

Published in final edited form as:

Am J Transplant. 2011 August ; 11(8): . doi:10.1111/j.1600-6143.2011.03635.x.

Relative Resistance of Human CD4⁺ Memory T Cells to Suppression by CD4⁺CD25⁺ Regulatory T Cells

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Abstract

Successful expansion of functional CD4⁺CD25⁺ regulatory T cells (T_{reg}) *ex vivo* under good manufacturing practice conditions has made T_{reg}-cell therapy in clinical transplant tolerance induction a feasible possibility. In animals, T_{reg} cells home to both transplanted tissues and local lymph nodes and are optimally suppressive if active at both sites. Therefore, they have the opportunity to suppress both naïve and memory CD4⁺CD25⁻ T cells (Tresp). Clinical transplantation commonly involves depleting therapy at induction (e.g. anti-CD25), which favors homeostatic expansion of memory T cells. Animal models suggest that T_{reg} cells are less suppressive on memory, compared with naïve Tresp that mediate allograft rejection. As a result, in the context of human T_{reg}-cell therapy, it is important to define the effectiveness of T_{reg} cells in regulating naïve and memory Tresp. Therefore, we compared suppression of peripheral blood naïve and memory Tresp by fresh and *ex vivo* expanded T_{reg} cells using proliferation, cytokine production and activation marker expression (CD154) as readouts. With all readouts, naïve human Tresp were more suppressible by approximately 30% than their memory counterparts. This suggests that T_{reg} cells may be more efficacious if administered before or at the time of transplantation and that depleting therapy should be avoided in clinical trials of T_{reg} cells.

Keywords

Cell therapy; human; memory T cells; naïve T cells; regulatory T cells; suppression

Introduction

Transplantation is the treatment of choice for most end-stage solid organ diseases, with tangible benefits to survival and quality of life. Immunological recognition of polymorphisms in histocompatibility proteins leads to graft rejection between HLA-mismatched individuals. This necessitates the use of broad-spectrum pharmacological

immunosuppression (IS) that renders recipients susceptible to accelerated cardiovascular disease, metabolic complications, life-threatening infections and malignancy (1). In the long-term, immunosuppressive drugs are both directly toxic to transplanted tissues and inefficient at preventing chronic allograft rejection. The establishment of clinical tolerance to engrafted tissues to minimize or eliminate long-term IS is therefore, a key research objective.

Natural regulatory T cells (T_{reg}), expressing high levels of the IL-2 receptor chain α , CD25 are considered by many as ideal candidates for cell therapy for the induction of transplantation tolerance. Although their physiological function is the maintenance of tolerance to self-components, preventing autoimmune disease, T_{reg} cells can regulate CD4⁺CD25⁻ T cells (Tresp) responses to both cognate antigen and polyclonal stimuli *in vitro* and mediate allograft tolerance in experimental animals (2). In humans, numbers of graft-infiltrating T_{reg} cells during rejection correlate positively with lower inflammatory responses and better renal outcomes (3). The ability to expand autologous T_{reg} cells *ex vivo* under good manufacturing practice conditions (4) has made T_{reg} -cell therapy in protocols of tolerance induction a feasible possibility in the near future.

T-cell immunity, in particular, CD4⁺ T cells plays a central role in allograft rejection. Priming of naïve alloreactive T cells occurs in local lymph nodes (LN) before migration of effector cells to the transplanted tissue (5). In experimental animals, fate-tracking of injected antigen-specific T_{reg} cells reveals that they home to both transplanted tissues and the local draining LN (6) and that they need to act within both the LN and the tissue to effectively induce tolerance (7). In this way, injected T_{reg} cells have the opportunity to suppress both naïve and memory Tresp. Clinical transplantation commonly involves the use of depleting induction agents (e.g. anti-CD25 or Campath; Ref. 1), which favor expansion of memory and prolonged depletion of naïve T cells (8). Animal models suggest that T_{reg} cells have a lower capacity to suppress memory, compared to naïve Tresp that mediate allograft rejection (9). As a result, it is important to define in the context of human T_{reg} -cell therapy how effective T_{reg} cells will be in regulating naïve and memory Tresp. This information will inform the design of clinical trials of T_{reg} -cell therapy, as it will determine whether T_{reg} cells should ideally be administered before or after transplantation and with or without depleting agents. Therefore, we sought to compare the suppressive capacity of human T_{reg} cells, both freshly isolated and expanded *ex vivo* for cell therapy, on peripheral blood naïve (defined as CD45RA expressing) and memory (defined as CD45RO expressing) Tresp.

Materials and Methods

Cell separation and fluorescence-activated cell sorting

CD25⁺ and CD25⁻ CD4⁺ T cells were separated from human buffy coats as previously described (10). Naïve and memory Tresp were negatively selected using anti-CD45RO (Caltag, Burlingame, CA, USA) and anti-CD45RA, respectively (Diaclone Research, Besaucon, France) and anti-mouse magnetic beads (DynaL Biotech, Oslo, Norway). CD4⁺ T cells stained for CD4, CD25 and CD127 using a Human Regulatory T Cell Sorting Kit™ (BD-Biosciences, San Jose, CA, USA) were FACS sorted to CD4⁺CD25⁻ and CD4⁺CD25^{hi}CD127^{lo} cells, respectively, using a FACSAria (BD). Cell sorting efficiency for the populations of interest was routinely over 95%.

Expansion of T_{reg} -cell lines

Human CD4⁺CD25⁺ T cells isolated from peripheral blood mononuclear cell (PBMC) of healthy subjects were plated at 1×10^6 /mL in X-vivo 15 (Lonza, Basel, Switzerland) supplemented with 5% human AB serum (HS) (Biosera, Ringer, East Sussex, UK)

containing 100 nM Rapamycin (LC Laboratories, Woburn, MA, USA). Cells were activated with anti-CD3 and anti-CD28 coated beads (Dynal) at a bead:cell ratio of 1:1. IL-2 (1000 IU/mL; Proleukin[®], Novartis, Surrey, UK) was added at day 2 postactivation and replenished every 2 days. Beads were removed by magnetic adherence every 10 days postactivation and fresh anti-CD3 anti-CD28 beads (1:1 ratio), Rapamycin (100 nM) and IL-2 (1000 IU/mL) were added. Expanded cells were used for further analysis 30 days postactivation.

Cell culture and proliferation assays

Syngeneic Tresp (naïve and memory) and T_{reg} cells were incubated alone and in coculture in 96 well U-bottomed plates (Techno Plastic Products, Zurich, Switzerland) in 250 µL of complete medium (RPMI 1640; Invitrogen, Paisley, UK) with 50 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine (PSG; PAA Laboratories GmbH, Pasching, Austria) with 10% HS. Cells were activated with either anti-CD3/CD28 beads at bead:cell ratios from 0 to 0.8 chosen from previous experience, or by wells coated with anti-CD3 (2 µg/mL) and anti-CD28 (0–2 µg/mL; both R&D, Leeds, UK) monoclonal antibodies. All conditions were set up in triplicate for 3, 5 and 7 days before supernatant recovering and addition of 1 µCi/well ³H-thymidine (GE Healthcare, Bucks, UK). Cells were recovered 20 h later and incorporated thymidine measured using a 1205 Betaplate liquid scintillation counter (LKB Wallac, Turku, Finland). Alternatively, assessment of proliferation by flow cytometry on days 3 and 5 was done by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution of Tresp stained with 1 µM (CFSE; Invitrogen). 7-Amino-actinomycin D (7-AAD; BD) was added for 30 min at room temperature before flow cytometry and CFSE dilution of gated 7AAD⁻ cells was assessed. Cells were acquired in a total volume of 250 µL PBS containing 20 µL of Perfect-Count[®] microspheres (Cytognos, Salamanca, Spain) on a FACScalibur running CellQuest software (BD). Acquisition was limited by gating on Perfect-Count[®] microspheres, collecting 7500 events for each sample. The number of acquired cells in each 7-AAD⁻ CFSE peak was multiplied by the ratio of acquired to added counting beads prior to analysis. The numbers of nonproliferating (cells in the first peak) and proliferating precursors were calculated using standard formulae (11) (Figure S1) and the former expressed as the percentage of nonproliferating precursors from the total number of cells added. Suppression was expressed as the difference in frequency of nondividing precursors. Cell death was estimated by the sum of the proliferating and nonproliferating CFSE⁺ precursors subtracted from the number of cells originally added to the culture, expressed as a percentage of the latter.

Suppression of activation markers

FastImmune Regulatory T-cell Function kit[™] (BD) was used to estimate FACS sorted T_{reg} suppression of the activation marker CD154 on FACS sorted Tresp at 7 h, according to the manufacturer's instructions. Tresp were additionally stained with CD45RA-FITC and CD45RO-APC-H7 (both BD) before data acquisition on an LSRII (BD) running FACSDiva software. Percentage suppression of CD154 on gated memory (CD4⁺CD25⁻CD45RO⁺CD45RA⁻) and naïve (CD4⁺CD25⁻CD45RO⁻CD45RA⁺) Tresp was calculated according to the protocol from the manufacturer.

Cytokine estimation

IFN- γ , IL-2 and IL-10 in supernatants of cell cultures were estimated using a multiple cytokine assay kit (Meso Scale Discovery, Gaithersburg, MD, USA) according to the manufacturer's instructions. Data was acquired on a SECTOR[™] Imager 2400 (MSD).

Data analysis and statistics

Flow cytometric data was analyzed with FlowJo (Treestar Inc., Ashland, OR, USA). Statistical analysis was carried out on GraphPad Prism (Graph-Pad software Inc., San Diego, CA, USA) and Microsoft Excel 2008 (Microsoft Corporation, Redmond, WA, USA). Parametric and nonparametric data were calculated as the mean \pm SD and median (interquartile range, IQR), respectively. For comparison of parametric data, paired and unpaired *t*-tests were used (for paired and unpaired data sets). Comparison of central tendency for nonparametric data sets was made by using the Wilcoxon signed rank test for paired data, respectively. For comparisons of trends in proliferation between naïve and memory T cells, a repeated measures ANOVA was used for each condition (volume of expander beads) over the three time points. Regression lines of percentage suppression with increasing stimulation for naïve and memory cells were compared using an analysis of covariance (ANCOVA) at an interaction depth of 1. In all cases, *p* value of less than 0.05 was taken as denoting statistical significance.

Results

CD4⁺CD25⁺T_{reg} cells exert a greater suppressive effect on naïve than memory Tresp

Naïve (CD45RA⁺) and memory (CD45RO⁺) Tresp were activated in parallel with increasing anti-CD3 and anti-CD28 stimulation and dose–response proliferation was measured on days 3, 5 and 7. Proliferation of naïve Tresp was greater than that of memory Tresp at higher anti-CD3/CD28 stimulation (Figure S2), due to greater sensitivity to costimulation in the former and greater cell death in the latter (Figure S3). However, the kinetics of proliferation across the three time-points was the same in naïve and memory Tresp [comparing the two populations by repeated measures ANOVA at each level of stimulus (anti-CD3/CD28 bead:Tresp ratio; *p* > 0.05, not shown)]. Therefore, like time-point were directly compared for T_{reg}-mediated suppression.

Naïve and memory Tresp were cocultured in the presence and absence of 1:1 ratio of syngeneic T_{reg} cells and activated with increasing anti-CD3 and anti-CD28 stimulation (bead:Tresp ratio 0–0.8). Proliferation and suppression of proliferation was initially measured by ³H-Thymidine incorporation on days 3, 5 and 7. As expected, little suppression was evident on day 3 (2.4 \pm 11.9% vs. 16.8 \pm 12.7% for memory and naïve Tresp, respectively *p* NS, data not shown) but significant suppression was seen on days 5 (Figures 1A and B) and 7 (Figure 1C). Regression lines of suppression against stimulus on days 5 (Figure 1B) and 7 (Figure 1C) showed divergence of the regression lines for naïve and memory Tresp, which reached statistical significance on day 7 where on average the proliferation of naïve Tresp was 20–30% more suppressed than memory cells (Figure 1C). To confirm, CFSE-labeled naïve and memory Tresp were activated in the presence and absence of 1:1 syngeneic T_{reg} cells with increasing anti-CD3 and CD28 stimulus and proliferation estimated on days 3 and 5 as previously (Figures 1D-F). As before, suppression was consistently higher in naïve than memory Tresp (Figures 1D-F), being apparent at both days 3 and 5 (although more pronounced at the latter). This difference was not accounted for by an increase in Tresp death in the presence of T_{reg} cells (median increase in cell death –3% and –1% for naïve and memory Tresp, respectively, *p* NS).

To quantify differences in susceptibility to suppression between naïve and memory Tresp, the two populations were cultured alone and in coculture with T_{reg} cells in 1:1, 1:2, 1:3, 1:4, 1:8, 1:16 and 1:32 ratio (Figure S4) at constant Tresp numbers. Suppression of ³H-Thymidine incorporation on day 5 was calculated as per standard methods (Figure S4). For comparison of suppression, we used the IC₅₀ (T_{reg} cells:Tresp ratio needed to cause 50% suppression) and maximum suppression obtained at any ratio (*S*_{max}) were calculated for

each population as per the methods described in (Figure 2A; Ref. 12). A clear difference was seen between the suppression of naïve and memory Tresp, with the memory population having a higher IC_{50} (mean T_{reg} : Tresp ratio of 1.05 ± 0.08 vs. 0.28 ± 0.14 ; Figure 2B) and lower S_{max} (mean $50.9 \pm 1.5\%$ vs. $68.2 \pm 7.3\%$; Figure 2C) than naïve cells.

Both the 3H -Thymidine incorporation and CFSE dilution methods assess suppression at “late” time points and are, therefore susceptible to confounding variables such as T_{reg} -cell death and exhaustion of culture medium. To remove these confounders, we measured suppression through a nonproliferation dependent assay, upregulation of the activation markers CD154 at 7 h. FACS-sorted Tresp were activated in the presence or absence of cell-sorted syngeneic T_{reg} cells at Tresp: T_{reg} cell ratio from 1:1 to 32:1 and suppression of CD154 on naïve and memory Tresp was analyzed by flow cytometry (Figures 3A and B). Similarly, in the absence of T_{reg} cells expression of CD154 on naïve Tresp was greater than on their memory counterparts (both had negligible expression at baseline; Figure 3B). T_{reg} cells suppressed expression of this marker on naïve, compared to memory Tresp to a greater extent (Figure 3B). In dose–response, the S_{max} was again consistently higher for naïve compared to memory Tresp (mean 52.2 ± 5.6 vs. 34.0 ± 7.3); the IC_{50} for naïve Tresp was 0.89 ± 0.2 whereas that for memory Tresp could not be calculated as 50% suppression was not achieved in any experiment (Figures 3C and D).

In addition to proliferation, effector function is an important consequence of T-cell activation. Therefore, the T_{reg} -cell suppression of cytokine production by naïve and memory Tresp activated with anti-CD3/CD28 beads for 3, 5 and 7 days in the presence or absence of 1:1 ratio of T_{reg} cells was also assessed. In the absence of T_{reg} cells, IL-2 concentrations in supernatants of naïve and memory Tresp were comparable at each time point, with peaks on day 3 (Figure 4A). IL-2 production from both populations was suppressed by T_{reg} cells. That from naïve Tresp was consistently more suppressed than that seen in supernatants from memory Tresp. At each time point, naïve Tresp IL-2 production was at least 25% more suppressed by T_{reg} cells (Figure 4B). IFN- and IL-10 were almost exclusively produced from memory Tresp (data not shown), making direct comparison of suppression invalid. As expected, T_{reg} cells suppressed IFN- but not IL-10 production from memory Tresp (data not shown).

T_{reg} cell lines expanded for cell therapy have greater suppressive effect on naïve, compared to memory Tresp

In the above series of experiments, T_{reg} cells were freshly isolated from PBMC. However, for clinical cell therapy T_{reg} cells must first be expanded *in vitro* and then infused into patients as part of a tolerance induction protocol. Large-scale expansion of T_{reg} cells that retain suppressive ability and stably express FOXP3 *in vitro* is possible through expansion in the presence of the mTOR inhibitor Rapamycin (13). To test whether expanded T_{reg} cells retain the same suppressive profile on naïve and memory Tresp as freshly isolated T_{reg} cells, the latter were expanded *in vitro* in the presence of Rapamycin. These cells retained high expression of CD25 and FOXP3 and were more potently suppressive than freshly isolated T_{reg} cells (data not shown). CFSE-labeled naïve and memory Tresp were activated *in vitro* alone and in the presence of dose–response titration of either freshly isolated or expanded T_{reg} -cell lines. Suppression was calculated on day 5 and the IC_{50} and S_{max} determined as before (Figures 5A and B). Both the freshly isolated and expanded T_{reg} -cell line showed a reduced ability to suppress memory when compared to naïve Tresp, although both were suppressible (IC_{50} 2.0 vs. 0.79 for fresh and 0.61 vs. 0.39 for T_{reg} -cell line; S_{max} 33.2 vs. 55.0% for fresh and 56 vs. 75% for T_{reg} -cell line; Figure 5C). Further-more, T_{reg} cells expanded in the presence of Rapamycin demonstrated an increased suppression of both naïve and memory Tresp compared to freshly isolated T_{reg} cells.

Discussion

T_{reg} cells are promising agents for cell therapy in the clinical induction of tolerance to transplanted tissues. Although T_{reg} cells certainly inhibit naïve T cell responses *in vitro* and *in vivo* and can prevent allograft rejection in naïve mice (2) studies of skin transplantation in animal models suggest that they have a lower capacity to suppress memory than naïve CD4⁺ T cells mediating graft rejection (14). Here we have shown that human T_{reg} cells also have greater capacity to suppress naïve, compared to memory Tresp proliferation. This pattern was true of both freshly isolated and *in vitro* expanded, therapeutically relevant T_{reg} cells, although T_{reg} cells expanded in the presence of Rapamycin were more suppressive on both populations than freshly isolated T_{reg} cells.

To remove confounders relating to length of cultures or purity of bead-selected T_{reg} cells, we confirmed these findings by cell sorting and measuring surface expression of the early activation marker CD154 that is upregulated on naïve and memory Tresp 7 h after activation. In this setting, T_{reg} cells also exerted greater suppression of naïve than memory Tresp. To our knowledge, although there is a previous report on this subject, the definition of memory and naïve T cells in the publication were based on *in vitro* activation of CD4⁺CD25⁻ with alloantigen and no comparisons of the two were made (15).

Naïve and memory Tresp produce effector cytokines upon activation and reactivation, respectively (Th1, Th2 and Th17) whose activity determines outcomes. In these series of experiments, cytokine concentrations were used as surrogate markers for effector function. The only cytokine directly comparable between naïve and memory Tresp IL-2 was also more suppressed in the naïve population than the memory one by T_{reg} cells indicating that T_{reg} cells may have greater ability to suppress naïve Tresp cytokine production.

Although naïve and memory Tresp were enriched by negative selection, it is unlikely that cross-contamination could explain the differences observed between the populations. This is because the purities were generally greater than 90% for each population (data not shown) and the results for the two populations were clearly different. In addition, the FastImmune assay was carried out on FACS sorted cells.

Memory CD4⁺ T cells are clearly important in allograft rejection (16), having the ability to circulate through both secondary and nonlymphoid tissues, mount immune responses within the latter and survive independently of peripheral low-grade stimulation by MHC-self peptide complexes (17). Naïve T cells (defined as CD45RA⁺ expressing) may also be directly involved in allograft rejection. The pre-transplant frequency of alloreactivity between naïve and memory T cells are equal (18,19) and CD45RA⁺ T cells can not only be identified, but sometimes dominate in biopsy materials from acutely rejecting human organs (20). Because injected tissue-specific T_{reg} cells home to both transplanted tissue and local draining LN (6) and are optimally suppressive when functioning within both LN and tissue (7) they have the opportunity to regulate both naïve and memory Tresp. Greater suppressive ability of T_{reg} cells on naïve compared to memory Tresp has potential implications for clinical cell therapy given that induction therapy for human transplantation routinely involves protocols of depleting antibodies, resulting in expansion of memory T cells during reconstitution (8). The efficacy of expanded T_{reg} cells following transplantation may, therefore be less than anticipated. These findings suggest that one possibility to maximize the efficiency of T_{reg}-cell therapy is to consider T_{reg}-cell infusion either before or concurrently with transplantation without the use of depleting therapy for induction. Arguably, regulating the priming of naïve Tresp may also have a greater benefit on long-term allograft survival than regulating memory Tresp at a later time point. In addition, the difference in susceptibility to suppression may indicate a therapeutic window for adoptive T_{reg}-cell therapy in which naïve

alloreactive responses could be inhibited but pathogen-specific memory T cell responses might be preserved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. The authors acknowledge the support of the MRC Centre for Transplantation.

Funding source: This work was funded by grants from the Medical Research Council (to BA, PM, SJ, MH-F and RIL), National Institute for Health Research (JC), the British Heart Foundation and Guy's and St Thomas' Charity Trust (MH-F, RIL and GL).

Abbreviations

7AAD	7-amino-actinomycin D
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
CFSE	Carboxyfluorescein diacetate succinimidyl ester
FOXP3	Forkhead box P3
HLA	Human Leukocyte Antigen
HS	Human Serum
IC50	Half maximal Inhibitory Concentration
IS	Immunosuppression
LN	Lymph node
mTOR	Mammalian Target of Rapamycin
PBMC	Peripheral Blood Mononuclear Cells
Th	helper
Treg	Regulatory T cell
Tresp	Responder T cells

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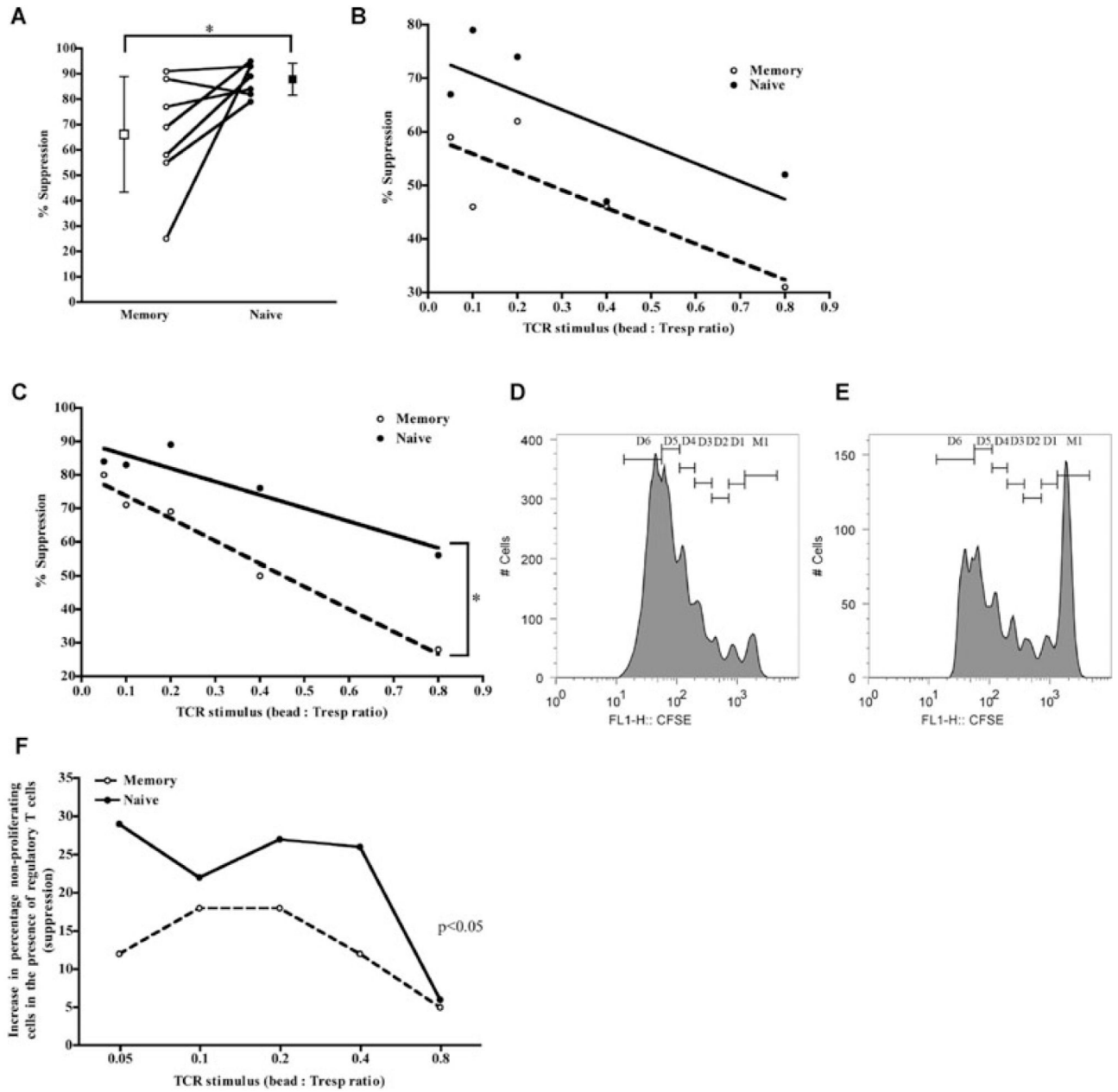


Figure 1. T_{reg} cells suppress naïve more than memory Tresp

(A–C) Suppression of ³H-Thymidine incorporation in naïve and memory Tresp by T_{reg} cells at 1:1 ratio. (A), plot showing pooled data from seven independent experiments taken from day 7 with a bead:Tresp ratio of 0.2. Mean percentage suppression against increasing stimulus for days 5 (B) and seven (C). Solid lines show regression for naïve while dashed lines indicate those for memory Tresp; *p < 0.05. (D and E) Suppression of CFSE dilution in Tresp by T_{reg} cells at 1:1 ratio. (D and –E) Representative CFSE profiles of Tresp in the presence (E) and absence (D) of 1:1 T_{reg} cells. (F) Suppression, expressed as the increase in the number of Tresp not dividing. (D and E) are representative examples, from day 5 of culture, from two independent experiments. Due to differences in absolute numbers between the two experiments, representative, rather than pooled data, have been shown.

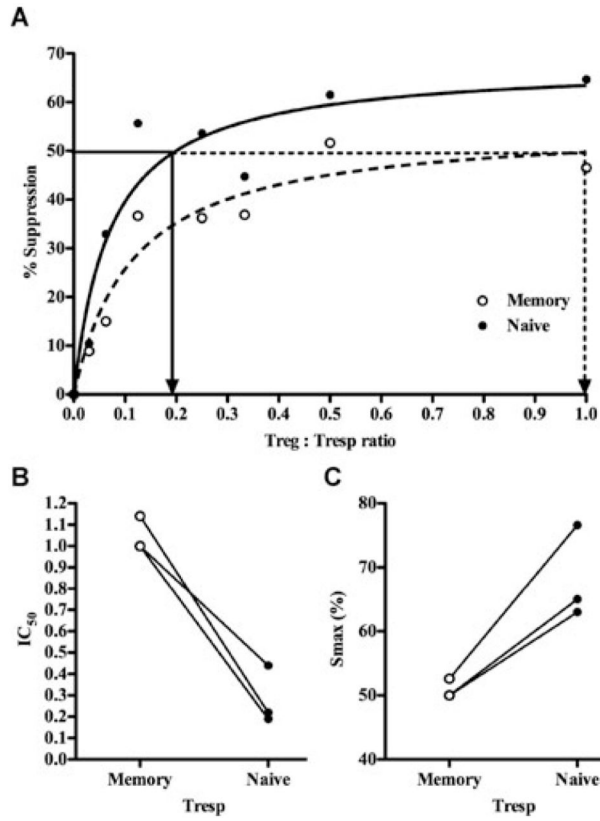


Figure 2. Suppression of naïve and memory Tresp by T_{reg} cells in dose-response
(A) Representative example from three independent experiments of suppression of ³H-Thymidine incorporation by naïve and memory Tresp at different T_{reg} cell:Tresp ratios (but constant bead:Tresp ratio of 0.2) for 5 days. Solid and dashed arrows show the IC₅₀ for naïve and memory Tresp respectively. (B and C) cumulative IC₅₀ (B) and S_{max} (C) for the three experiments. Lines join paired data.

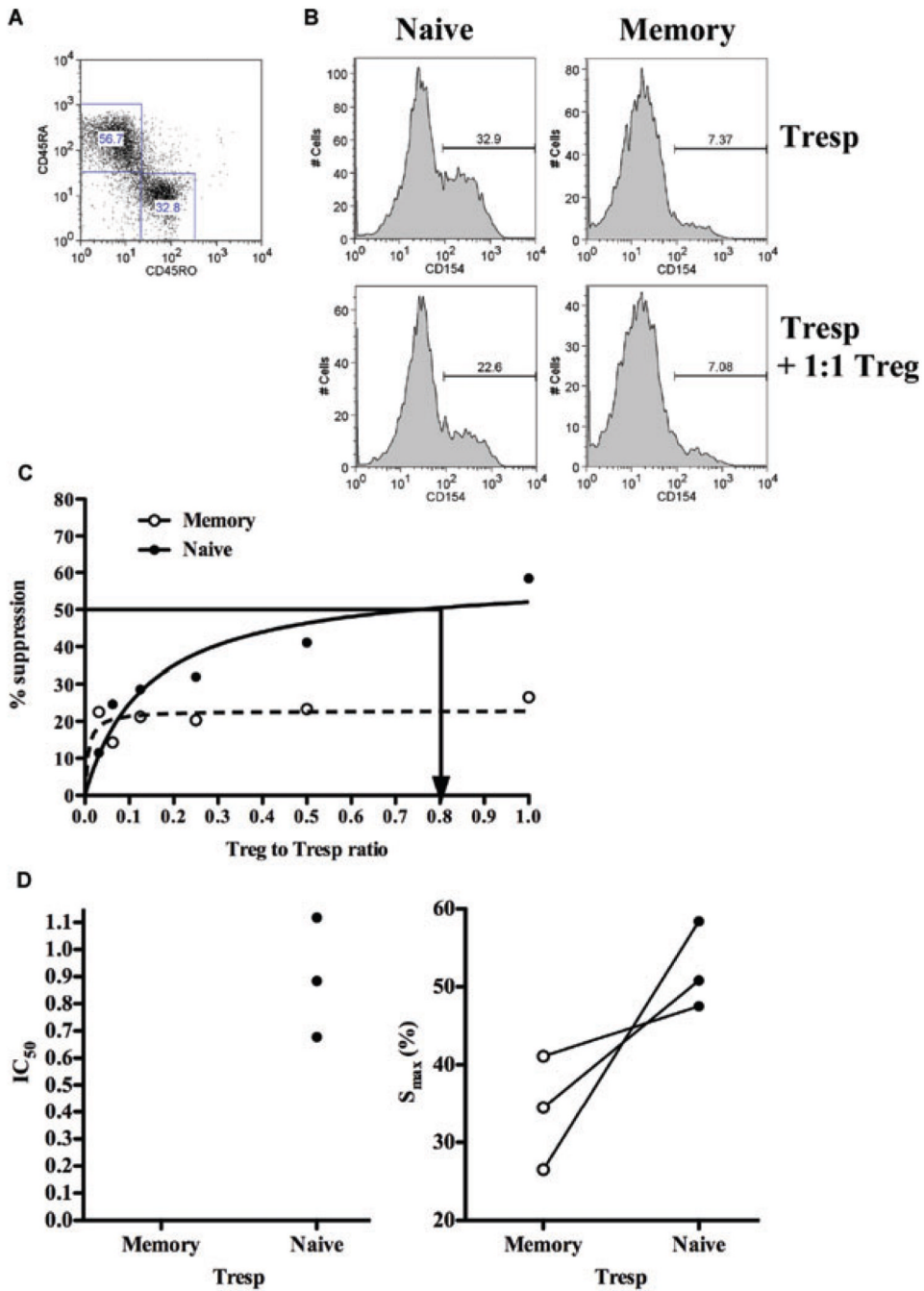


Figure 3. T_{reg}-cell-mediated suppression of early activation markers on naïve Tresp is greater than on memory Tresp
 (A) CD45RA and CD45RO gating strategy to identify naïve and memory Tresp respectively in cocultures of FACS-sorted Tresp and T_{reg} cells. (B), CD154 expression on naïve and memory Tresp after 7 h of stimulation (bead:Tresp ratio of 0.2) in the presence and absence of T_{reg} cells. (C), percentage suppression of CD154 expression on Tresp by T_{reg} cells at increasing T_{reg} cell:Tresp ratios. (A–C) are representative examples from three independent experiments. (D), cumulative IC₅₀ (left panel) and S_{max} from the three experiments.

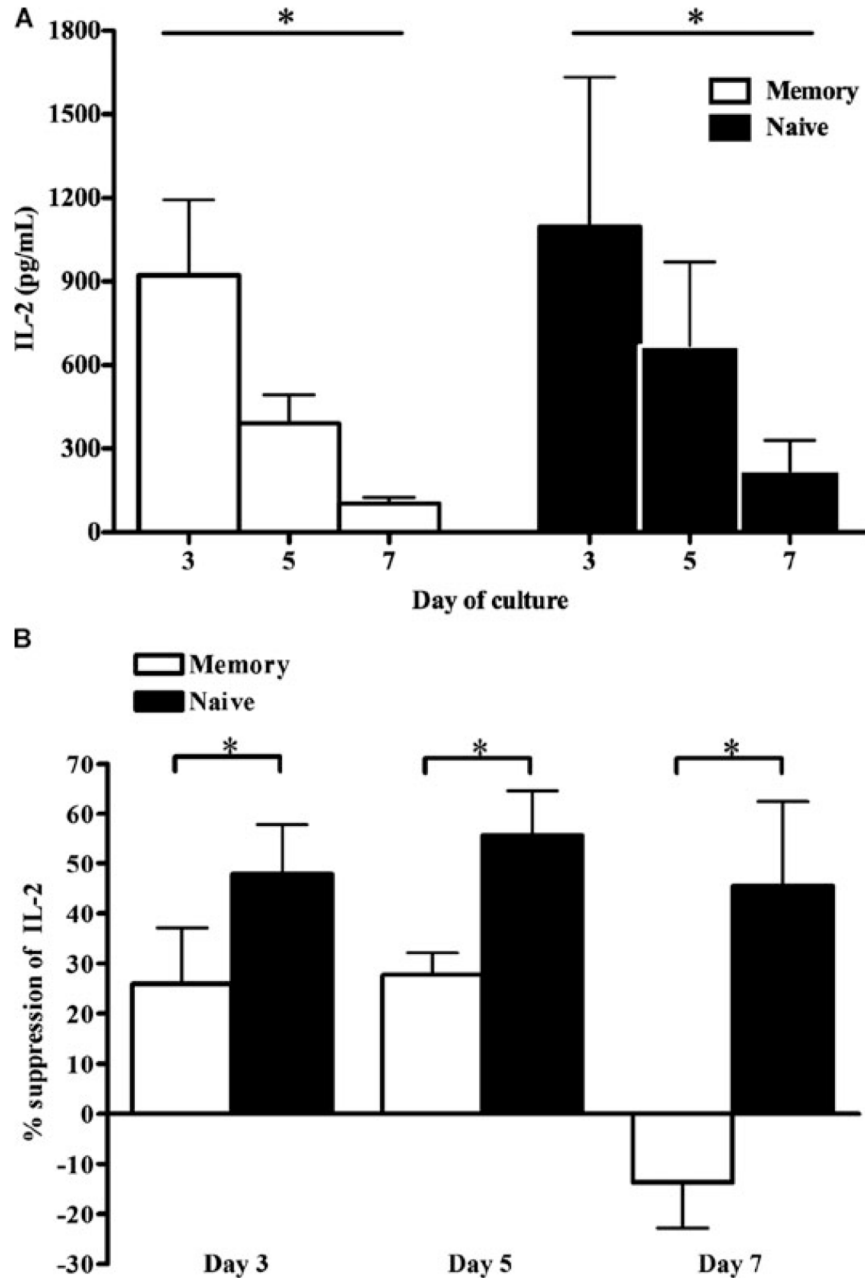


Figure 4. IL-2 production from naïve Tresp is more regulated by T_{reg} cells than those from memory Tresp

(A) IL-2 concentrations in supernatants of naïve and memory Tresp polyclonally stimulated with anti-CD3/CD28 beads (bead:Tresp ratio of 0.2) *in vitro* at days 3, 5 and 7. (B) Percentage suppression of IL-2 production from naïve and memory Tresp by T_{reg} cells at 1:1 ratio on days 3, 5 and 7. All graphs show pooled (mean ± s.d.) results from three independent experiments; *p < 0.05.

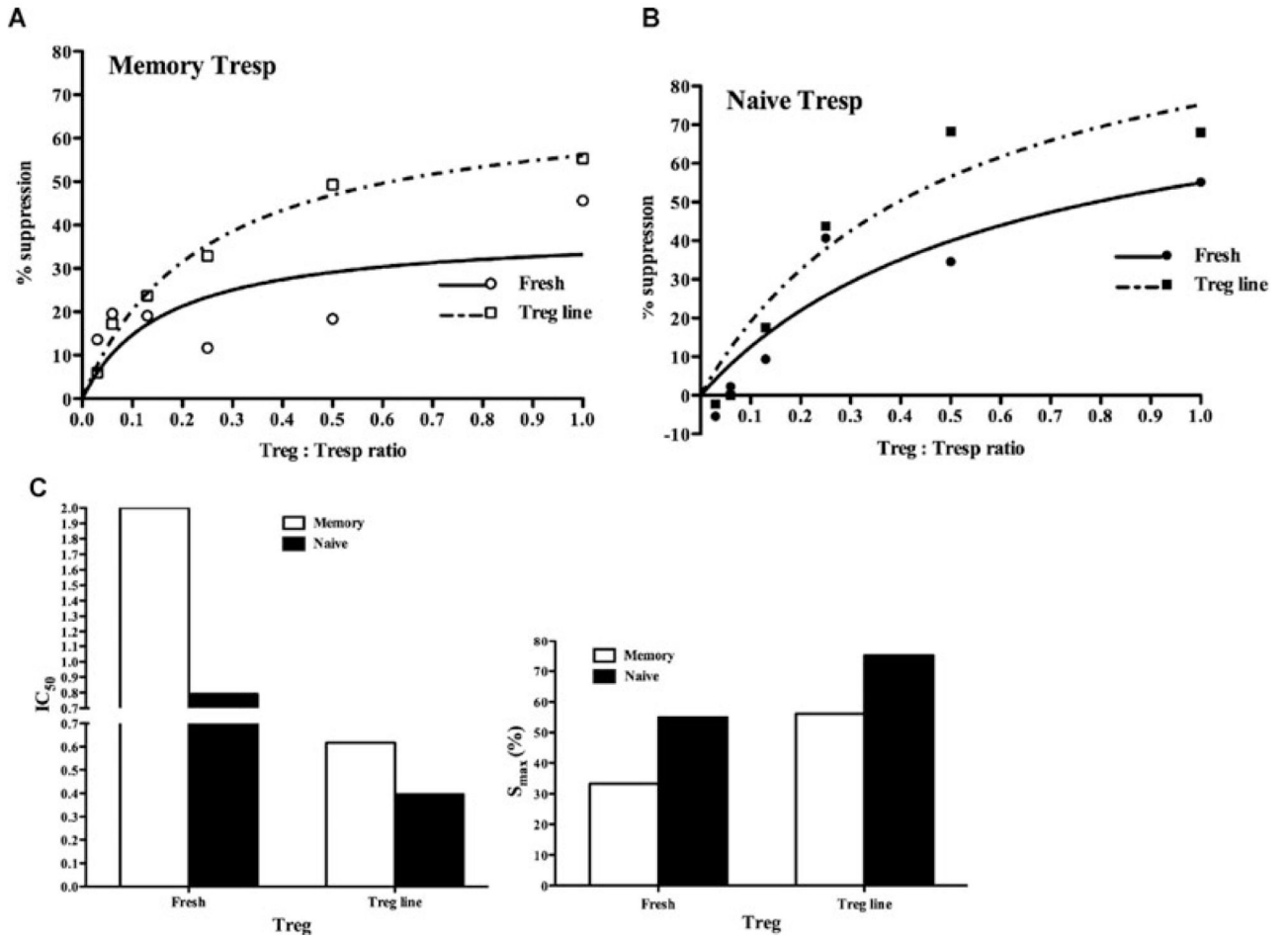


Figure 5. Rapamycin-expanded T_{reg}-cell lines suppress naïve Tresp more than memory Tresp (A) Percentage suppression of CFSE dilution in memory (A) and naïve (B) Tresp by fresh T_{reg} cells and a T_{reg}-cell line generated *in vitro* in the presence of Rapamycin. (C) IC₅₀ (left panel) and S_{max} (right panel).