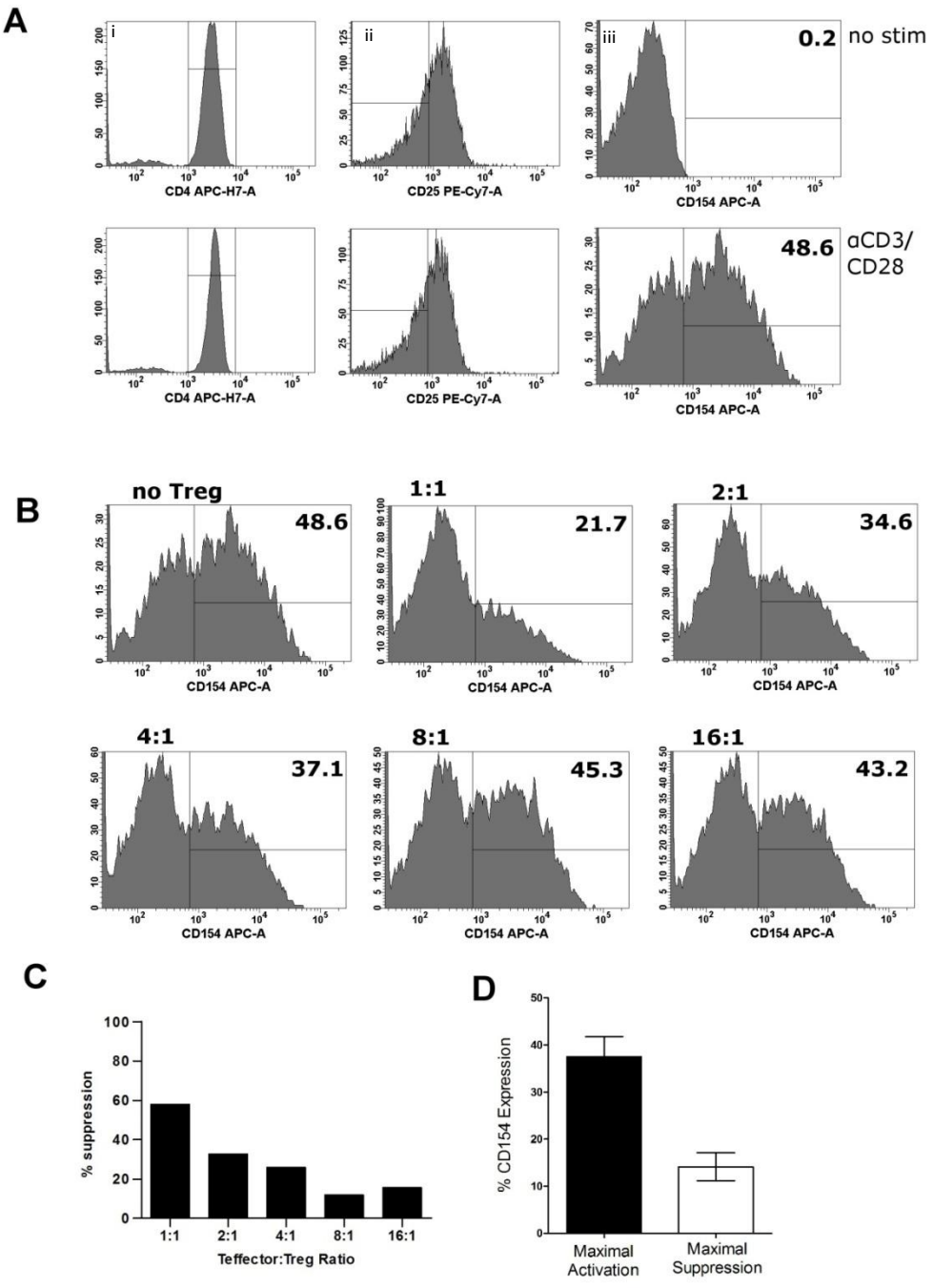

Figure 2: Expanded cord blood Tregs suppress Teffector cell CD154 expression. (A) Gating strategy for measuring CD154 expression on $CD4^+$ $CD25^-$ cells in the absence of CD25+Treg. $CD4^+$ cells are isolated (i), gated for low CD25 expression (ii) and then analysed for CD154 expression (iii). The CD154 positive gate is set using the unstimulated control. (B) Suppression of CD154 expression in $CD4^+$ $CD25^-$ by CB Tregs. Co- culture at various ratios of Teffector:Treg, one representative experiment (n=8). (C) Percent suppression of CD154 expression by CB Tregs, one representative experiment (n=8). (D) The range of CD154 induction by stimulation with anti- CD3/CD28 beads (maximal activation), and in the presence of Tregs at 1:1 ratio (maximal suppression). Average of 8 experiments with SEM.

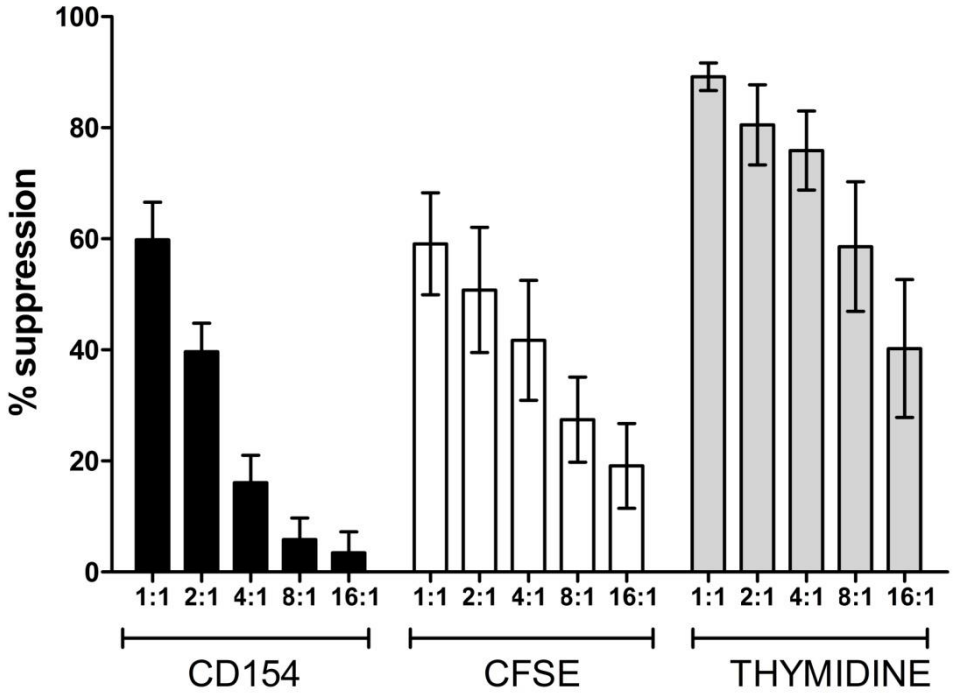

Figure 3. CD154 assay measures comparable suppressive function to CFSE and Thymidine Assays. Identical CB Treg samples were utilised, n=6, and percentage suppression was calculated for various Teffector:Treg ratios. Average of 6 experiments, with SEM.

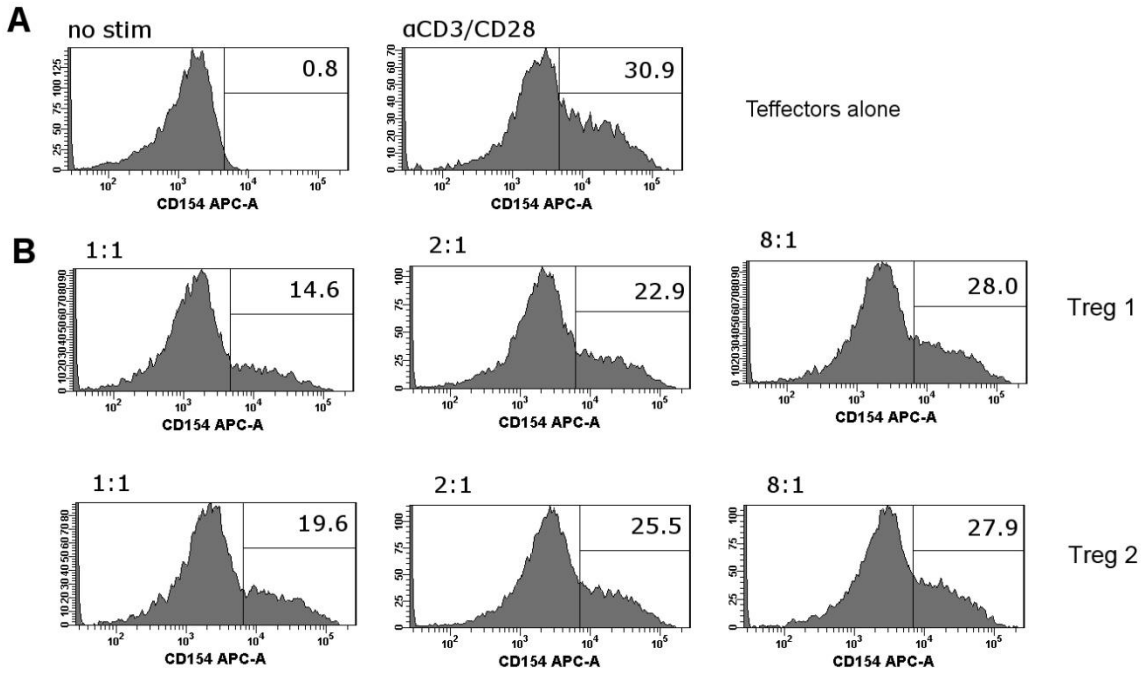

Figure 4. Adult PB Tregs suppress CD154 expression in Teffector cells. Adult Tregs were able to suppress CD154 induction in unmatched $CD4^+$ $CD25^-$ cells. (A) Teffector cells upregulate CD154 in response to stimulation with anti-CD3 and anti-CD28 beads. (B) Suppression by two representative Treg populations shown at various Teffector:Treg ratios n=4.

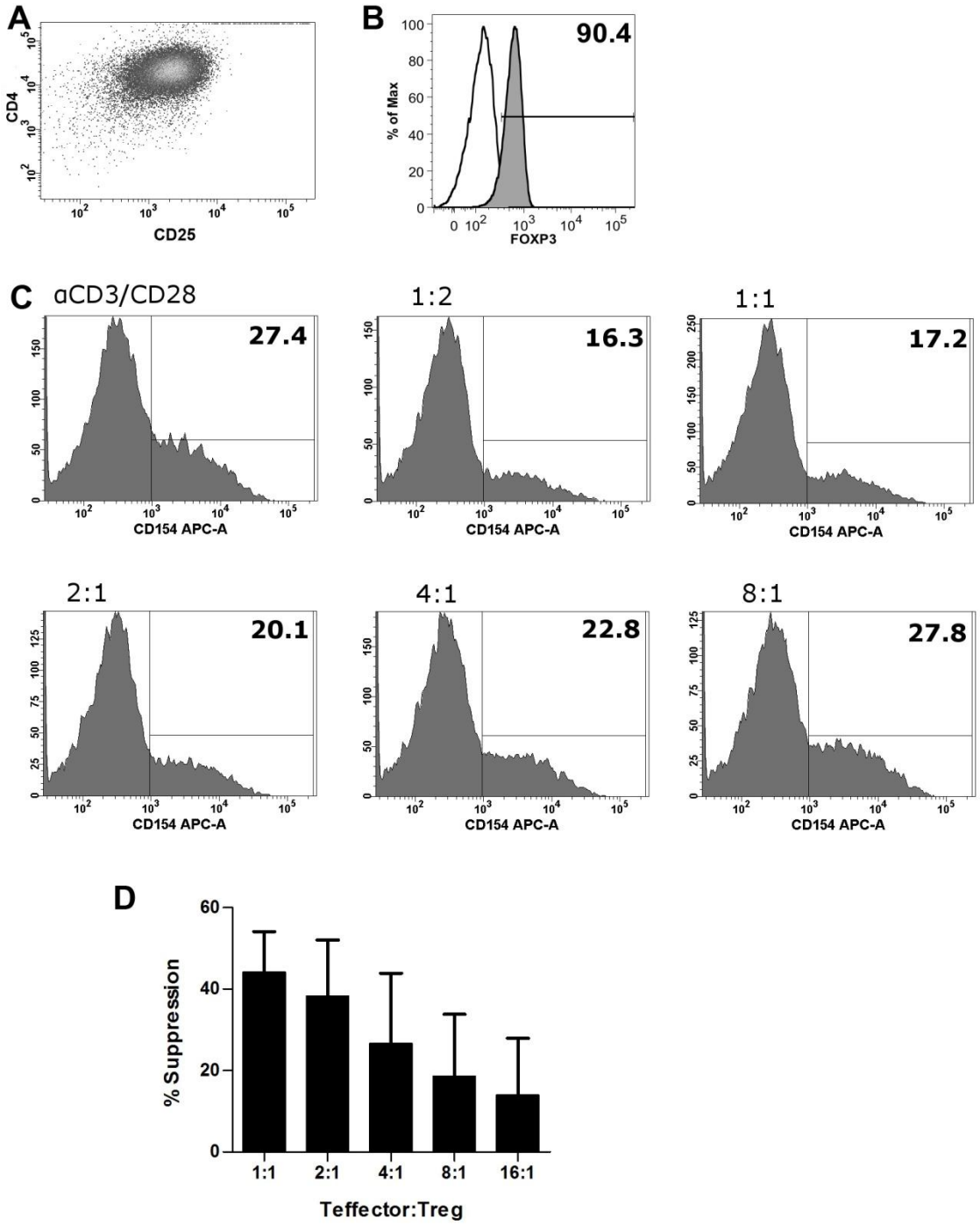

Figure 5: CD154 assay can measure suppressive function 'Treg- like' cell line KARPAS-299 cell line. (A) KARPAS-299 cells express CD4 and CD25, and (B) express FOXP3. (C) KARPAS-299 cells suppress CD154 expression in Teffector cells at various Teffector:Karpas-299 ratios, one representative experiment shown. (D) Percentage Suppression by KARPAS-299 cells measured by CD154 assay, average of three experiments with SEM.

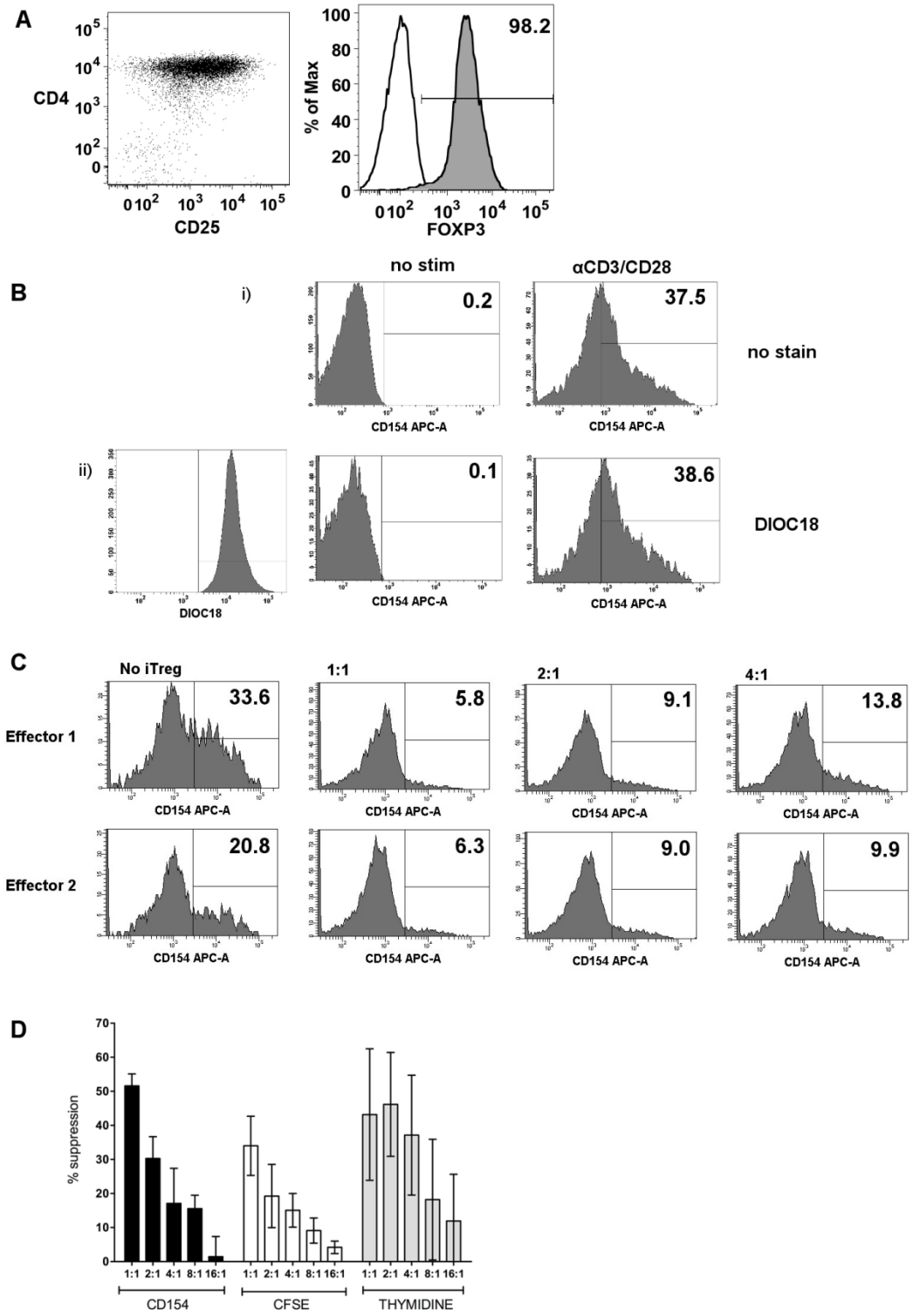

Figure 6: Membrane labelling of Teffector cells enables measurement of suppressive function by induced Tregs (iTreg). (A) CB induced Treg cultures show a range of CD25 expression and express high levels of FOXP3. (B) Staining $CD4^+$ $CD25^-$ Teffector cells with $DIOC_{18}(3)$ dye does not alter CD154 induction in response to antiCD3/CD28 beads. Using $DIOC_{18}(3)$ labelled Teffectors allows for resolution of Teffector cells from iTregs. (C) The addition of iTregs caused suppression of CD154 expression at various Teffector:Treg ratios in the $DIOC_{18}(3)^+$ fraction; two representative experiments, (n=4).

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