PERIPHERAL GENERATION OF REGULATORY T CELLS IN HEALTH AND DISEASE

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DEDICATION

I would like to dedicate this work to my parents, Jothiprakasam and Mythili, for their sacrifices, support and encouragement for everything I wanted to pursue in life

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PERIPHERAL GENERATION OF REGULATORY T CELLS IN HEALTH AND DISEASE

by

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$CD4^+CD25^+FOXP3^+$ regulatory T cells (T_{reg}s) form an important arm of the immune system responsible for suppressing untoward immune responses. They play a role in autoimmunity, allergy, asthma, transplantation, tumors and infectious diseases. T_{reg}s are increased in the peripheral blood of chronic hepatitis C virus-infected patients and their depletion in-vitro increases anti HCV responses when measured by a sensitive CFSE-based flow cytometric proliferation assay. The CFSE-based assay, developed and validated by my laboratory, has a greater ability to detect low frequency and low avidity type T cell responses in the chronic HCV patients that are difficult to measure using ex-vivo assays. Using this

assay in a cross sectional study, I showed that anti-viral immune responses are attenuated in untreated chronic HCV patients and are increased after anti-viral treatment with Interferon and Ribavirin. Interestingly, increase in anti-viral immune responses after T_{reg} depletion was not seen in patients who were successfully treated with interferon and ribavirin. These results suggest that anti-viral therapy may be acting by modulating anti-viral immune responses.

T_{reg}s can be thymically derived (natural T_{reg}s) or peripherally induced (adaptive T_{reg}s). FOXP3 expression and in-vitro suppressive activity are considered unique hallmarks of this dedicated and stable lineage of regulatory cells. Initial evidence indicated that it is a specific marker of natural T_{reg}s. It was thought that $FOXP3^+$ T cells cannot arise in the periphery from naïve $CD4^+CD25^-$ T cells. However, using allostimulation of CFSE stained T cells and polycolor flow cytometry, I showed that virtually all human $CD4^+CD25^-FOXP3^-$ T cells and $CD8^+CD25^-FOXP3^-$ T cells attain a transient $FOXP3^+CD25^+$ state during activation. In this state of activation, these cells possess the classic phenotype of T_{reg}s, in that they express similar markers and inhibit in-vitro proliferation of autologous $CD4^+CD25^-$ T cells. This state is characterized by suppressed IFN-γ production and robust TNF-α and IL-10 production. Interestingly, the great majority of the activated cells eventually downregulate FOXP3 expression, with a concomitant drop in suppressive ability. However some of the $FOXP3^+$ T cells continue to maintain FOXP3 expression suggesting that activation might be a mechanism of producing $FOXP3^+$ Regulatory T cells in the periphery during viral infections like chronic HCV infection. Transient FOXP3 expression in activated T cells might also be a mechanism of fine tuning excessive immune activation. These results show

that, in humans, FOXP3 expression and T_{reg} functionality are not exclusive features of a stable or unique lineage of T cells, but may also be a transient state attained by almost all T cells. These results warrant caution in interpreting human studies using FOXP3 and suppressive activity as readouts and suggest that attempts to induce "T_{reg}s" may paradoxically result in induction of effector T cells, unless stability is confirmed.

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PUBLICATIONS

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ABBREVIATIONS

μg – micrograms

μl – microliters

μM – microMolar

γδ T cells – Gamma Delta T cells

Ab – Antibody

Ag – Antigen

APC – Antigen Presenting Cell

APC – AlloPhycoCyanin

BSA – Bovine Serum Albumin

CTL – Cytotoxic T lymphocyte

cDNA – complementary DNA

CD – Cluster of differentiation

CFSE – 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester

DC – Dendritic cell

DMSO – Dimethyl Sulfoxide

DNA – DeoxyRiboNucleic acid

EDTA – EthyleneDiamineTetraAcetic acid

EVR – Early viral Responder

FACS – Fluorescence Activated Cell Sorter

FBS – Fetal Bovine Serum

FOXP3 – Forkhead box P3

FITC – Fluorescien IsothioCynate

HBSS – Hanks Balanced Salt Solution

IFN-γ - Interferon Gamma

IBD – Inflammatory Bowel Disease

IL-2 – Interleukin 2

IL-10 – Interleukin 10

MLR – Mixed Lymphocyte Reaction

mRNA – messenger RNA

MS – Multiple Sclerosis

MHC – Major Histocompatibility Complex

ml – MilliLiter

MAB – Monoclonal Antibody

NKT cells – Natural Killer T cells

NR – Non Responder

PFC – Polycolor Flow Cytometry

PerCP - Peridinin-chlorophyll-protein

PE – PhycoErythrin

PMA - Phorbol 12-myristate 13-acetate

PBS – Phosphate Buffered Saline

RA – Rheumatoid Arthritis

RT-PCR – Reverse Transriptase Polymerase Chain Reaction

SEB – Staphylococcal Enterotoxin B

SVR – Sustained Viral Responder

T_{reg} – Regulatory T cell

TN – Treament Naive

TNF-α - Tumor Necrosis Factor Alpha

TGF-β - Transforming Growth Factor beta

TCR – T Cell Receptor

TSLP – Thymic Stromal lympho Poietin

CHAPTER ONE: INTRODUCTION

CENTRAL AND PERIPHERAL TOLERANCE

A central problem in immunology is how the body maintains immune responses against foreign antigens while being tolerant to self-antigens. Two concepts explain how the immune system distinguishes self from non-self; central tolerance and peripheral tolerance. T cells bearing a multitude of TCR's arise through a process of positive and negative selection in the thymus in the fetal life. Thymocytes are selected based on their self-reactivity. Thymocytes that do not react with any antigen are deleted due to neglect. Thymocytes strongly reactive with self are also deleted. These mechanisms, called clonal deletion, constitute central tolerance. Clonal deletion ensures broad T cell reactivity against pathogens while excluding potentially autoreactive T cells from the periphery. However, many self-reactive cells have been found in the periphery. Hence, it is thought that self-reactive T cells might survive thymic deletion mechanisms and can persist later in life. These self reactive T cells have the potential to cause autoimmunity if they are reactivated. Hence the need for multiple regulatory mechanisms that can suppress these autoreactive cells and prevent them from causing damage. These suppressive mechanisms constitute the peripheral tolerance. It has been hypothesized that failure of these suppressive mechanisms leads to autoimmunity.

REGULATION OF THE IMMUNE SYSTEM

Suppressor T cells

It had been suggested by RK Gershon and colleagues as early as in 1971 that there might be population of T cells, which can suppress other T cells by a dominant tolerance mechanism (2). They found that irradiated mice that received a mixture of bone marrow and thymocytes had weaker responses to sheep RBC's than mice who received bone marrow only. Other key studies showed that day 3 thymectomy resulted in the development of autoimmune oophoritis and thyroditis (3, 4). These autoimmune diseases could be prevented by transferring thymocytes from syngeneic mice. It was later determined that loss of T cells from the thymus were responsible for the autoimmune disease (5-7). Evidence from other models indicated that both $CD4^+$ and $CD8^+$ T cells were capable of suppression. However, the mechanism of suppression was not clear. Sakaguchi and colleagues showed that a negatively selected $CD4^+CD5^+$ subset was capable of causing autoimmune disease when transferred into athmyic mice. Co-transferring un-depleted T cells seemed to protect from autoimmune disease. Hence, soluble factors secreted by suppressor T cells might play a role in the mechanism of suppression. The MHC restriction of the suppressor T cells was not clear and many studies suggested that a hypothetical I-J region on chromosome 17 between I-E_α and I-E_β was important for suppressive ability. However when the mouse MHC was sequenced it became clear from molecular studies that the hypothetical I-J region could not be present in the predicted MHC region. Hence, the existence of suppressor T cells came to be questioned and it quickly became the dirty "S" word in immunology (8).

RE-INCARNATION OF SUPPRESSOR T CELLS:T_{REG}S

Suppressor T cells have had a re-incarnation as regulatory T cells in recent years primarily through some seminal work by Sakaguchi and colleagues. Their experiments revealed that a subset of $CD4^+$ T cells expressing the IL-2 receptor alpha chain CD25 is crucial in maintaining tolerance to the self in the periphery (9-11). CD25 expressing T cells constituted 10% of $CD4^+$ and 1% of all T cells in mice. $CD4^+$ T cells expressing CD25 appear in the periphery on the third day. Day 3 neonatal thymectomy eliminate these CD25 expressing populations and abolished the protective effect of CD25 expressing populations (11). Transfer of $CD25^-$ cells from 3-day-old mice or adult mice into adult athymic nude mice can produce autoimmune disease in the recipient nude mice (10). They also showed that removal of $CD25^+$ T cells increased responses against both self and non-self antigens. Hence, they hypothesized that the CD25 expressing subset prevents the activation of responses to both self and non-self antigens in the periphery. Identification of the $CD4^+CD25^+$ T cell subset was a major advance in the field, which allowed us to study regulatory cells in detail. Though CD25 was the best surface marker for regulatory T cells, its use was limited by the fact that it was also expressed on activated T cells in humans and mice. Other suggested markers like CTLA-4, GITR, and CD44 also could not accurately identify T_{reg}s from activated T cells. Hence, the search for even more specific markers for T_{reg}s continued.

FOXP3 as a marker to identify natural thymically generated T_{reg}s

Early reports in humans suggested that an X-linked disorder was associated with fatal multiple autoimmune dysfunction in infancy (12). It was also noted that the scurfy mutant

mice suffered from multiple autoimmune diseases similar to the day 3 thymectomized mice. The scurfy mutation was a spontaneous mutation (that arose in 1959) in an X linked gene which resulted in lymphocytic infiltration and enlargement of multiple organs (13). Eventually it was determined mutations in the gene encoding scurfin (Foxp3) was responsible for the syndrome (14). *Foxp3* gene from transgenic mice was able to rescue mice from the autoimmune phenotype when they were crossed into scurfy mutant mice. Positional cloning studies in humans also identified mutations in FOXP3 as the cause of IPEX syndrome (15-17). Eventually FOXP3 was shown to be crucial in the function and development of regulatory T cells in mice and humans (18-21). FOXP3 was highly expressed in $CD4^{+}CD25^{+/high}$ T cells. Retroviral transduction of FOXP3 converted naïve T cells in both humans and mice to express regulatory phenotype and function. Deletion of FOXP3 in adult mice also led to a similar autoimmune disorders showing the continuing importance of FOXP3 expressing cells later in life (22). FOXP3 belongs to the forkhead family of DNA binding transcription factors. Other members of this family like Foxo3a, Foxj1 and Foxn1 all have play important role in T cells and NFkB signaling as evidenced by the immunological phenotypes of their knockouts. However, FOXP3 had some limitations with its use as a marker for T_{reg}s. FOXP3 could only be identified intracellularly after permeabilization of cells. Hence, its current use is limited to phenotyping of the dead cells and not functional studies with live FOXP3 expressing cells. We can however isolate enriched populations of $FOXP3^{+}$ T cells based on other markers like CD25 and then use them in functional studies after checking their FOXP3 expression in an aliquot of the sample.

PROPERTIES OF T_{REG}S

Early in-vivo experiments on suppressor T cells suggested a soluble factor mediated suppression. However, further elucidation of the mechanism of suppression was not possible at that time. Development of an in-vitro T cell suppression assay has allowed us to dissect this process in-vitro (23, 24). In this system, regulatory $CD4^+CD25^+$ T cells are co-cultured with responder $CD4^+CD25^-$ T cells in varying ratios. Robust suppression is seen with a 1:1 or even a lower ratio depending on the strength of the primary stimulation and co-stimulation used. Polyclonal or antigen specific activation through the TCR was necessary for the suppression. Though activation was specific, suppression seemed to be non specific (25). Suppressor T cells were able to suppress the proliferation of both $CD4^+$ and $CD8^+$ T cells.

T_{reg}s were also found to be anergic in both proliferation and cytokine secretion in restimulation experiments in-vitro. Though T_{reg}s are anergic in-vitro, studies have shown that they do proliferate in-vivo (26, 27). High dose IL-2 can break the anergy and abrogate the suppression mediated by T_{reg}s in the in-vitro suppression system (28). T_{reg}s also do not seem to have a defined single antigen specificity in peripheral blood. They are polyclonal rather than monoclonal in their TCR repertoire. They are more specific against self-antigens rather than foreign antigens suggesting that their primary function might be to limit immune responses against the self in the periphery (29). Their TCR repertoire is very similar to that of naïve T cells reflecting perhaps the similarity in their development (30).

MECHANISMS OF SUPPRESSION

The exact mechanism of suppression continues to elude us. The mechanisms of suppression seems to differ between in-vitro and in-vivo systems (Fig. 2) (28). Contact seems to be necessary for suppression in-vitro because suppression is abolished when the suppressor and the effector cells are separated by a semi-permeable membrane that allows soluble factors to cross but not cells (31). Supernatants from in-vitro suppression assays also cannot mediate suppression. However, it is not clear, if T_{reg}s act by modifying APC's or if they directly modulate responding cells. These experiments also leave open the possibility that a short acting or a membrane bound soluble factor might be mediating the suppression.

Introduction of antibodies against TGF-β or IL-10 did not prevent suppression in in-vitro assays. T_{reg}s from IL-10 or TGF-β deficient mice can still suppress arguing against a role for these cytokines in the suppression (31). However, surface TGF-β seems to be necessary for suppression. Nevertheless, IL-10 has been shown to be important for suppression in an inflammatory bowel disease model and TGF-β has been shown to be important in other disease models. Anti IL-10 or anti TGF-β treatment abrogated suppression by regulatory T cells and resulted in autoimmune disease in both models.

Though the apparent difference between in-vitro and in-vivo is puzzling, a short range acting secreted or membrane bound cytokine has been suggested to be responsible for the suppression as a compromise (32). There are also reports suggesting that membrane bound TGF-β is more important for suppression. Multiple non-redundant mechanisms of

suppression or the presence of non-homogenous T_{reg} population may also be the cause of these conflicting findings.

ORIGIN OF T_{REG}S

Evidence from experiments in mice indicates that T_{reg}s arise in the thymus (33). T_{reg}s are formed from double positive thymocytes at the same time as the single positive naïve T cells through similar mechanisms. They arise at the double positive stage when $CD4^+CD8^+$ thymocytes undergo positive and negative selection on the thmyic cortical epithelium. They require TCR and MHC class II interactions for them to arise in the thymus. T cells, which have a moderate/high affinity, preferentially go on to become regulatory T cells (34, 35). However, the close parallel between the origin of T_{reg}s and naïve T cells in the thymus also suggest that there might not be anything intrinsically different between the two cell lineages (30).

Mouse studies indicate that de-novo origin of T_{reg}s is possible under special conditions. Hence, two models are proposed to explain the origin of T_{reg}s. In one model T_{reg}s arise in thymus and are expanded in the periphery. While in the other model T_{reg}s arise de-novo in the periphery and persist during antigenic stimulation. Most of the evidence in mice is indicative of a model where thymic origin would be predominant. In humans, evidence for the thymic origin is limited. Thymic organ culture studies indicate that T_{reg}s arises from Hassall's corpuscles expressing TSLP (Thymic stromal lymphopoietin), which then activate dendritic cells to form $FOXP3^+$ T cells. (36). However, the use of a strong T cell activator like TSLP without other controls could merely means that strong T cell activation by activated dendritic cells can result in the formation of $FOXP3^+$ T cells.

Moreover, in humans, FOXP3 can be expressed on activated $CD4^+CD25^-$ T cells. $FOXP3^+$ T_{reg} can also be formed on activation by TGF-β raising the question whether peripheral induction of T_{reg}s is possible. The source and origin of T_{reg}s in humans is currently a very controversial topic. Most immunologists lean towards the view that $FOXP3^+$ T_{reg}s arise from the thymus in humans. The exact contribution of thymically derived and peripherally derived T_{reg}s in the periphery is not known (32).

DIFFERENT SUBSETS WITH REGULATORY PROPERTIES

In recent times, other subsets of cells have also been known to possess the capability to regulate T cells (Fig.1) (32). The mechanism of suppression varies from subset to subset. Many of these cells can be induced in the periphery under special conditions. TGF-β plays a crucial role in the induction of cells with regulatory properties. In humans and in mice, culturing in the presence of TGB-β converts naïve FOXP3- T cells into FOXP3 expressing $CD4^+CD25^+$ regulatory T cells (37-40). The strength of antigenic stimulation and the maturation status of DC's are other conditions that modulate the formation of cells with regulatory properties. Low dose antigenic stimulation also induced the formation of regulatory T cells in the periphery (41, 42) The exact relationship between peripherally induced regulatory T cells and the classical FOXP3 expressing $CD4^+CD25^+$ T_{reg}s is not clear.

Other $CD4^+$ T cells that possess regulatory capacity include the previously known IL-10 secreting Tr1 and TGF-β secreting Th3 T cells. Both Tr1 and Th3 subsets are not known to express FOXP3. Tr1 cells are produced following TCR stimulation with cognate antigen in the presence of IL-10 (43). They can also be produced by culturing in the presence of

dexamethasone and vitamin D (44). They are characterized by secretion of IL-10, which mediates the suppression. They are induced in-vivo as a response to infection in the periphery and hence it is thought that they may play important roles in preventing local immunopathology following an immune response. Tr1 cells were also found to prevent colitis in mice and man.

Oral tolerization regimens have been found to induce suppressor T cells in the past (45, 46). Special tolerizing properties of gut APC's are thought to be important in the induction of suppressor T cells by oral antigens. TGF-β secreting Th3 cells were later found to be induced by oral tolerization regimens. The suppression of both Tr1 and Th3 cells can be blocked by addition of anti TGF-β or anti IL-10 antibodies.

The suppressor T cells of the past were predominantly $CD8^+$ T cells. Current resurgence of regulatory T cells has mainly focused on $CD4^+$ regulatory T cells though there are a few reports about $CD8^+$ regulatory T cells. The negative connotation associated with suppressor T cells of the past has probably hindered research on $CD8^+$ Regulatory T cells. Evidence from EAE models in mice indicate that a Qa-1 restricted subset of $CD8^+$ T cells have regulatory properties (47). Qa-1 molecules on activated CD4+ T cells present self-peptides to $CD8^+$ T cells. Some of these $CD8^+$ T cells then acquire the capability of suppressing those Qa-1 expressing $CD4^+$ T cells. This subset of cells may also be active in humans through the analogous non-classical HLA-E molecule. The exact mechanism of suppression of Qa-1 restricted $CD8^+$ T cells has not been elucidated. Cytotoxic mechanisms might turn out to be more important for suppression by $CD8^+$ regulatory T cells.

$CD8^+CD28^-$ T cells are another subset of $CD8^+$ T cells which are capable of suppressing $CD4^+$ T cells (48, 49). They arise under allostimulatory condition and express FOXP3 unlike other regulatory $CD8^+$ T cells. However, their mechanism of suppression seems to differ from other subsets. They seem to suppress by inducing immunoglobulin like transcripts ILT-3 and 4 in APC's.

Other subsets with regulatory properties include the NKT cells, γδ T cells and the $CD4^-CD8^-$ double negative T cells (32, 50). Hence, the property of suppression is shared by numerous subsets of cells. Their mechanisms of induction and suppression and their unique identifying characteristics are very heterogeneous. The field of regulatory T cells continues to grow and become complicated. A unified theory of suppression has been suggested to assimilate all these subsets into classifications based just on the property of suppression (32).

ROLE OF T_{REG}S IN VARIOUS DISEASES

T_{reg}s have been hypothesized to be involved in many models of autoimmune diseases, allergy, asthma, tumors, transplantation, graft vs. host disease and infectious diseases. In fact, as explained earlier, most of the initial research and discovery of T_{reg}s were done in mouse models of autoimmunity like EAE. Data from most of the models of autoimmune disease in mice indicated a strong role for naturally occurring T_{reg}s in the prevention, progression or therapy of the disease. In humans, there is a dysregulation in either the number or function of regulatory T cells in many autoimmune disorders like multiple sclerosis, juvenile idiopathic arthritis, rheumatoid arthritis and polyglandular syndrome type II (51). Autoimmune diseases are one of prime targets for T_{reg} modulation currently.

Mouse models of tumors clearly indicate a role for T_{reg}s in cancer (52). Tumor immunity in fibrosarcoma and melanoma models was shown to be suppressed by T_{reg}s (53, 54). In humans, increased frequency of $FOXP3^+$ T_{reg}s were found in patients with solid tumors like ovarian and head and neck malignancies (55, 56) or hematological malignancies like lymphomas and leukemias (57, 58). Hence, in contrast to the situation in autoimmunity, it has been hypothesized that presence of regulatory T cells suppresses the immune response against tumors and allows the tumor to grow. Hence, presence of T_{reg}s is detrimental to the host in this situation and their depletion early in the disease course or locally from the tumor would aid in clearing the tumor (59, 60).

IL-10 secreting regulatory T cells are involved in viral, bacterial and parasitic infections in mice (61, 62). Co-transfer of regulatory T cells was found to prevent immune responses against pathogens in an inflammatory bowel disease model. It was also noted that bacterial, viral and fungal infections induced FOXP3 expressing regulatory T cells. Depletion of regulatory T cells before the infection allowed the pathogen to be cleared but also caused immune damage to tissues. Based on these experiments, it has been suggested that regulatory T cells might actually be limiting an immune response against the pathogen. In chronic infection models like the friend virus model, such a regulation allows the pathogen to persist in the body (63, 64). In humans, too, there seems to be a dysregulation in the number or function of regulatory T cells in HCV, HBV, EBV and HIV infections (65-70). In HIV infection there is conflicting evidence as to the role of T_{reg}s in the disease progression.

STRATEGIES TO MODULATE T_{REG}S

Great interest in T_{reg}s can be attributed to their global involvement in varied disease process and potential for therapeutic manipulation in those diseases. While T_{reg} augmentation might be useful in autoimmunity, transplantation, allergy and asthma, T_{reg} depletion might be of use in infectious diseases, tumors and graft vs host disease.

T_{reg}s can be augmented by two approaches. Firstly, they can directly be expanded in-vivo through treatment with various agents. In fact, there is evidence to indicate that many agents found to be effective in the treatment of autoimmunity, transplantation and allergy may actually be working by inducing T_{reg}s. Many allergen desensitization therapies induce immune deviation from a Th2 to an IL-10 producing T_{reg} phenotype (71). Glucocorticoids, immunosuppressive drugs, vitamin D and mycophenolate mofetil can all induce IL-10 secreting regulatory T cells (72, 73). Glatiramer acetate, a commonly used immunomodulatory agent for the treatment of relapsing remitting multiple sclerosis, also induces regulatory T cells (74, 75) .

The second way of increasing T_{reg}s in the body would be to isolate them from peripheral blood and then expand them in-vitro using various agents and then therapeutically use them to achieve the desired effect. Protocols have been developed for efficient isolation of $CD4^{+}CD25^{high}$ cells from PBMC's using magnetic beads and/or flow sorting and expanding them in-vitro by using combinations of anti-CD3/anti-CD28, IL-2, anti-CD28 superagonist etc (76-80). However, many of these approaches indiscriminately expand T_{reg}s with varying antigen specificities. Hence, efforts are also being made to expand antigen specific T_{reg}s by culturing the presence of the relevant antigen. Potential problems with all of

these approaches include the expansion of effector T cells due to impurity of the starting population and the need for extensive manipulation in-vitro before introducing them in-vivo.

T_{reg} depletion can be achieved by using various agents to deplete them in-vivo. Particularly depletion of T_{reg}s in tumor using anti-CD25 monoclonal antibodies, anti CTLA-4 antibodies holds great promise (59, 81). However, these approaches carry a risk of removing activated effector $CD25^{+}$ T cells along with the regulatory T cells in humans. In addition, continuous or repeated treatment may be necessary to maintain a T_{reg} deficient state.

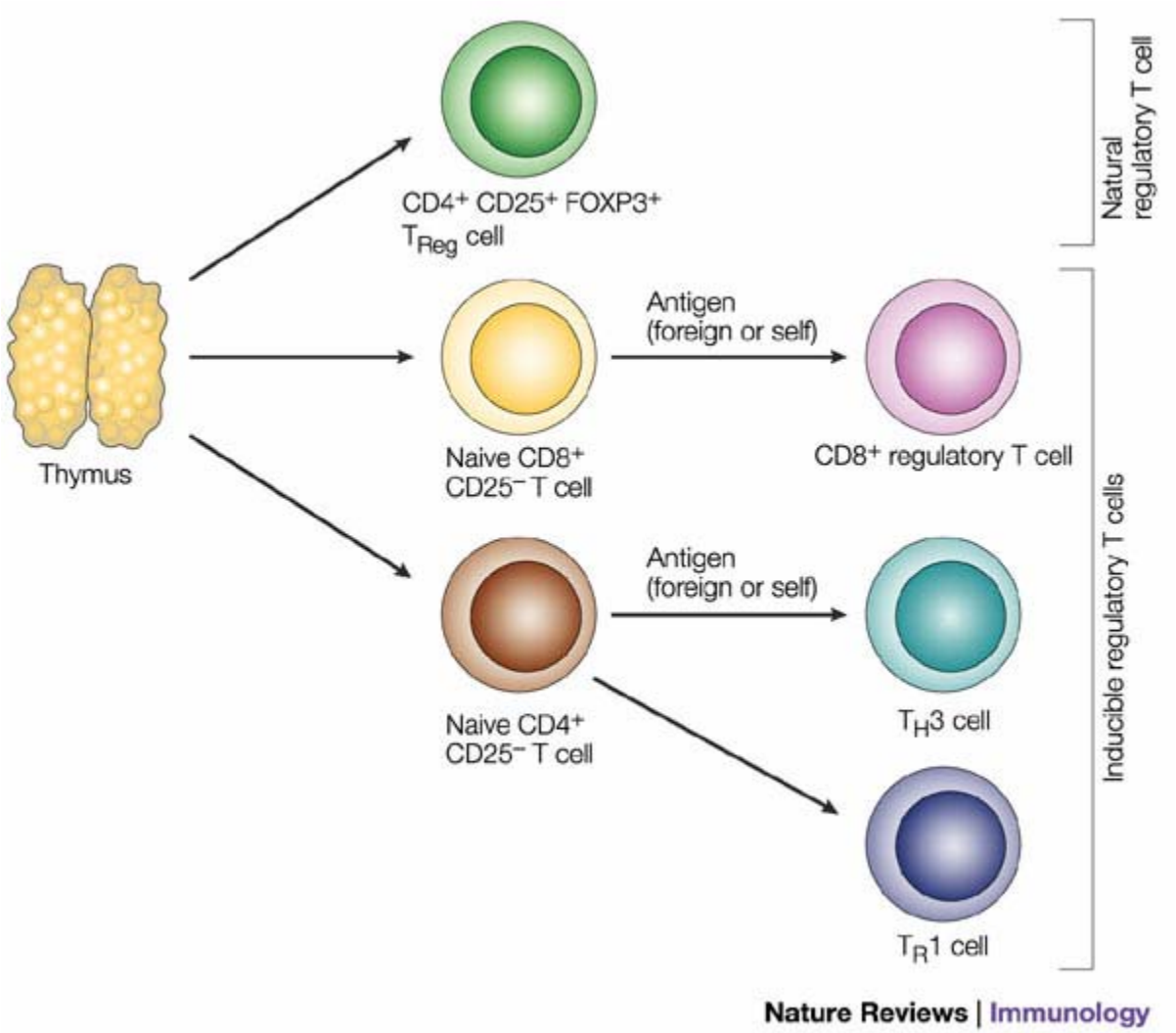

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Figure 1. Natural and inducible regulatory T cells. Natural regulatory T cells express the cell-surface marker CD25 and the transcriptional repressor FOXP3 (forkhead box P3). These cells mature and migrate from the thymus and constitute 5–10% of peripheral T cells in normal mice. Other populations of antigen-specific regulatory T cells can be induced from naïve $CD4^{+}CD25^{-}$ or $CD8^{+}CD25^{-}$ T cells in the periphery under the influence of semi-

mature dendritic cells, interleukin-10 (IL-10), transforming growth factor-β (TGF-β) and possibly interferon-α (IFN-α). The inducible populations of regulatory T cells include distinct subtypes of $CD4^+$ T cell: T regulatory 1 (Tr1) cells, which secrete high levels of IL-10, no IL-4 and no or low levels of IFN-γ; and T helper 3 (Th3) cells, which secrete high levels of TGF-β. Although $CD8^+$ T cells are normally associated with cytotoxic T-lymphocyte function and IFN-γ production, these cells or a subtype of these cells can secrete IL-10 and have been called $CD8^+$ regulatory T cells.

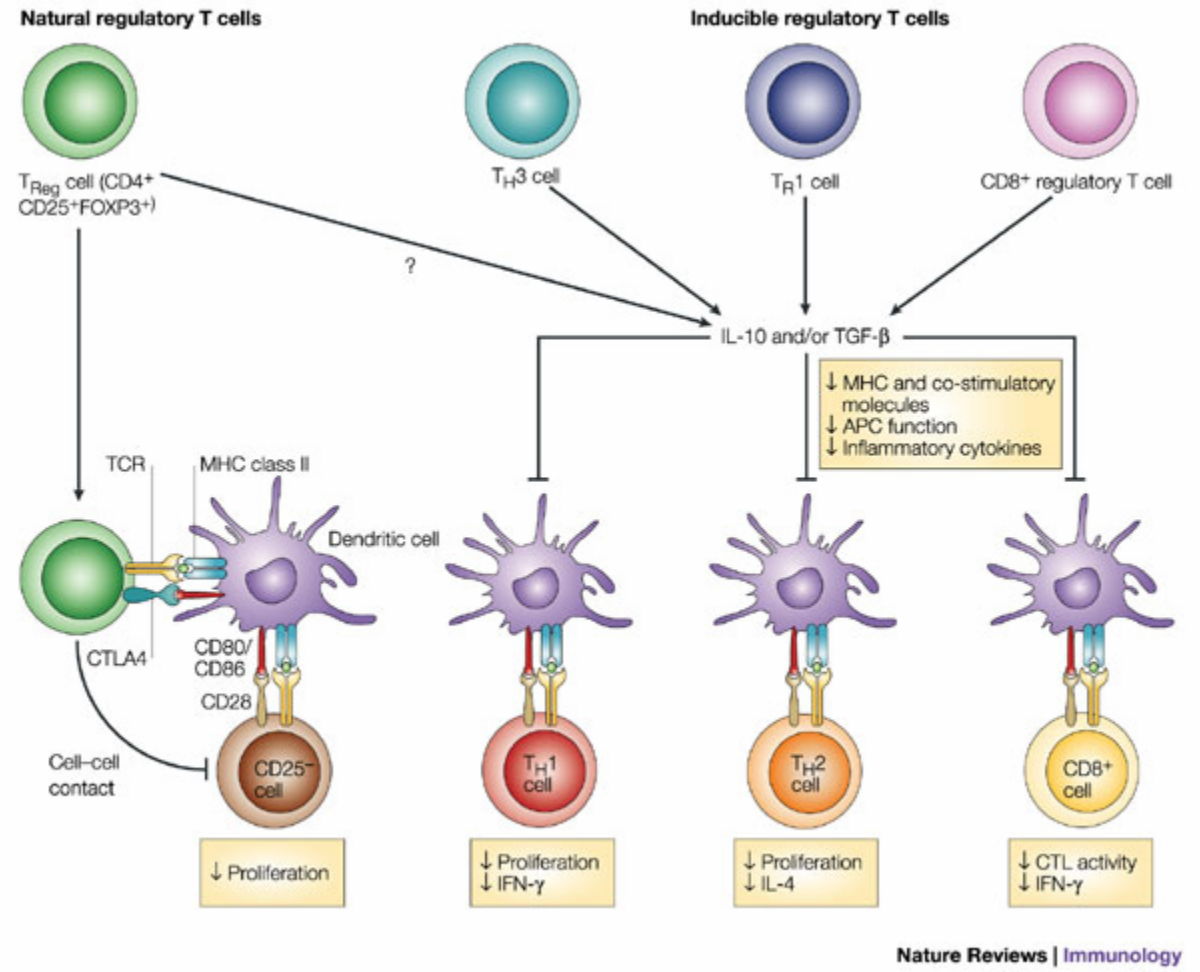

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Figure 2. Targets of regulatory T cells and mechanisms of suppression.
$CD4^+CD25^+FOXP3^+$ natural regulatory T cells (T_{reg} cells) inhibit the proliferation of $CD25^-$ T cells. The mechanism of suppression seems to be multifactorial and includes cell–cell contact. $CD4^+CD25^+$ T_{reg} cells express cytotoxic T-lymphocyte antigen 4 (CTLA4), which interacts with CD80 and/or CD86 on the surface of antigen-presenting cells such as dendritic cells , and this interaction delivers a negative signal for T-cell activation. There is also some evidence that secreted or cell-surface TGF-β or secreted IL-10 might have a role in suppression mediated by natural regulatory T cells. Natural killer T (NKT) cells (not shown) and inducible populations of regulatory T cells, which include T regulatory 1 (Tr1) cells, T helper 3 (Th3) cells and $CD8^+$ regulatory T cells, secrete IL-10 and/or TGF-β. These

immunosuppressive cytokines inhibit the proliferation and cytokine production by effector T cells like Th1 cells, Th2 cells and $CD8^+$ cytotoxic T lymphocytes, either directly or through their inhibitory influence on the maturation and activation of DCs or other APCs.

CHAPTER TWO: METHODOLOGY

CHRONIC HCV INFECTED PATIENTS AND HEALTHY SUBJECTS.

Healthy volunteers and patients with chronic genotype 1 HCV infection were recruited at the Aston and Parkland liver clinics, UT Southwestern Medical Center, as approved by the UT Southwestern IRB. I performed cross-sectional evaluation of 72 subjects, including 28 treatment-naive patients (TN's), 8 early virologic responders (EVR's), 12 sustained virologic responders (SVR's), 18 clinical non-responders (NR's) and 6 healthy subjects. EVR's and SVR's were defined using standard criteria where EVR was defined as a minimum 2-log decline in viremia during the first 12 weeks of treatment and SVR was defined as absence of detectable viremia at 6 months after the end of treatment (83). As part of standard care, patients were treated with a combination of pegylated Interferon 2b and Ribavirin for 48 weeks. EVR's were recruited between 24 and 48 weeks of ongoing therapy and all had viral loads below detectable limits at these time points. SVR's and NR's were recruited between 6 and 30 months after the last dose of interferon. Patients with HIV/HCV co-infection, known immune suppression or other liver diseases were excluded from the study. The predominant modes of infection were injection drug use and blood transfusion. Detailed information and summary of the baseline patient characteristics is provided in tables 1 and 2, respectively. None of the parameters analyzed showed significant differences across the patient groups. 6 HCV-seronegative, healthy subjects were also recruited for these studies (84). It was difficult to follow patients longitudinally due to a high drop out rate in

patients undergoing treatment due to complications, side effects, non-response and other factors.

CELL PREPARATION AND BEAD SORTING

PBMC were isolated from fresh buffy coats from healthy blood donors using Ficoll-Hypaque Plus (GE Healthcare Biosciences AB, Uppsala, Sweden) density gradient as per the manufacturer's protocol. "Untouched" $CD4^+$ T cells and $CD8^+$ T cells were negatively selected using negative selection kits and AutoMACS (DEPLETE program) from Miltenyi Biotech Inc, Auburn, CA as per the manufacturer's protocol. Negatively selections beads were used as much as possible in order to avoid any unwanted effects that may occur on T cells if CD4 or CD8 were used directly to select them. Total $CD3^+$ T cells were negatively selected using MagCellect negative selection kits from R&D systems Inc, Minneapolis, MN as per the manufacturer's protocol (Fig. 3). All of these were negatively selected to greater than 85% purity. $CD25^+$ T cells were depleted from the purified $CD4^+$ and $CD8^+$ T cells using CD25 microbeads and AutoMACS (Miltenyi Biotec) automatic cell separator (DEPLETES program) to greater than 95% purity (Fig. 3). CD25 bead selection was also used to isolate transient T_{reg}s from activated T cells cultures (Fig. 4). 10 µl (microliters) of CD25 microbeads per 10 million cells were used for positive selection while 20 µl of CD25 microbeads was used for depletion. CD45RO microbeads from Miltenyi biotech were used per the manufacturer's protocol for CD45RO depletion (depletion greater than 95%) (Fig. 5). CD3 microbeads from Miltenyi biotech were used per the manufacturer's protocol to deplete T cells from PBMC to enrich for antigen-presenting cells (APC) (Fig. 3). The $CD3^+$ T-cell-

depleted population was irradiated at 3000 rads before being used as APC's. Aliquots of $CD4^+CD25^-$ T cells and CD3-depleted cells were frozen for use at later time points as responders and APC's, respectively. 10-50 million aliquots of cells were frozen in freezing media containing RPMI, 10% DMSO, 40% heat inactivated filtered human serum, 0.7% 1 M Hepes buffer, and 0.7% L-glutamine. Such aliquots of cells were first gradually cooled in isopropanol baths in a -80C freezer before moving them for long term storage at -270C in liquid nitrogen freezers.

HCV AND CMV PEPTIDE POOLS

The HCV and CMV peptide pools used in these studies are outlined in tables 3 and 4, respectively**.** Sets of HCV 18-mer peptides (overlapping by 11) representing the entire genotype 1a or 1b virus were obtained from the NIH AIDS Reference and Reagent program (catalog nos. 7620 and 8681). 18-mer peptides have been shown to induce both $CD4^+$ and $CD8^+$ T-cell responses in-vitro assays (85). A total of 441 peptides representing genotype 1a (based on the consensus H77 sequence) and 434 peptides representing genotype 1b (based on the consensus J4 sequence) were divided into 9 pools (Table 2). The stock pools were prepared in DMSO and used in the assays at a final concentration of 2.5 μg/ml per peptide. The genotype 1b pools were used in patients infected with genotype 1b HCV, whereas the 1a pools were used for patients with genotype 1a and where the genotype 1 subtype could not determined. The CMV peptide pools (Table 3), a kind gift from Dr. Joseph Casazza, consisted of 14 known HLA class I-restricted epitopes (Pool 1) and 5 known class II-restricted epitopes (Pool 2) (86-91). Staphylococcal enterotoxin B (SEB) at 1 μg/ml (Sigma) served as the positive control in all experiments.

CFSE BASED FLOW CYTOMETRIC PROLIFERATION ASSAYS

Antigen-specific $CD4^+$ or $CD8^+$ T cell responses were detected and quantified by flow cytometric proliferation assays, utilizing the green fluorescent dye, 5 (and 6)-carboxyfluorescien diacetate succinimidyl ester (CFSE – Invitrogen, CA), as described previously (75, 92). Cells were first suspended at 1×10^6 cells /ml in phosphate-buffered saline (PBS) and incubated at 37°C for 7 min with 0.25 μM CFSE for staining. Further staining was stopped by quenching with heat inactivated, filtered human serum. Cells were washed twice with PBS to remove any excess CFSE. Cells were then suspended in 5% human media at 1×10^6 cells/ml and cultured with the appropriate antigens. The staining concentration was intentionally kept at a minimum to avoid CFSE staining associated cell death during long term culture. As more than 1 μl DMSO was added per ml of culture in some experiments involving HCV and CMV antigens, cells were washed in warm media twice following 18 hours of culture, and re-suspended in 1 ml of media. On different days post culture, cells were washed and stained with fluorescently tagged antibodies against CD3 PerCP, CD4 PECY5.5, CD8 APC (BD Biosciences) along with other appropriate antibodies. They were fixed in 1% Para formaldehyde (BD) before data acquisition on a flow cytometer. Flow cytometric data was acquired on a BD FACS Calibur/BD LSRII flow cytometers using BD Cellquest/BD FACS DIVA software and analyzed using FlowJo software (Ashland, OR). The detailed gating and analysis strategy used to analyze CFSE stained populations is described later. As previously described (92), a response was considered positive if it satisfied two criteria based on ratio and difference, compared to the no antigen background.

We required that the response be at least twice the background and at least 0.50% greater in magnitude.

EX-VIVO INTRACELLULAR CYTOKINE FLOW CYTOMETRY ASSAYS

Cytokine flow cytometry assays for the detection of antigen-specific IFN-γ responses were performed as described previously (75, 93). PBMC were suspended in 5% human media (RPMI 1640 supplemented with glutamine, 5% human AB serum, penicillin and streptomycin) and cultured with the indicated antigens. The cells were cultured for a total of 6 hours, with the last five hours in the presence of Brefeldin A (BFA - Sigma-Aldrich, St Louis, MO) in 5 ml polypropylene FACS tubes. At the end of the culture period they were washed with washed with FACS buffer (10X PBS, 1% BSA, 0.1% Sodium azide) for 10 minutes at 1400 RPM (Rotations per Minute). The cells were re-suspended in volume of 100 ul and stained with fluorescently tagged antibodies for surface CD3 PerCP and CD8 FITC (BD Biosciences, San Diego, CA) for 30 minutes. The surface stain was washed off with FACS buffer and followed by permeabilization for 10 minutes with permeabilization buffer (30% Tween 20 + FACS Lyse- Becton Dickinson, Franklin Lakes, NJ). After two washes, intracellular staining was done with 20 ul of IFN-γ APC and CD69 PE for 30 minutes. Cells were washed again and fixed in 1% Para formaldehyde (BD). Flow cytometric data were acquired on a BD FACSCalibur/BD LSRII flow cytometer using BD Cellquest/BD FACS DIVA software and were analyzed using FlowJo software (Ashland, OR). A minimum of

100,000 total events were acquired for analysis. Proliferating and non-dividing cells were initially gated through a broad gate based on their forward and side scatter properties. They were then gated using their CD3 and CD4 expression. $CD3^+/CD8^-$ T cells were designated as $CD4^+$ T cells for convenience a few experiments in chapter three. For cytokine flow cytometry experiments described in chapter three, a dot plot of CD69 vs IFN-γ was finally used to calculate the percentage of cytokine secreting cells who are also $CD69^+$. CD69 staining was used to identify recently activated IFN-γ secreting T cells. In a subset of chronic HCV patients, I evaluated TNF-α or IL-10 instead of IFN-γ. A response was considered positive if it was at least twice the background (no antigen) and 0.05% greater in magnitude. These values were arrived at based on our previous experience with PBMC's from healthy individuals.

FLOW CYTOMETRIC FOXP3 STAINING

Human FOXP3 staining kits from eBiosciences, San Diego, CA were used to stain for intracellular FOXP3 as per the manufacturer's protocols. PCH101 anti-FOXP3-PE or anti-FOXP3-AlexaFluor 700 were used to stain for total FOXP3, while FJK-16S anti-FOXP3-PE was used to stain for full-length FOXP3. Special permeabilization buffers and longer intracellular staining protocols developed and standardized by the manufacturer are needed to stain for intranuclear FOXP3. I found that the previously described intracellular staining protocols stain only the induced FOXP3 expressing populations in activated cultures in-vitro and not the natural FOXP3 expressing populations occurring ex-vivo in PBMC's.

FLOW CYTOMETRIC DATA ACQUISITION

Flow cytometric data described in chapter four was acquired on a 4-Laser, 17-color custom BD LSRII using FACSDiva software (Fig. 6). At least 200,000 events were acquired in most experiments. Higher numbers of collected events allowed me to visualize, gate and display populations clearly. Linear uncompensated data was then transferred as FCS 3.0 files and analyzed after compensation and transformation using FlowJo version 6.4.1 (TreeStar, San Carlos, CA), as recommended (94, 95). FCS 3.0 as opposed to FCS 2.0 is a newer method of storing data where even the data in the negative axis are stored along with the relevant uncompensated data files. This allows recompensation and transformation by the analyzing program over and above the compensation of the data acquiring machine. Recompensation and transformation at the time of analysis is essential to avoid PFC associated artefacts.

FLOW CYTOMETRIC DATA DISPLAY – BI-EXPONENTIAL AND TRANSFORMATION

Previously used flow cytometric dot plots suffered from a significant draw back in display in that it was not possible to visualize cell populations lying below the axis. Even thought it is not possible for a cell to express negative amounts of a marker, the flow cytometric graph can show such an artefact. This artefact arises due to the inherent errors and limitations of flow cytometers. The error in calculating the position of a cell can be either above or below the axis. Bi-exponential display is a newer method of display available in recent versions of FlowJo and FACS DIVA where the X and Y axes start in the negative range below zero. This display allows one to visualize even cells lying below the axis. This

display allows us to accurately compensate since the whole population and its mean can be visualized. This method of display was used in most of my graphs.

Transformation is another technique used to overcome data display errors that arise to problems inherent in flow cytometry. It is known in flow cytometry that data spread occurs in certain channels and dot plots as one goes away from the zero on the log display scale. This is because there is a greater spread of data at higher values compared to lower values again due to inherent limitations of the data collected by a flow cytometer. This data spread can result in cell populations being buried below the axis. This can lead to errors in drawing the negative/control gates during analysis besides resulting in very few cells on the actual display plot. Transformation can compress these populations for better visualization by compressing the display axis. Both bi-exponential display and transformation do not change the actual proportion of cells in any gate or any other parameter of data; they just result in a more aesthetic display.

I have also used color coded contour plots ("Pseudo color plots") to display my data in all of my flow cytometric plots. Color contour plots are a combination of black and white dot plots and contour plots. They are used to overcome the limitations in dot plots and contour plots. In dot plots, the density of cell populations cannot be visualized so one cell on the plot could potentially represent any number of cells. The limitation of contour plots is that outlying populations cannot be visualized so small populations like cytokine secreting populations can be missed. Pseudo color plots overcome these limitations by displaying both information in a single plot.

FLOW CYTOMETRIC DATA ANALYSIS

First the forward (FSC) vs. side scatter (SSC) plot was used to draw an initial gate on lymphocytes and proliferating lymphocytes. This gate was also used to exclude dead cells and other debris. Then the CFSE vs. CD4 or CD8 plot was used to accurately gate on high staining true $CD4^+$ and $CD8^+$ T cells respectively. The CFSE vs. CD25 plot was used to exclude dead cells which may have been included in the previous gates due to non specific staining for CD4 and CD8 by dead cells. I found that such a gating strategy helped me to gate out dead cell populations and accurately gate in on the population of interest. Cut-offs for positive populations were determined by using either fluorescence minus one (FMO) staining for polychromatic flow cytometry (PFC), no stimulus background staining or isotype control staining, as appropriate (1, 95). FMO controls are stained with all the conjugates in the panel except the one of interest. It allows us to assess the contribution of other markers and conjugates on the channel to interest. It is essential to have FMO controls in order to draw accurate gates for antigen negative populations. FMO controls are superior to isotype controls in PFC. In addition to all the above controls, intra-experiment controls are even more crucial in determining the negative populations in some experiments. Hence multiple levels of machine and experiment controls were used in my experiments. Samples from experiments involving readouts at different days were run with the same appropriate instrument and compensation settings to ensure comparability. An integrated multicolored display platform similar to the one shown was sometimes used to identify the phenotypic characteristics and origin of the population of interest (Fig. 7). The proliferation platform in

FlowJo was used to back-calculate the precursor frequency of the dividing cells by CFSE dilution.

POLY COLOR FLOW CYTOMETRY (PFC)

Flow cytometry using more that 6 colors constitutes polycolor flow cytometry. It is very different from flow cytometry using less than 6 colors in that it is more complicated and presents new problems and challenges. More controls were needed to ensure results obtained are not artefactual. 6-10 color phenotyping panels with appropriate FMO controls were set up using different combinations of CTLA-4-PECY5, CCR7-PECY7, CD4-PECY5.5/APC (Caltag), CD8-Pacific Blue/APC, CD25-PE,APC or APC-CY7, CD62L-PE or PECY5, CD27-APCCY7, CD28-PECY5 or Biotin+ Streptavidin Qdot 655 (Invitrogen), CD127 (IL-7R) APC (R&D systems), CD45RA-FITC and CD45RO-APC (all antibodies from BD Biosciences unless indicated otherwise). For cytokine studies, antibodies against IFN-γ APC, TNF-α FITC, IL-2 PE, IL-10 PE, IL-4 PE were used. All antibodies were titrated to optimize their staining concentrations for my experimental conditions. Same concentrations of antibodies were used to ensure comparability in different experiments.

In order to arrive at the appropriate staining combinations and panels, the following general guidelines were adhered to. The number of antigens and conjugates used were kept to the minimum necessary to answer the question posed to avoid the complications of PFC. The lasers and the fluorochromes detected by the machine are first taken in consideration. Then the conjugates available for all the desired antigens are also noted down. The next problem is to how to select various antigens for different conjugates. As a rule of thumb, high density common antigens like CD3, CD4, CD8 etc were used in rare colors like pacific

blue, alexa 700 etc, while rare low density antigens like CD27, CD28, CCR7 were used in common conjugates of PE, APC etc. Important antigens in my experiment like CD25, FOXP3, CD4 and CD8 were measured off of different lasers to minimise interference. However the correct combination can only be arrived at by starting with a four color panel which is known to work and then working up one new conjugate at a time. Even when all the theoretical criteria are satisfied, some combinations may interfere with one another for other reasons. Hence it is a matter of trial and error in creating new combinations and panels. New panels should also be extensively standardized to ensure there is not interference in the main flow cytometry plots used for display or analysis. Antibodies were also sometimes switched and used in different conjugates to ensure results were reproducible

FLOW SORTING

Flow cytometric sorting of $CFSE^{low}$ dividing vs. $CFSE^{high}$ non-dividing populations by CFSE was performed on a FACS Vantage (with FACS Diva upgrade) to greater than 95% purity (Fig. 8) at the flow cytometry core in the department of pathology. Cells were stained only with CFSE before sorting to minimise the effects of other staining antibodies on the cells. Gates on the sorter were adjusted to give the highest possible purity without compromising the yield. Slow sorting was also done to maximise yield in the given conditions. A post-sort run was performed to assess purity, which was greater than 95%. Sorted cells were used fresh in new experiments evaluating T_{reg} function. A few sorted cells (10,000 – 50,000) were collected in 1.5 ml sarstedt tubes, pelleted, and frozen at –80°C for subsequent molecular analyses for FOXP3 and cytokine expression studies.

INDUCTION OF TRANSIENT T_{REG}S AND STIMULATING ANTIGENS

Anti-CD3 or allostimulation was used to induce formation of transient FOXP3 expressing T_{reg}s from $CD4^+CD25^-FOXP3^-$T cells, $CD8^+CD25^-FOXP3^-$ T cells and $CD3^+CD25^-FOXP3^-$ T cells. An irradiated CD3 depleted and enriched in APC population was used in anti-CD3 and allostimulated cultures to provide the necessary co-stimulation. Irradiation was performed to ensure none of those cells can proliferate and express FOXP3. Irradiation also ensured their death at later stages of T cell activating cultures so that they can now be easily gated out during analysis. Gating at later time points was particularly a problem because irradiated APC's lie in the same region occupied by CFSE ^{low} activated dividing T cells. Allostimulus was used because it stimulates only a proportion of the T cells in the culture resulting in the clear induction of a FOXP3 expressing population distinct from a non FOXP3 expressing non responding population. Anti-CD3 (BD Biosciences) was used at a concentration of 1 μg/ml/million cells. No IL-2 or co-stimulation was used in experiments involving transient T_{reg}s to keep extraneous factors to a minimum. The effects of these agents on FOXP3 expression and function are not known. Anti-CD28 (BD Biosciences) was used at 1 μg/ml/million cells along with anti-CD3 as positive control in initial cytokine secretion experiments in HCV patients. Whole CMV lysates (50 μg/ml, Microbix Biosystems, Ontario, Canada), Tetanus Toxoid (20 μg/ml; Accurate Chemical and Scientific) were used as antigens to stimulate PBMC's.

3H-THYMIDINE BASED SUPPRESSION ASSAYS

Suppression assays were performed in duplicate in 96-well U bottom plates (Corning, NY). 50,000 $CD4^+CD25^-$ T cells (Responders) were cultured with 50,000 irradiated APC

and anti-CD3 in a total volume of 200 µl/well. Varying ratios ranging from 1:16 to 2:1 of induced or ex-vivo purified T_{reg}s to responder T cells were added to the cultures. The cultures were pulsed with 0.25 µCi ^{3}H-Thymidine per well in H-5 media on day 4 and cultured for a further 16-24 hrs. They were harvested on day 5 in an automatic 96 plate harvester onto filter papers. An automated counter (Wallac Betaplate liquid scintillation counter - PerkinElmer Life Sciences, Wellesley, Massachusetts, USA) was used to read their radioactivity levels and consequently determine their proliferation. Results are expressed in cpm or cpm (background subtracted). Experiments with very low baseline proliferation counts were not considered in the final analysis.

QUANTITATIVE REAL-TIME PCR

Total RNA was isolated using the QIAamp RNA mini kit (Qiagen, Valencia, CA) as per the manufacturer's protocols. Random hexamers were used to convert mRNA into cDNA using SuperScript First-Strand Synthesis system from Invitrogen as per the manufacturer's protocols. Isoforms of FOXP3 were then amplified using 5'-GCCCTTGGACAAGGACCCGATG-3' as the sense and 5'-CATTTGCCAGCAGTGGGTAGGA-3' as the antisense primers (Invitrogen), allowing for detection of FOXP3 isoforms (96). Relative expression of total FOXP3 was determined by normalizing to β-actin expression in SYBR-Green based real time PCR reactions, as described previously (75, 92). Briefly, 25 µL PCR reactions were performed on the Strategene MX-3000P in 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U Taq (Amersham), 800 nM forward and reverse primers, and a 1:50 000 dilution of SYBRGreen I nucleic acid gel stain 10 000 x (Molecular Probes, Eugene, OR). The following thermocycler

conditions were used: 95°C for 10 minutes x 1; 94°C for 45 seconds, 60°C for 1 minute, and 78°C for 7 sec x 45 cycles. The SYBR Green fluorescence was read during the extension phase of the reaction. After amplification, melting analysis was performed by heating the reaction mixture from 55°C to 98°C at the rate of 0.5 C/s. The specificity of the product was confirmed by melt curve analyses as well as visualization of product size on 3% agarose gels. IFN-γ, IL-10, IL-4 and TNF-α messages were also quantified using published protocols and primers (92).

STATISTICAL ANALYSIS

Results were analyzed with unpaired student *t* tests, Fisher's exact test or Pearson correlation, where appropriate. *P* values less than 0.05 were considered statistically significant.

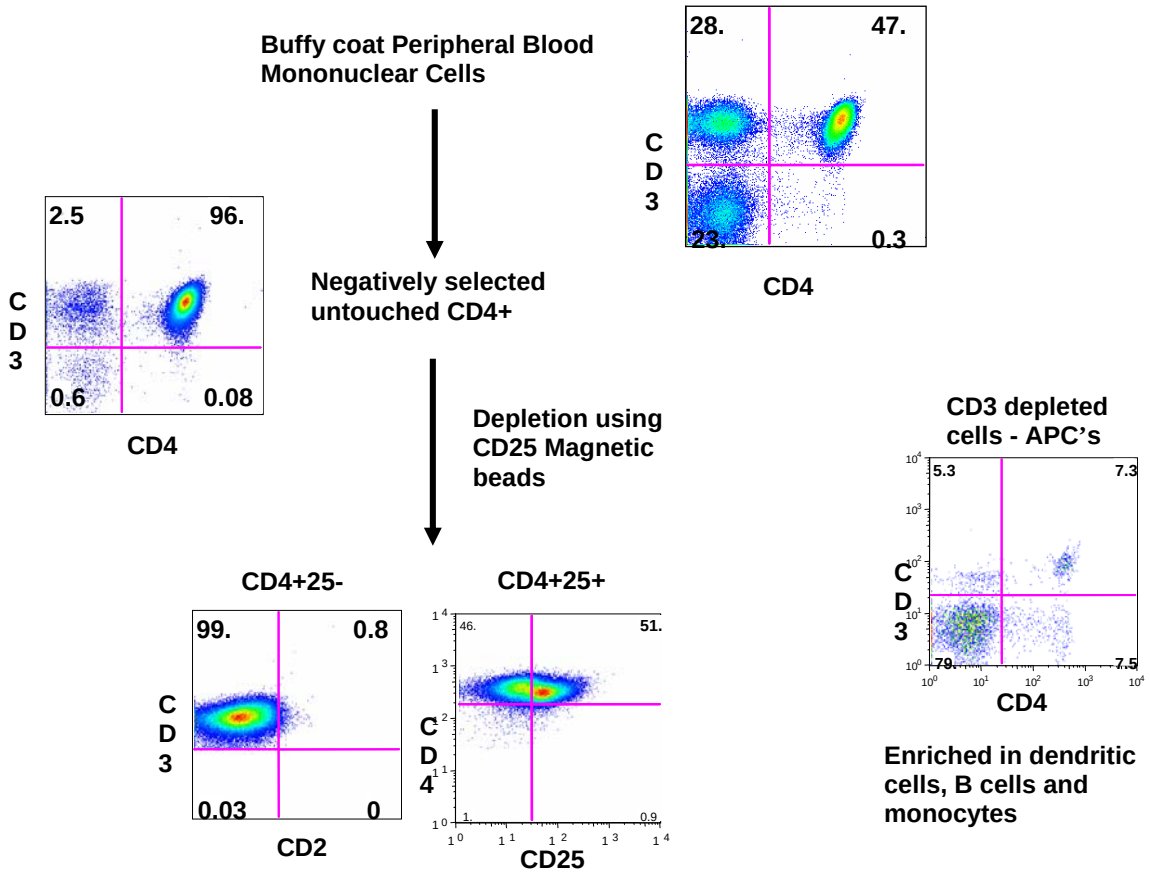


Figure 3. Isolation of T cell populations using magnetic beads. Magnetic beads and AUTOMACS from Miltenyi were used to isolate cell populations to greater than 90% purity. PBMC's were isolated from peripheral blood using Ficoll-Paque and then subjected to negative selection to obtain untouched $CD4^+$ T cells. $CD4^+$T cells were then subjected to CD25 positive selection to obtain the required $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell populations. In parallel a CD3 depleted population enriched in antigen presenting cells was obtained at the same time which was either used fresh or frozen for later use.

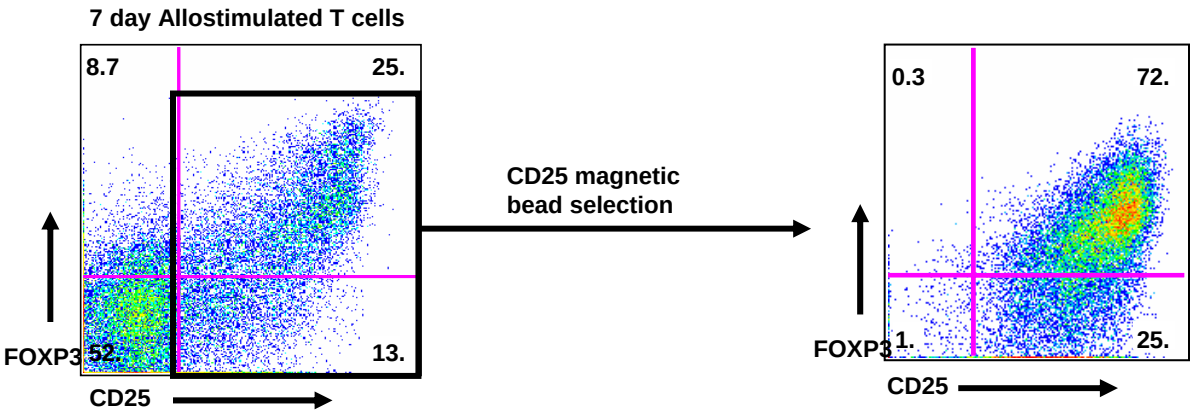


Figure 4. Isolation of CD25$^+$ T cells from allostimulated CD4$^+$CD25$^-$ T cell cultures. A magnetic bead selection strategy was used to positively select CD25 expressing activated T cells from allostimulated CD4$^+$CD25$^-$ T cells. This strategy allowed us to reproducibly isolate enriched populations of FOXP3 expressing activated T cells during days 2-6 of culture. In this representative example greater than 75% of positively selected cells are CD25$^+$ out of which 75% are FOXP3$^+$.

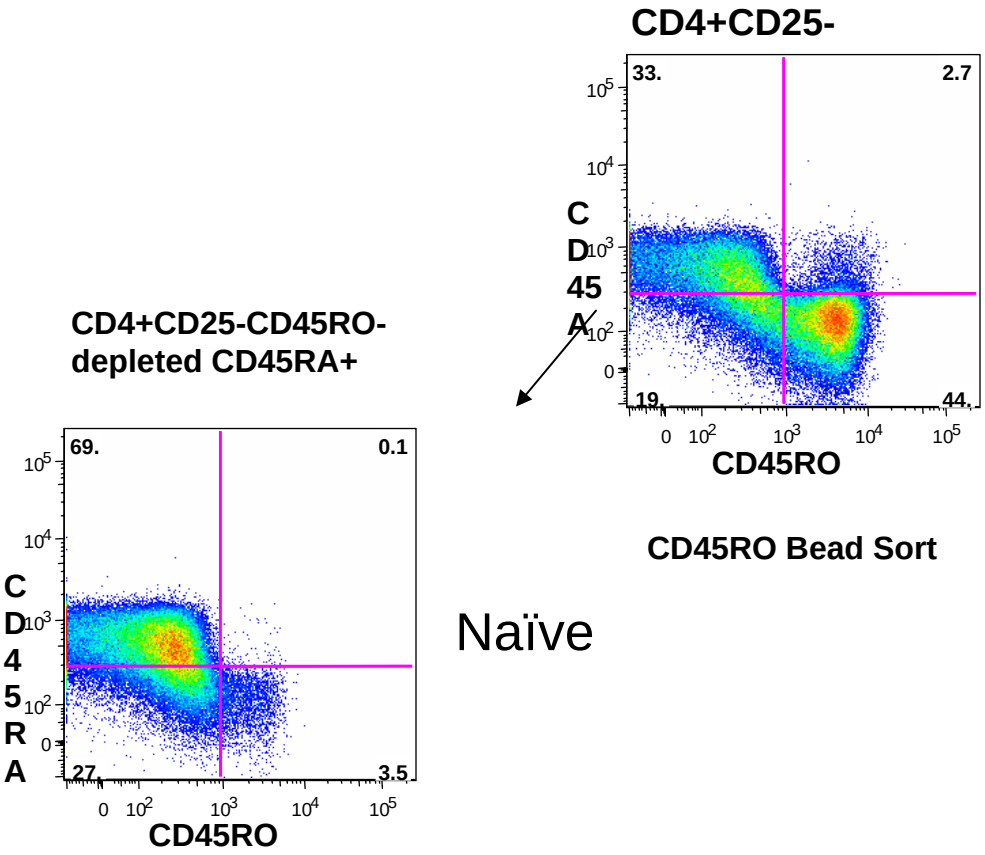
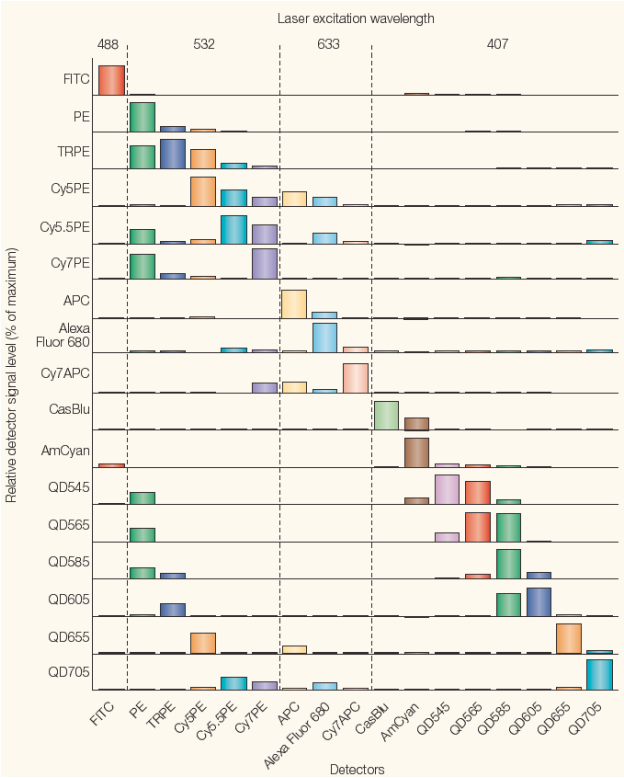


Figure 5. Isolation of predominantly naïve CD4$^+$CD45RA$^+$ T cells by depletion of CD4RO$^+$ T cells. CD45RO magnetic beads were used to deplete CD45RO$^+$ T cells from CD4$^+$CD25$^-$ T cells to obtain a naïve CD4$^+$CD25$^-$CD45RA$^+$CD62L$^+$ T cell population. In this representative example there was greater than 95% depletion of CD45RO expressing cells. The separated populations were used to set up transient T_{reg} inducing cultures as described previously.



BD LSR2 lasers and colors

Blue laser - 488nm
CFSE
CD3 FITC

Green laser - 532nm
FOXP3/CD62L/CD45RO PE
CTLA-4 PE-CY5
CD4 PE-CY5.5
CCR7 PE-CY7

Red Laser - 633nm
CD25 APC
CD27/CD25 APC-CY7
FOXP3/CD3 ALEXA700

Violet laser - 407nm
CD8/CD3/FOXP3 PACIFIC BLUE
CD28 QDOT 655

Modified and Reprinted by permission from Macmillan Publishers Ltd: Perfetto P, Roederer, M, et al., Seventeen color flow cytometry: Unravelling the immune system, Nat Rev Immunol 4, 648-55 (2004) (1)

Figure 6. Polychromatic flow cytometry using the BD LSRII – Lasers and conjugates used. 10 color flow cytometry was used to accurately and simultaneously phenotype the transient regulatory T cells. A custom built 4 laser, 17 color BD LSRII was used to collect polychromatic flow cytometry. Conjugates were selected to minimise interference with other colors and lasers. Important parameters like CD25, FOXP3 and CFSE were measured using conjugates detected by different lasers to ensure results were not an artefact caused by interference between different lasers. Multiple combinations of conjugates was also tested to the obtain the best possible combination giving the clearest results.

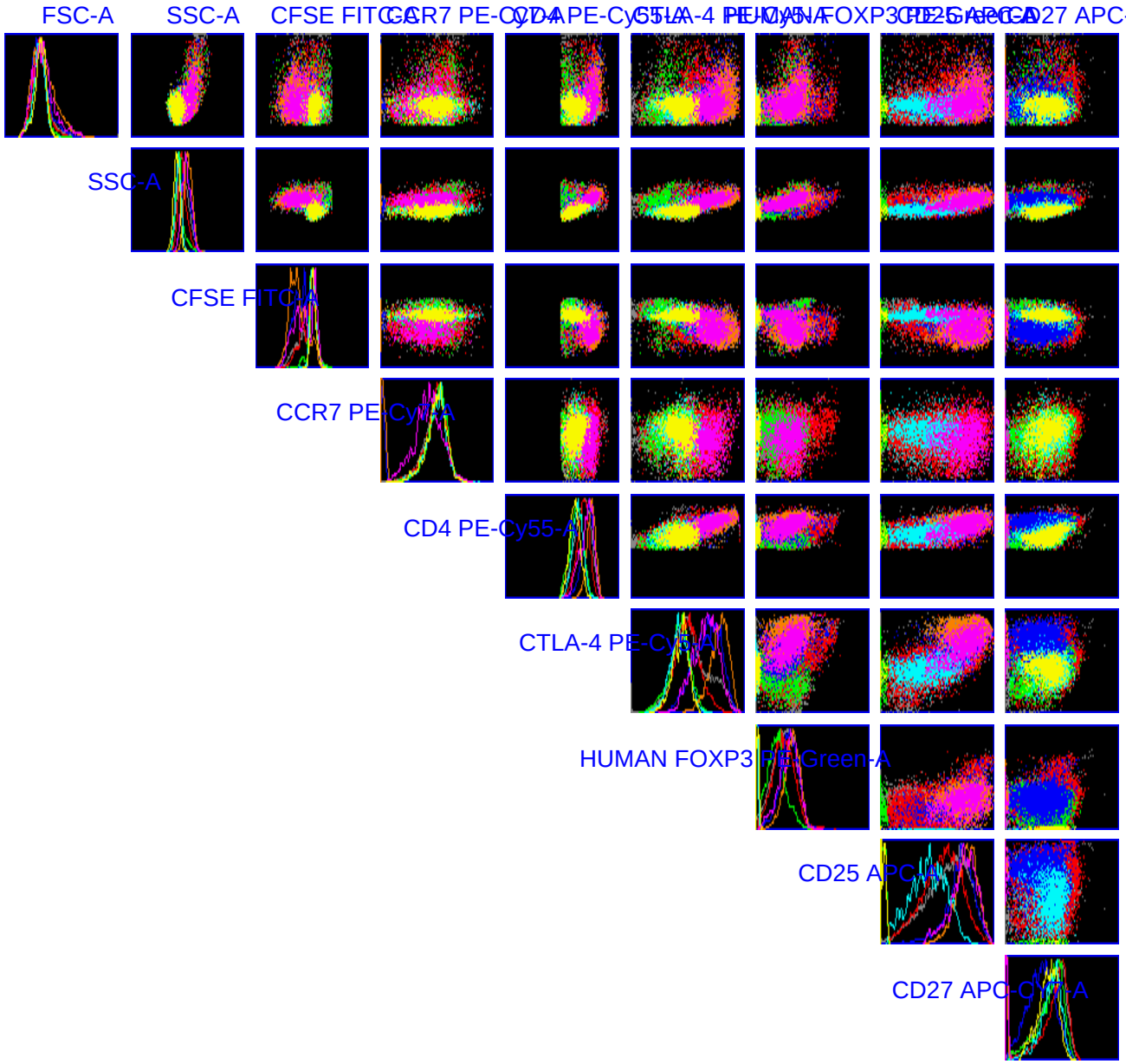


Figure 7. Integrated display platform to simultaneously visualize at all the measured parameters in polycolor flow cytometry. This image was obtained using the Flow Jo display platform. It helps one to simultaneously to look at all the measured parameters.

Different populations could also be painted using different colors to accurately display their phenotypic status.

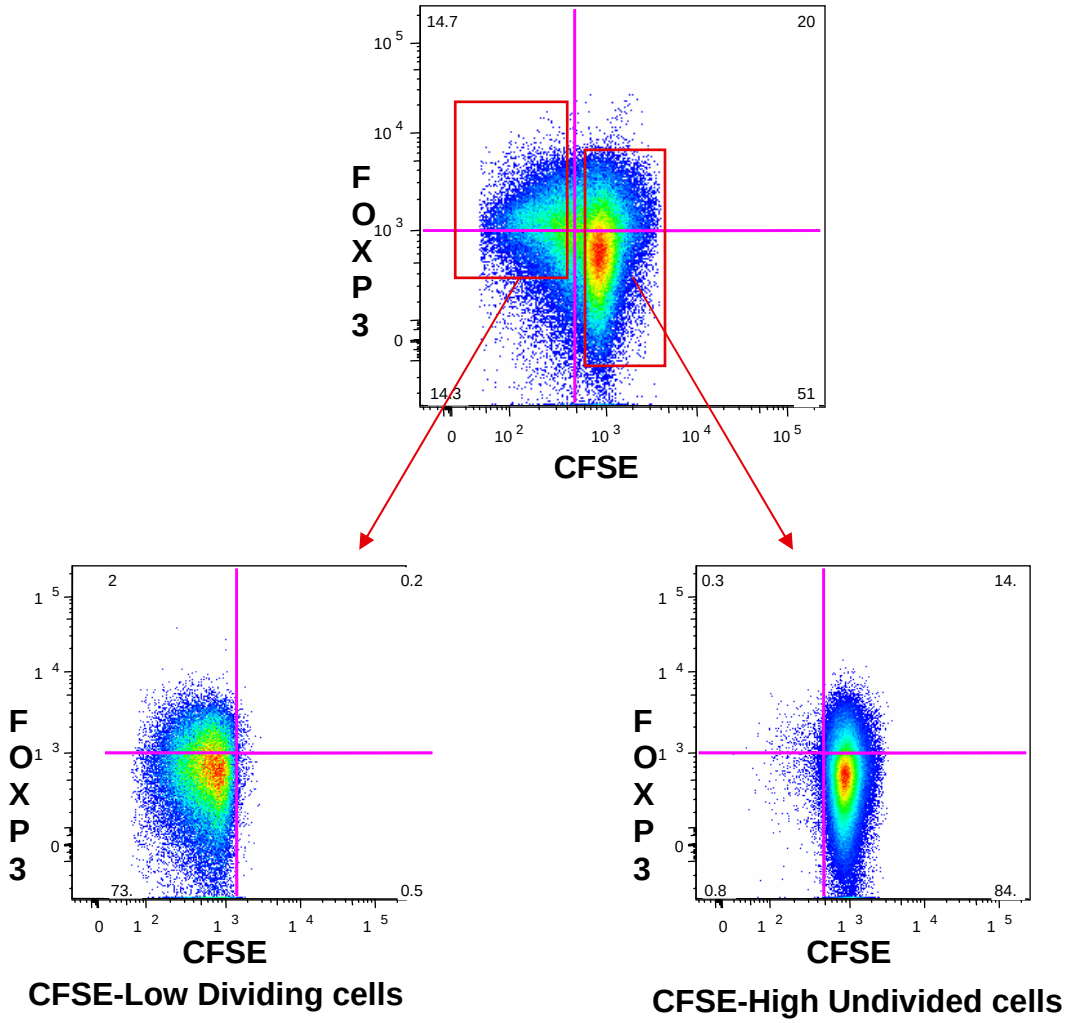


Figure 8. Flow sorting using CFSE dilution. A flow sorting strategy using CFSE dilution was used to obtain activated and non activated T cell populations. The activated population would express abundant FOXP3 at its peak around 4-6 days depending on the stimulus. FOXP3 would be down regulated in the activated dividing population at later time points post stimulation. Such a strategy was more accurate than a $CD25^{+}$ T cell positive selection strategy to isolate $FOXP3^{+}$ T cell at different stages of their formation since CD25 expression varies after stimulation.

Table 1. Detailed clinical data of all patients

PT ID	Age	Sex (M/F)	Race (C/B/ H)	HCV Geno-type	Viral load (*10 5 IU/Ml)	AST	ALT	Histology
TN's								
V355	52	M	C	1		73	204	GR 1-2, ST 1-2
V211	50	F	C	1a				
V516	46	F	C	1a	2.83	29	46	GR 2, ST 2
V817	44	F	C	1		49	79	GR1-2, ST 0-1
V381	59	M	C	1a	>5	82	125	GR 2, ST 4
V299	57	M	H	1a	>5	70	76	GR 2, ST 4
V567	42	F	C	1	1.54	45	63	GR2, ST 0-1
V627	45	M	C	1b	>5	54	104	GR2, ST1
V800	55	M	C	1a	>5	74	71	GR2-3, ST -3
V351	43	F	B	1	140	115	183	GR 1, ST1-2
V226	56	F	B	1b	>5	21	20	GR1-2, ST 2
V734	63	M	B	1	27.2	234	158	GR3, ST -4
V956	34	M	C	1	0.0285	17	18	GR0-1, ST1
V983	46	M	B	1b	>5	92	55	GR 3, ST 2
V451	33	M	C	1	2.8	45	74	GR1, ST1
V598	56	M	B	1	2.91	67	67	GR2, ST 2-3
V834	42	M	C	1b	13.6	45	79	GR1-2, ST 0-1
V712	40	M	C	1b	>5	157	444	GR2, ST 1
V853	30	M	B	1	58.8	26	35	GR1, ST1
V921	56	F	C	1b	2.07	23	26	GR1, ST1
V936	48	M	H	1b	37.1	65	100	GR1-2, ST1
V133	40	M	C	1	28.8	25	30	GR2, ST1
V717	48	F	C	1	8.6	49	69	GR2, ST1
V729	62	F	B	1b	3.42	29	31	GR1, ST 2-3
V699	46	M	B	1	31.6	94	55	GR 2-3, ST 4
V323	31	M	B	1	2.74	27	29	GR1, ST1
V572	49	F	B	1b	30.7	35	27	GR2-3, ST 3
V552	41	M	C	1	0.25	32	41	GR2, ST2
Mean	46.93	18/10	16/10/2			62.00	85.52	

PT ID	Age	Sex (M/F)	Race (C/B/ H)	HCV Geno-type	Viral load (*10 5 IU/Ml)	AST	ALT	Histology
EVR'S								
V867	38	M	C	1a	1.89	121	207	GR3, ST3
V078	47	F	B	1a	11.2	92	61	GR1, ST1
V808	34	M	C	1a	3.41	46	102	GR2, ST 2-3
V337	54	M	H	1a	>5	53	78	GR1-2, ST2
V221	51	M	C	1b	30.9	112	113	
V756	52	M	H	1a	1.61	215	395	GR1, ST1
V322	44	F	C	1	1.27	38	61	
V451	33	M	C	1	2.8	45	74	GR1, ST 1
Mean	43.78	7,2	6,1,2					
SVR'S								
V390	48	F	C	1	7.25	24	17	
V867	38	M	C	1a	1.89	121	207	GR3, ST3
V879	52	F	C	1b	8.36	93	176	GR2-3, ST2
V913	49	F	C	1a	1.91	137	104	GR1, ST1
V274	44	M	C	1a	1.62	78	106	GR1, ST4
V635	62	M	C	1a	0.0005	147	236	
V673	49	F	C	1b	0.294	63	93	GR1-2, ST2-3
V808	34	M	C	1a	3.41	46	102	GR2, ST 2-3
V966	46	F	H	1a	8.5	42	72	
V285	57	M	C	1	6.52	207	236	GR3, ST3-4
V066	44	M	H	1	0.86	124	227	GR2, ST3
V523	44	M	C	1	>5	31	51	GR1-2, ST3
Mean	47.25	7,5	10,0,2			92.75	135.58	
NR'S								
V475	43	F	H	1a	1.62	68	58	
V442	41	M	B	1	3.15	62	132	GR2, ST2
V671	60	M	C	1b	>5	36	67	GR1, ST1-2
V465	44	M	B	1a	32.3	59	93	GR2, ST2
V598	56	M	B	1a	2.91	67	67	GR2, ST 2-3

V732	61	M	B	1a	20.1	64	45	GR2, ST 2-3
V287	55	M	B	1	>5	95	81	
V432	51	M	B	1	>5	44	107	GR2, ST2
V182	47	F	C	1	5.6	91	78	GR2, ST4
V772	52	M	C	1		54	39	GR2, ST2
V253	52	M	H	1		65	100	
V154	50	F	C		8.5	24	19	GR2, ST2
V618	48	M	C	1b	5.11	67	112	GR2, ST3
V683	49	M	C	1a	2.63	49	58	
V178	51	M	C	1a	6	24	30	
V791	50	M	C	1		19	20	GR2, ST3
V499	49	F	C	1a				
V760	55	M	C	1a	35.2	28	18	
Mean	50.78	14,4	10,6,2			53.88	66.12	

Table 2. Summary of Patient Characteristics

Characteristics [A]	**TN**	**EVR**	**SVR**	**NR**
Age (y); Median (Range)	46 (30-63) (n=28)	45.5 (33-54) (n=8)	47 (34-62) (n=12)	50.5 (41-61) (n=18)
Sex (Male/Female)	18/10	6/2	7/5	14/4
Race (C/AA/H) [B]	16/10/2	5/1/2	10/0/2	10/6/2
HCV genotype (1/1a/1b) [C]	14/5/9	2/5/1	4/6/2	8/8/2
Viral Titer ($\times 10^5$ IU/ml) [D] **Median (Range)**	>5 (0.03-140)	3.1 (1.27-30.9)	2.7 (0.0005-8.5)	5.1 (1.62-35.2)
AST (U/L) Mean; Median (Range)	49 (17-234)	72 (38-215)	85.5 (24-207)	59 (19-95)
ALT (U/L) Mean; Median (Range)	67 (18-444)	90 (61-395)	105 (17-236)	67 (19-132)
Histology [E]				
Grade	2 (0-4)	1.5 (1-3)	2 (1-3)	2 (1-2)
Stage	1.5 (0-4)	1.5 (1-3)	3 (1-4)	2 (1-4)

A. Patient characteristics before treatment. Some parameters were not available on all cases.

B. C=Caucasian; AA=African American; H=Hispanic

C. All genotype 1. 1a and 1b are shown wherever discrimination was possible.

D. As these were performed by different assay systems, these data were not used for any analysis.

E. Scored according to Batts and Ludwig (84)

Table 3. HCV peptide pools

Pool number	1a peptide no	Amino acids	Protein subunit
1	1-55	1-389	Core + E1
2	56-106	379-746	E2
3	107-148	736-1040	P-7+ NS2
4	149-194	1030-1355	NS3-1
5	195-240	1345-1667	NS3-2
6	241-287	1657-1978	NS4a+NSb
7	288-339	1968-2342	NS5a-1
8	340-390	2332-2669	NS5a-2+NS5b-1
9	391-441	2659-3011	NS5b-2

Table 4. CMV peptide pools

Peptide No.	Peptide location	HLA restriction	Sequence
CMV-1	IE85-99	A1, Bw6, B8	LVKQIKVRVDMVRHR
CMV-2	IE198-207	B8	DELRRKMMYM
CMV-3	IE199-207	A1	ELRRKMMYM
CMV-4	IE309-317	B7	CRVLCCYVL
CMV-5	pp65417-25	B7	TPRVTGGGA
CMV-6	pp65418-426	B7	PRVTGGGAM
CMV-7	pp65495-503	A2	NLVPMVATV
CMV-8	pp65417-426	B7	TPRVTGGGAM
CMV-9	pp65265-274	B7	RPHERNGFTV
CMV-10	pp65155-163	B52	QMWQARLTV
CMV-11	pp65512-521	B12 (B44)	EFFWDANDIY
CMV-12	pp65173-181	B35	NQWKEPDVY
CMV-13	pp65186-195	B35	FVFPTKDVAL
CMV-14	pp65401-410	B35	TSGSDSDEEL
MV-1	pp65361-375	DR11	PQYSEHPTFTSQYRIQ
MV-2	pp65485-500	NA	PPWQAGILARNLVPMV
MV-3	pp65511-	DR52	QEFFWDANDIYRIFA

	524		
MV-4	pp65$_{041-054}$	DR15	LLQTGIHVRVSQPSL
MV-5	pp65$_{281-295}$	DR53	IIKLPGKISHIMLDVA

CHAPTER THREE: SUCCESSFUL ANTIVIRAL THERAPY REVERSES SUPPRESSION OF ANTIVIRAL T-CELL RESPONSES IN CHRONIC HCV INFECTION

INTRODUCTION

HCV infection is a major global health problem with over 200 million individuals infected worldwide (97). Nearly 70-85% of infected individuals are unable to clear the virus, resulting in chronic infection with the potential to develop cirrhosis, end-stage liver disease and hepatocellular carcinoma (83). A strong, multi-specific and sustained T-cell response against the virus is associated with a self-limited course after acute infection (98-100). In contrast, chronic HCV infection is characterized by attenuated $CD4^+$ and $CD8^+$ HCV-specific T-cell responses (100-103). The attenuation of these responses makes their detection and characterization by immunophenotypic assays a technological challenge. To address the role of these responses in HCV infection, the vast majority of previous studies have focused on a limited number of T-cell epitopes in the context of common HLA haplotypes, such as HLA-2, with some assumption of immunodominance based on binding algorithms (99, 101, 104-108). Studies from HIV and HCV have shown that these limited epitopes do not represent the overall immune response (109, 110). The overlapping peptide pool approach provides a way to comprehensively assess $CD4^+$ and $CD8^+$ T-cell responses without any bias towards HLA haplotype (111). Short-term ELISPOT assays using overlapping peptide libraries show good sensitivity but lack important phenotypic information about the responding cells. Thus, it becomes important to adapt or develop immunophenotypic assays that can reliably detect these responses using unbiased peptide libraries.

The biologic reasons for the attenuation of these responses are not entirely clear and may include failure of antigen presentation, T-cell exhaustion and dysfunction, viral mutations, intra-hepatic modulation or increased immune regulation (65, 99, 112-119). While the role of HCV-specific T-cell responses in the spontaneous resolution of HCV infection is unquestioned, their involvement and dynamics during antiviral therapy are poorly understood. Moreover, it is unclear whether the factors that attenuate these responses during chronic infection can be reversed by therapy.

In the current study, I used two flow cytometric approaches to evaluate antiviral $CD4^+$ and $CD8^+$ T-cell responses in cross-sectional cohorts of HCV-infected patients at various stages of disease and therapy, including treatment-naive patients (TN's), early virologic responders (EVR's), sustained virologic responders (SVR's) and clinical non-responders (NR's). One approach was the CFSE based proliferation assay while the other was the standard intracellular cytokine secretion assay. Our hypothesis would be that the proliferation-based assay was more sensitive at the detection of these responses compared to the cytokine secretion assay. I also hypothesized that there would be differences in T-cell responses between the different patient groups which are responsible for their different clinical responses to the treatment.

RESULTS

The CFSE-based proliferation assay detects HCV-specific $CD4^+$ and $CD8^+$ T cell responses with greater sensitivity, compared to ex-vivo cytokine flow cytometry

The vast majority of prior studies addressing HCV-specific T-cell responses have focused on few viral epitopes with presumed immunodominance in the context of certain HLA haplotypes. While ELISPOT assays using unbiased serial peptide pools for T-cell stimulation show good sensitivity, they lack immunophenotypic information. Thus, I used two flow cytometric approaches to evaluate HCV-specific T-cell responses in genotype 1-infected patients, employing genotype- and subtype-specific serial peptide pools representing the entire virus. One approach was a standard ex-vivo cytokine flow cytometry assay designed to detect IFN-γ responses after a 6-hour culture. This type of assay system has been extensively used to study antiviral T-cell responses in chronic viral infections such as HIV and CMV (93, 109, 120-122). The other approach was a 7-day CFSE-based flow cytometric proliferation assay, which would allow for amplification of antigen-specific T-cell responses, while maintaining the advantage of immunophenotyping. In previous studies, we have adapted and standardized this assay system to detect autoreactive $CD4^+$ and $CD8^+$ T-cell responses (75, 92), which are also difficult to detect in ex-vivo assays.

I performed these assays on PBMC from 72 subjects, including 28 TN's, 8 EVR's, 12 SVR's, 18 NR's and 6 HCV-seronegative healthy individuals. Figure 9 shows a

representative result from a single HCV-infected patient (in this case, an SVR). In the cytokine flow cytometry assay, this patient showed no detectable HCV-specific $CD4^+$ or $CD8^+$ T-cell response to pools 1, 2 or 6, whereas there was a detectable $CD4^+$ response to the CMV peptide pool. Stimulation with the superantigen, SEB, induced detectable responses in both $CD4^+$ and $CD8^+$ T cells (positive control). In contrast to the ex-vivo cytokine assay, the CFSE-based proliferation assay was able to detect robust $CD4^+$ T-cell responses to HCV peptide pools 1, 2 and 6 (black populations in Fig. 9). In addition, I also found $CD8^+$ T-cell responses to pools 1 and 2 (red/dark gray populations, specifically gated for $CD4^-/CD8^+$ T cells). There were $CD4^+$ and $CD8^+$ responses to both CMV as well as SEB.

A compilation of all the assays (Figure 10) demonstrated that the CFSE-based assay has far greater sensitivity in detecting HCV-specific T-cell responses in every patient cohort. For example, in treatment-naïve patients, the cytokine assay detected 0 of the possible 180 $CD4^+$ T-cell responses (20 patients × 9 pools). In contrast, the CFSE-based assay, which was performed on 8 additional patients, was able to detect 66 (26.2%) of 252 responses (28 patients × 9 pools); $P=8.3 \times 10^{-18}$. Thus, in each cohort and for every comparison, the CFSE-based assay, which allows for in-vitro amplification of the response, performed significantly better at detecting HCV-specific T-cell responses.

This pronounced difference in the assays was not due to the restricted readout of IFN-γ used in the cytokine assay. In a limited set of patients, I performed the assay to evaluate other cytokines, such as TNF-α or IL-10 or even the upregulation of activation marker CD69. In all cases, I was unable to reproducibly detect $CD4^+$ or $CD8^+$ responses over the background (data not shown). These findings are in keeping with prior reports that HCV-

specific T-cell responses are highly attenuated in patients who are chronically infected with HCV (100-103). Our results also demonstrate that HCV-specific $CD4^+$ and $CD8^+$ responses remain difficult to detect even in treated patients and require in-vitro amplification for efficient characterization.

We have shown in previous studies that the CFSE-based assay detects HLA-restricted, antigen-specific T-cell responses (92). Of note, HCV-specific T-cell responses were detected far less often in HCV-seronegative, healthy individuals. Thus, in the subjects tested, the cytokine assay detected no $CD4^+$ or $CD8^+$ T-cell responses (0/36 in both cases), while the CFSE-based assay detected only 4/54 (7.4%) $CD4^+$ and 4/54 (7.4%) $CD8^+$ responses. This suggests that the responses detected in HCV-infected patients predominantly represent proliferation of antigen-experienced T cells that could not be detected by the ex-vivo assay. Overall, these results establish the utility of this assay system in evaluating HCV-specific T-cell responses.

Clinical virologic responders (EVR's and SVR's) show significantly higher HCV-specific $CD4^+$ and $CD8^+$ responses, compared to TN's and NR's

I next asked whether there were any differences in HCV-specific T-cell responses across the different patient cohorts at various stages of therapy. With the help of the CFSE-based assay, I observed that successful antiviral therapy was characterized by a significantly higher proportion of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses (Figure 10). Thus, EVR's showed significantly higher $CD4^+$ and $CD8^+$ responses than TN's (P=0.0004 and 0.0008, respectively). Importantly, EVR's also showed significantly higher responses compared to NR's (P=0.00005 and 0.00006). Similar to EVR's, SVR's also showed

significantly greater HCV-specific $CD4^+$ and $CD8^+$ responses, compared to TN's ($p = 0.0008$ and 0.002) as well as NR's (P=0.00009 and 0.0001). There were no significant differences between EVR's and SVR's. There were also no statistically significant differences between TN's and NR's; however, there was an overall trend toward lower responses in NR's. Similar results were found between the different patient cohorts when the data was compared using the actual magnitude of proliferation (Figure 13). These results demonstrate the biologic/clinical relevance of the CFSE-based assay system. Thus, clinically successful antiviral therapy is accompanied by a significant enhancement of HCV-specific T-cell responses. In contrast, unsuccessful therapy is characterized by unchanged or diminishing responses to HCV.

EVR's and SVR's show a greater breadth of HCV-specific responses, corresponding to higher responses against Core/E1, NS3, NS4 and NS5b proteins

Studies of spontaneously recovered acute HCV patients have indicated a greater breadth of responses against multiple regions of the virus, compared to patients who could not clear the virus (98-100). Hence, I compared the breadth of the responses between the different patient cohorts by enumerating the number of pools that elicited positive responses in each patient (corresponding to different target regions of the virus). Figure 11 demonstrates the spread of responses in each patient cohort. As expected, these results matched the overall picture seen in Figure 9, such that EVR's showed a greater breadth of CD4+ and CD8+ T-cell responses compared to TN's (P=0.03 and 0.07) or NR's (P=0.02 and 0.02). SVR's also showed a similar trend, compared to TN's (P=0.07 and 0.01) and NR's

(*P*=0.04 and 0.03). Again, there were no significant differences between EVR's and SVR's. These results suggest a therapeutic induction of T-cell responses targeted against more regions of the virus.

I next evaluated whether the higher numbers of responses were targeted against any specific regions of the virus (Figure 12). A pool-wise analysis of responses showed significant differences across patient cohorts in responses to peptide pools 1, 5, 6 (corresponding to Core, E1, NS3 and NS4 regions of the virus) as well as some differences in pool 9 (NS5b). The most consistent differences were observed in responses to pool 1 (Core+E1 region) with significantly greater $CD4^+$ and $CD8^+$ responses in EVR's compared to TN's (*P*=0.06 and 0.002) or NR's (*P*=0.02 and 0.002), with a similar pattern in SVR's compared to TN's (*P*=0.03 and 0.04) or NR's (*P*=0.008 and 0.03). The carboxyl half of NS3 (pool 5) elicited significantly higher $CD4^+$ responses in EVR's compared to TN's and NR's (*P*=0.01 and 0.007) and higher $CD8^+$ responses in EVR's and SVR's, compared to NR's (*P*=0.07 and 0.02). SVR's also showed significantly higher $CD4^+$ and $CD8^+$ responses to the NS4a+NS4b peptides (pool 6), compared to TN's (*P*=0.007 and 0.01) and NR's (*P*=0.004 and 0.008). The EVR $CD8^+$ response to NS4a+NS4b was greater than that of NR (*P*=0.05). Finally, I also noted higher $CD4^+$ responses to the carboxyl end NS5b in EVR's than in TN's or NR's (*P*=0.04 and 0.08). These results show that the greater breadth of HCV-specific T-cell responses in successfully treated patients is targeted predominantly against certain regions of the virus, particularly the Core/E1, NS3 and NS4 proteins. Paucity of cells precluded further dissection of these responses to the epitope level in this study.

CMV-specific $CD4^+$ T cell responses are lower in TN's compared to all groups that have received therapy

In addition to evaluating HCV-specific T-cell responses, I also evaluated CMV-specific responses in the same patients as an additional control condition (see Methods and Fig. 9). I used two pools of a limited number of peptides with known HLA Class I and Class II restriction. Several interesting points emerged through the inclusion of these analyses. Firstly, in sharp contrast to the performance of the two assay systems in detecting HCV-specific responses, their ability to detect CMV-specific responses was comparable, particularly in the $CD8^+$ subset (Fig. 14). While there was a trend toward detection of more $CD4^+$ responses by the CFSE assay, none of these apparent differences reached statistical significance. Secondly, to my surprise, untreated patients showed a low proportion of detectable T-cell responses to CMV by both the cytokine and CFSE-based assays (Fig. 14). Thirdly, similar to HCV-specific responses, I found significantly higher $CD4^+$ T-cell responses to CMV in EVR's (P=0.002) and SVR's (P=0.02) using the CFSE assay. However, in contrast to their low HCV-specific responses (Fig. 10), NR's showed higher CMV-specific responses than TN's (P=0.04). There were no significant differences between NR's and SVR's or EVR's. There was a trend toward higher CMV-specific $CD8^+$ T-cell responses in SVR's, EVR's and NR's in the cytokine flow cytometry assay; however, this did not reach statistical significance. Thus, the predominant finding was an increase in $CD4^+$ T-cell proliferative responses to CMV in patients currently or previously receiving interferon/ribavirin therapy, regardless of their HCV outcome.

Depletion of $CD25^+$ cells increases HCV-specific $CD8^+$ T cell responses in TN's, but not SVR's

In the setting of chronic antigenic stimulation, several mechanisms may account for attenuation of responses. Recently, a great deal of attention has focused on $CD4^+CD25^+$ regulatory T cells, which may arise de novo or be induced as a result of antigenic stimulation (31, 123). It has been suggested in prior studies that suppression by induced $CD4^+CD25^+$ regulatory T cells causes decreased HCV-specific responses in chronic HCV infection (65, 119). However, it is not known whether such suppression can be reversed as a result of therapy**. I hypothesized that the enhanced HCV-specific T-cell responses seen in treated patients could be, in part, a result of such a reversal**. I addressed this hypothesis in a subset of 7 TN's and 5 SVR's.

First, I performed ex-vivo immunophenotypic analysis of PBMC to enumerate the proportion of circulating $CD4^+CD25^+$ T cells (Fig. 15; left panel). Similar to a prior report, I observed that patients with untreated chronic HCV infection showed a remarkably high proportion of $CD4^+CD25^+$ T cells. Thus, among gated $CD4^+$ T cells, the proportion of CD25+ T cells ranged from 44.9% to 71.0% (mean=54.98%). Interestingly, SVR's showed a significantly lower proportion of $CD4^+CD25^+$ T cells, ranging from 24% to 30.5% (mean=27.35%; P=0.005). This also corresponded to significantly higher mean fluorescence intensity for CD25 on gated $CD4^+$ T cells in TN's, compared to SVR's (data not shown).
I next asked whether depletion of these cells would affect HCV-specific $CD4^+$ or $CD8^+$ T-cell responses. $CD25^+$ cells were depleted from the PBMC of both TN's and SVR's using magnetic bead sorting, which resulted in over 95% depletion (Fig. 15, middle panels). The

vast majority of the depleted cells were $CD4^+$ (Fig. 15; right panels; over 70-90% in all cases). Figure 16 demonstrates the effect of in-vitro CD25 depletion on anti-viral T cell responses. Panels A and B show representative results from one treatment-naïve patient. Using bulk PBMC (Panel A), I could detect a $CD4^+$ response to HCV-pool 2 in this patient, along with $CD4^+$ and $CD8^+$ responses to CMV. There were no detectable responses to HCV-pool 1 (over background). After depletion of $CD25^+$ cells (Panel B), I could detect a robust $CD4^+$ response to HCV-pool 1 in this patient, along with clearly detectable CD8+ responses to pools 1 and 2. The CMV-specific responses were only minimally affected by this depletion. When the data from all experiments were put together, I observed that the predominant effect of CD25 depletion was a significant enhancement of HCV-specific $CD8^+$ T-cell responses in TN's (Panel C; P=0.049). At the level of the patient cohort, there were no significant differences in their HCV-specific $CD4^+$ responses. Of note, I did not observe such enhancement of responses in SVR's (Fig. 16; Panel C), corresponding to their lower proportion of $CD4^+CD25^+$ T cells (Fig. 15). In addition, no significant or consistent differences were seen in the CMV-specific responses in either TN's or SVR's (data not shown). These results suggest that HCV-specific $CD8^+$ T-cell responses are actively suppressed in untreated patients by $CD4^+CD25^+$ regulatory T cells. Moreover, such suppression can be reversed by successful antiviral therapy.

DISCUSSION

As HCV-specific T-cell responses are attenuated in chronic HCV infection (100-103, 115, 124, 125) and are difficult to detect in ex-vivo short-term assays, I adapted a novel combination of genotype-specific overlapping peptide pools and a CFSE-based proliferation assay. This allowed us to perform an unbiased evaluation of T-cell responses to HCV infection without restriction to specific HLA haplotypes or epitopes. I show here that this assay has far greater sensitivity at detecting HCV-specific $CD4^+$ and $CD8^+$ T-cell responses, compared to ex-vivo cytokine flow cytometry. These differences may reflect of the qualitative nature of the antigen-specific immune responses detected by the two assay systems. Cytokine flow cytometry detects high frequency, high avidity responses such as those in HIV and CMV, while the longer-term CFSE-based proliferation assay can amplify and detect even low frequency and/or low avidity responses (75, 92). Thus, patients with chronic HCV infection harbor HCV-specific responses against several regions of the virus and these can be detected by using this flow cytometric approach.

Importantly, this assay system also showed clinical and biologic relevance, in that it detected significantly greater $CD4^+$ and $CD8^+$ responses in EVR's and SVR's compared to both TN's and NR's. The host and viral factors that play a role in mediating viral clearance following interferon/ribavirin treatment are unclear. Genotype 1 infection, which accounts for 70-75% of all chronic HCV cases in the US, is associated with a low response rate of 42-46% compared to 76-82% for genotypes 2 and 3 (83). There is a rapid initial decline in viremia after the initiation of antiviral therapy, which is likely mediated by the direct antiviral effects of interferon and ribavirin. The later slower decline, which is important in

complete viral clearance, is thought to be mediated by immunological mechanisms. However, prior studies do not clarify the exact nature of these mechanisms. While a strong HCV-specific $CD4^+$ T-cell response is associated with viral clearance after therapy (125-127), the dynamics of $CD8^+$ responses is not clear. Different studies have come to conflicting conclusions with some finding an increase in responses after treatment (105, 128), while others a decrease or no change (107, 125). Most studies have relied on proteins or selected peptides derived from a single prototypical HCV 1a strain to study antigen-specific responses across all HCV genotypes. This is a significant confounding factor since the nucleotide sequences vary by as much as 31-34% between different genotypes and by 20-23% between different subtypes (83). Most of the putative dominant epitopes commonly studied also differ widely between genotypes and subtypes (129). The current studies used genotype and subtype-specific peptide pools and were restricted to genotype 1-infected patients. Using this approach to interrogate HCV-specific T cells, I show that not only enhanced $CD4^+$ responses, but also broad, multi-specific $CD8^+$ responses, are associated with successful therapeutic clearance of the virus. These are also the characteristics of a response that can clear virus spontaneously in HCV-infected individuals (98-100). Thus, in some respects, antiviral therapy probably induces complete viral clearance through a successful antiviral T-cell response.

In that regard, it is also interesting that the multi-specific responses in EVR's and SVR's are focused on certain regions of the virus. Responses against epitopes in Core and NS3 were initially thought to be associated with clearance of the virus in spontaneous resolution of acute HCV infection but later studies showed that responses are also directed

against epitopes spread throughout the viral polyprotein (98, 129). It is interesting that the targets I identified for therapeutically induced T-cell responses also focused on multiple regions of the virus. Importantly, my results demonstrate that HCV-infected patients still retain the T cells specific for these target regions and possess the ability to generate robust responses against these proteins. Thus, the knowledge that immune attenuation of these responses is reversible is important for the design of therapeutic vaccines.

I found no significant differences between T-cell responses in EVR's versus SVR's. This is not surprising as the majority of EVR's eventually become SVR's (83). However, this indicates that antiviral $CD4^+$ and $CD8^+$ T-cell responses are sustained in the majority of patients even 6 months to 3 years after their last dose of interferon. This is important with respect to potential for re-infection. Future longitudinal studies could address the duration of these responses and whether detection of these responses early in therapy is predictive of an eventual SVR.

While I was expecting to find low HCV-specific T-cell responses in untreated patients, I was surprised to see low CMV-specific T-cell responses in this group. HCV-infected patients are not known to be overtly immunosuppressed and do not show obvious evidence of CMV reactivation. However, it is entirely possible that HCV infection will cause subtle immunosuppression that goes beyond the attenuation of HCV-specific responses. While effects of certain HCV proteins on several immune functions have been suggested in-vitro, there are conflicting reports on the in-vivo effects of infection on different immune populations (112, 130-132). Some studies have suggested that chronic HCV infection may modulate the phenotype or function of T-cell responses to other viral

infections, such as CMV (133). In the current study, I found that patients treated with interferon/ribavirin have higher CMV-specific $CD4^+$ T-cell responses than untreated patients, regardless of their HCV status. Admittedly, my evaluation was performed with a limited pool of CMV peptides and thus, I cannot draw definitive conclusions as I can for HCV-specific responses. However, this exposes subtle immune features that underlie chronic HCV infection and support the notion of an in-vivo immune modulatory role of interferon/ribavirin therapy, as suggested in prior studies (134-139).

The biologic reasons for the attenuation of immune responses in HCV infection are not clear and may include failure of antigen presentation and priming (112, 113), T-cell exhaustion and dysfunction (99, 114, 115), viral escape mutations (117), impaired T-cell maturation (116) and intra-hepatic down regulation (118), among others. Some studies have suggested that increased function of $CD4^+CD25^+$ regulatory T cells (T_{reg}s) could be a mechanism in HCV infection (65, 119) and also in other chronic viral infections such as CMV and HIV (66, 69, 70). T_{reg}s in HCV-infected patients were found to be HCV-specific, secrete TGF-β and IL-10 and act by cell contact-dependent mechanisms (119). Similar to these reports, I also found a very high proportion of $CD4^+CD25^+$ T cells in chronically infected patients. Moreover, I show that depletion of these cells significantly increases HCV-specific $CD8^+$ T-cell responses, suggesting that these responses are actively suppressed by HCV-specific T_{reg}s (which would be stimulated in the bulk cultures by the relevant HCV peptide pool). Depletion did not have a significant effect on HCV-specific $CD4^+$ T-cell responses, which could be simultaneously evaluated in this assay. In addition, there were no consistent differences in CMV-specific responses before and after CD25 depletion,

suggesting that the modulation of CMV-specific responses in chronic HCV infection is probably not mediated through T_{reg} function.

More importantly, I found that the expansion and function of this population of HCV-induced T_{reg}s is reversible by antiviral therapy. Thus, successfully treated SVR's showed significantly lower numbers of $CD4^+CD25^+$ T cells. Moreover, CD25 depletion of their PBMC had no detectable effect on HCV-specific $CD4^+$ or $CD8^+$ responses. These results suggest that interferon/ribavirin therapy is capable of modulating the immune system such that the dysfunctional generation of HCV-induced T_{reg}s is curtailed, allowing for the development of effector $CD4^+$ and $CD8^+$ T-cell responses. My results, taken together with other reports, support a model where chronic HCV infection results in differentiation of HCV-specific $CD4^+$ T cells toward a regulatory phenotype, either through chronic antigenic exposure or subtle changes in antigen presentation or other immune functions. This dysregulation results in not only decreased $CD4^+$ responses to HCV, but also active suppression of (and lack of help for) $CD8^+$ T-cell responses. Through rapid clearance of virus, promotion of antigen presentation and/or direct action on T_{reg}s, interferon/ribavirin therapy may reverse this underlying dysregulation and allow HCV-specific $CD4^+$ T cells to differentiate into effector/helper cells. This would not only negate the active suppression of HCV-specific $CD8^+$ T cells but also provide help to support the development of a robust, multi-specific T-cell response. Thus, reversal of T_{reg}-mediated suppression is probably an important immunologic event in successful antiviral therapy.

In summary, my findings demonstrate the utility and relevance of the CFSE-based assay system in evaluating the biology of antiviral T-cell responses to HCV and other viral

infections. The study suggests that, similar to spontaneous viral clearance, the development of a multi-specific $CD4^+$ and $CD8^+$ T-cell responses is important in therapy-induced viral clearance as well. This is dependent, at least in part, on the reversal of active suppression mediated by $CD4^+CD25^+$ regulatory T cells.

Figures and Tables.

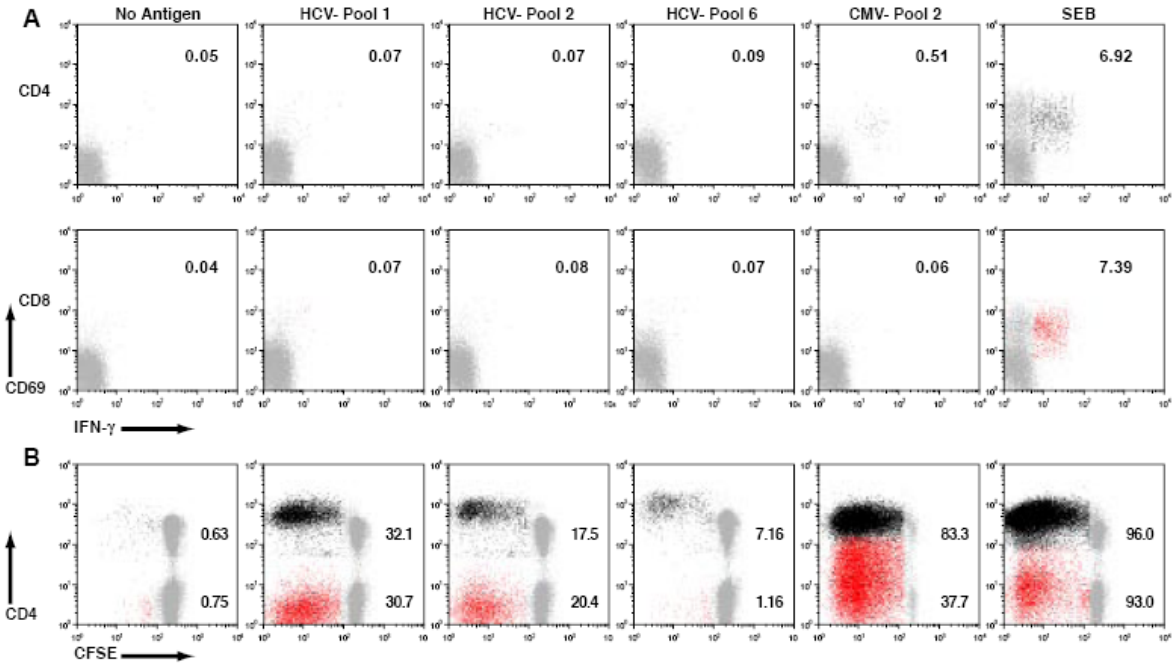


Figure 9. The CFSE-based proliferation assay shows greater sensitivity in detecting HCV-specific T-cell responses compared to ex-vivo cytokine flow cytometry. Ex-vivo cytokine flow cytometry assays and CFSE-based proliferation assays were performed to detect and quantify antiviral $CD4^+$ and $CD8^+$ T-cell responses in cross-sectional cohorts of HCV-infected patients at various stages of therapy (TN's, EVR's, SVR's and NR's). This figure shows representative results from a single patient, in this case an SVR. Panel A shows IFN-γ responses detected by the ex-vivo cytokine assay against selected antigenic pools (indicated). The top panels represent gated $CD3^+/CD8^-$ T cells (labeled CD4) and the middle panels represent $CD3^+/CD8^+$ T cells. The Y-axis shows CD69 expression, while the X-axis shows the IFN-γ response. The numbers represent the percentage of gated cells that were $CD69^+/IFN\text{-}\gamma^+$. The only positive responses in this patient were observed against the CMV pool (CD4 response) and the super antigen control, SEB.

Panel B shows results from the CFSE assay for the same patient against the same peptide pools. The data represent gated $CD4^+/CD8^-$ or $CD8^+/CD4^-$ T cells. CFSE staining is shown on the X-axis and CD4 staining on the Y-axis. The $CD4^-$ populations in the dot plots represent gated $CD8^+$ T cells. The gray populations to the right represent non-dividing cells. The numbers next to the darker populations represent the proliferating fraction of $CD4^+$ T cells (black) and $CD8^+$ T cells (red), i.e., the proportion of gated $CD4^+$ T cells (or $CD8^+$ T cells) that were proliferating on day of analysis. In contrast to the cytokine assay, the CFSE assay showed detectable $CD4^+$ T-cell responses to the three HCV peptide pools as well as $CD8^+$ T-cell responses to HCV pools 1 and 2.

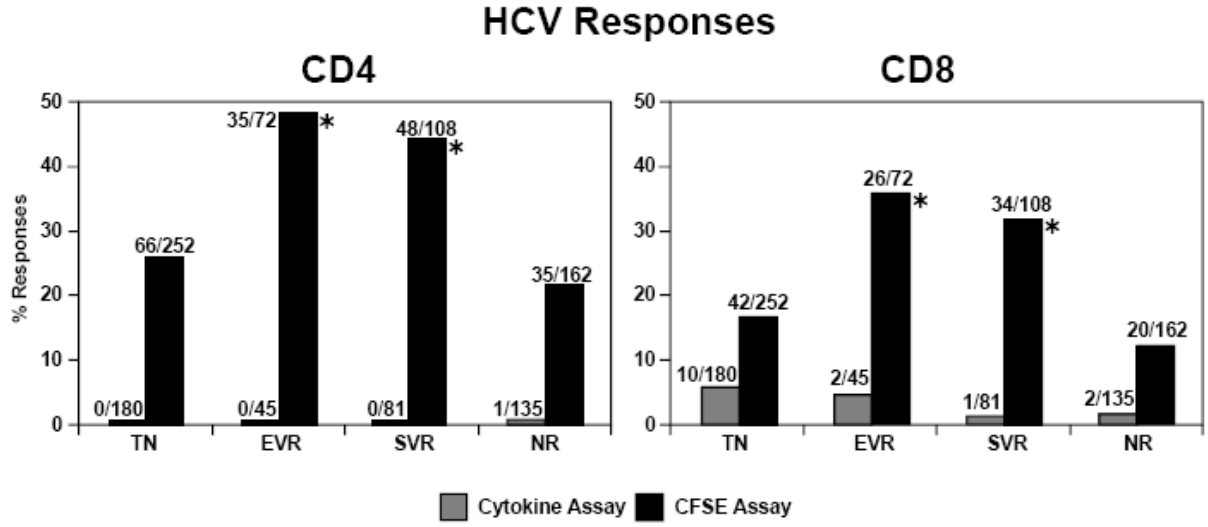


Figure 10. Successful antiviral therapy is characterized by significantly higher HCV-specific T-cell responses, detected by the CFSE assay. This is a compilation of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses detected by the cytokine and CFSE assays in the four cohorts of patients. The Y-axis represents the proportion of positive responses detected by each assay. The numbers above each bar represent the actual number of responses over the total possible responses in each cohort (i.e., 9 × no. of patients tested by the assay). In every instance, the CFSE assay could detect a far greater number of $CD4^+$ and $CD8^+$ T-cell responses in every cohort of patients. Furthermore, this assay revealed that EVR's and SVR's (clinical virologic responders) had significantly greater HCV-specific $CD4^+$ and $CD8^+$ T-cell responses, compared to TN's as well as NR's, as denoted by the asterisks.

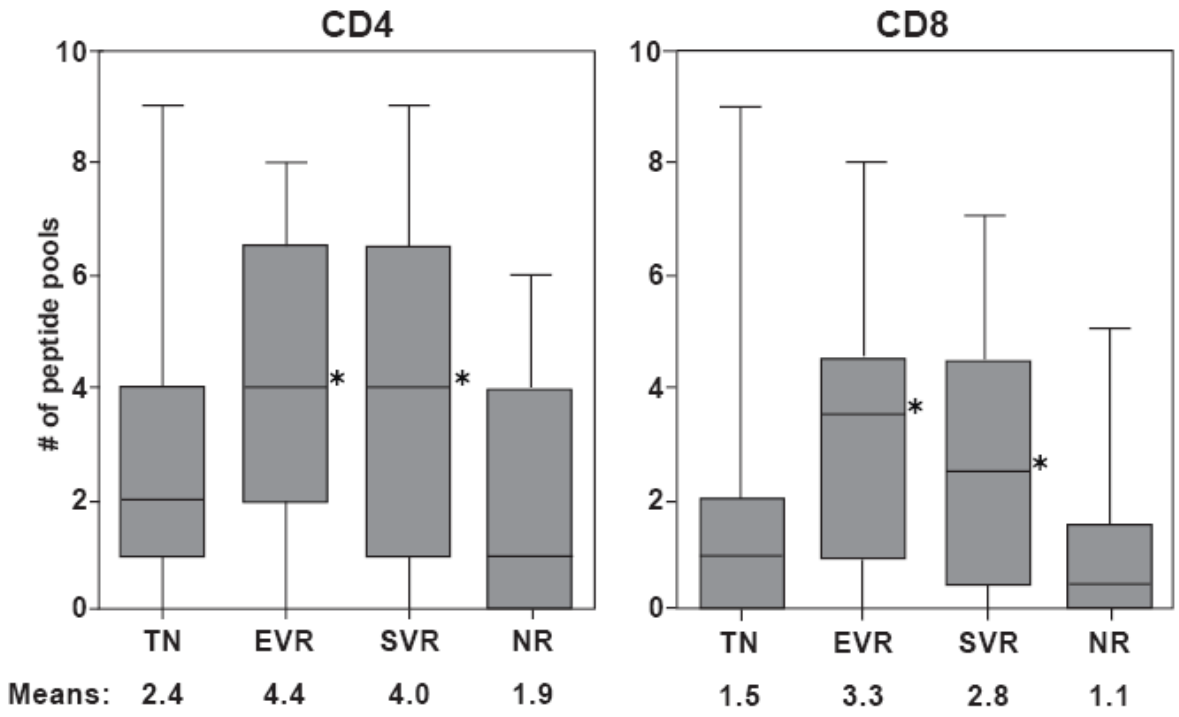


Figure 11. EVR's and SVR's show a greater breadth of HCV-specific T-cell responses than TN's or NR's. The Y-axis represents the number of peptide pools that elicited $CD4^+$ (left panel) or $CD8^+$ (right panel) responses in each patient. The bars and lines show the median and quartile ranges for each cohort. The numbers at the bottom represent the mean number of peptide pools to which each cohort responded. Asterisks indicate that EVR's and SVR's showed a greater breadth of $CD4^+$ and $CD8^+$ T-cell responses when compared to TN's or NR's.

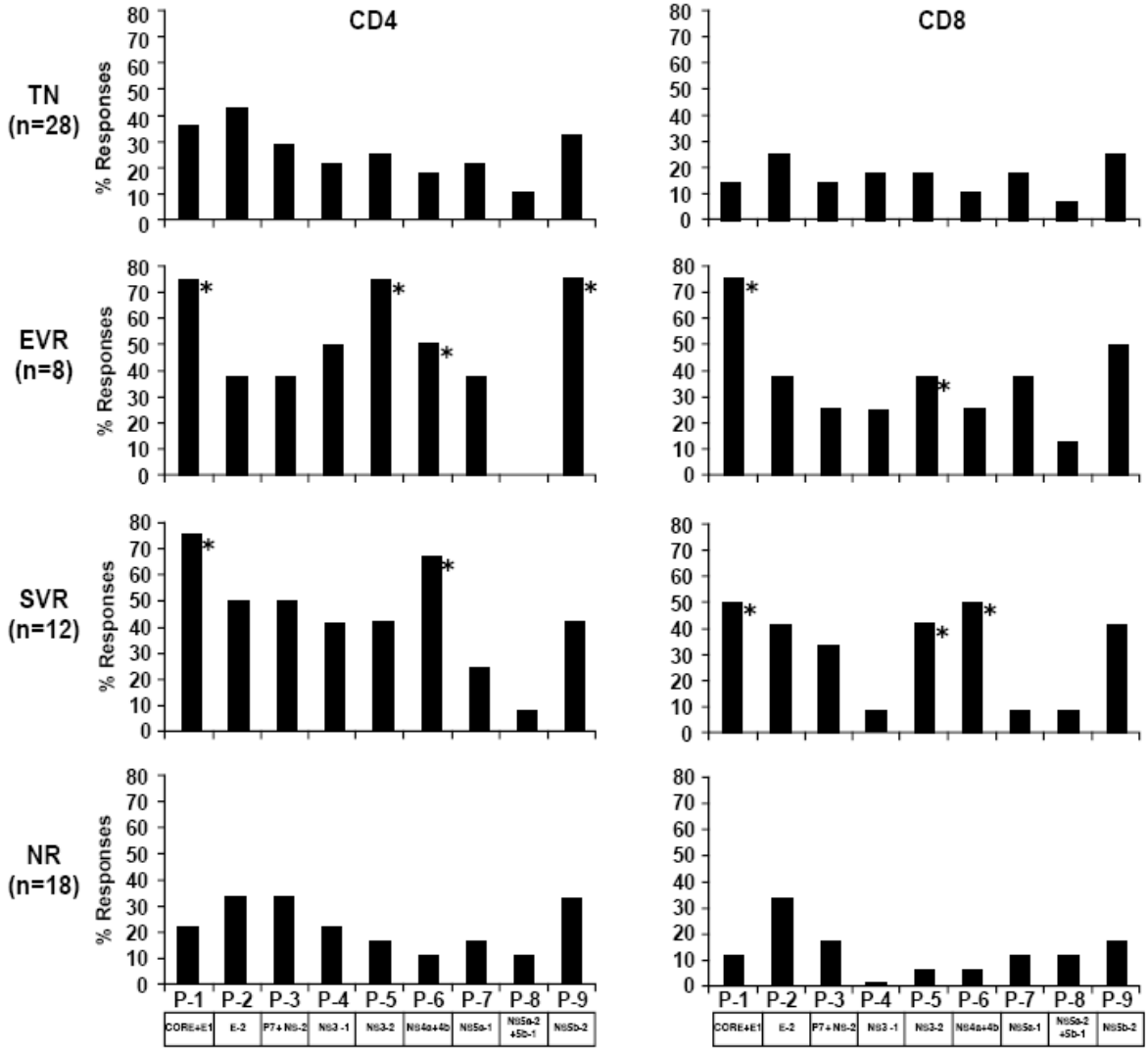


Figure 12. The enhanced responses from clinical virologic responders are focused on the Core, Envelope 1, NS3, NS4 and NS5b regions of HCV. A pool-wise comparison of $CD4^+$ and $CD8^+$ responses was performed. The X-axis shows the different HCV peptide pools representing different regions of the virus. Each bar represents responses against the specific pool in each patient cohort. Asterisks represent the significantly higher responses detected in EVR's and SVR's, which were focused on pools 1, 5, 6 and 9, which correspond to Core+Envelope1, NS3-2 (carboxyl half), NS4a+NS4b and NS5b-2 regions of the virus.

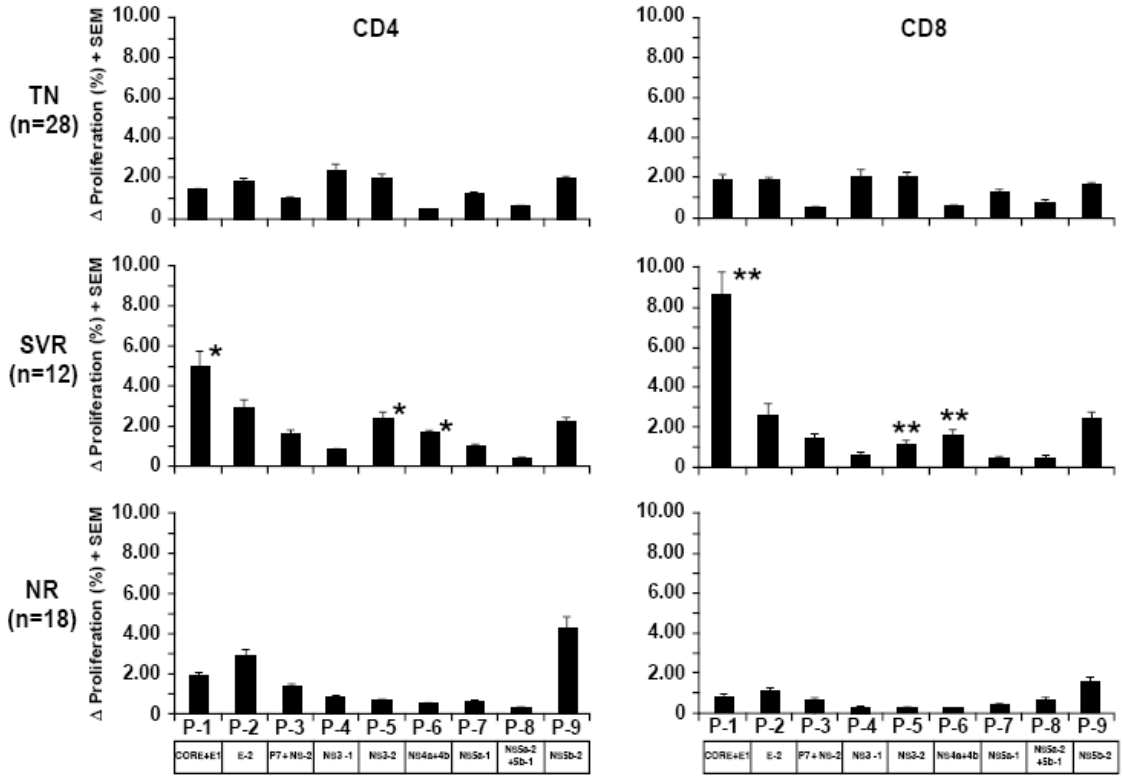


Figure 13. SVRs show significantly higher magnitude of proliferation of $CD8^+$ responses compared with TN patients and NRs. A pool-wise comparison of the magnitude of $CD4^+$ and $CD8^+$ responses was performed. The X-axis shows the different HCV peptide pools representing different regions of the virus. Each bar represents the mean D proliferating fraction (DPF), which is calculated by subtracting background percentage proliferation (no antigen) from the percentage proliferation to the specific antigenic pool. Double asterisks represent the significantly higher $CD8^+$ responses detected in SVRs compared with NRs, which were focused on pools 1, 5 and 6 (corresponding to core + Envelope 1, NS3 and NS4 regions of the virus). Single asterisks denote a similar trend in $CD4^+$ responses ($P < 0.09$).

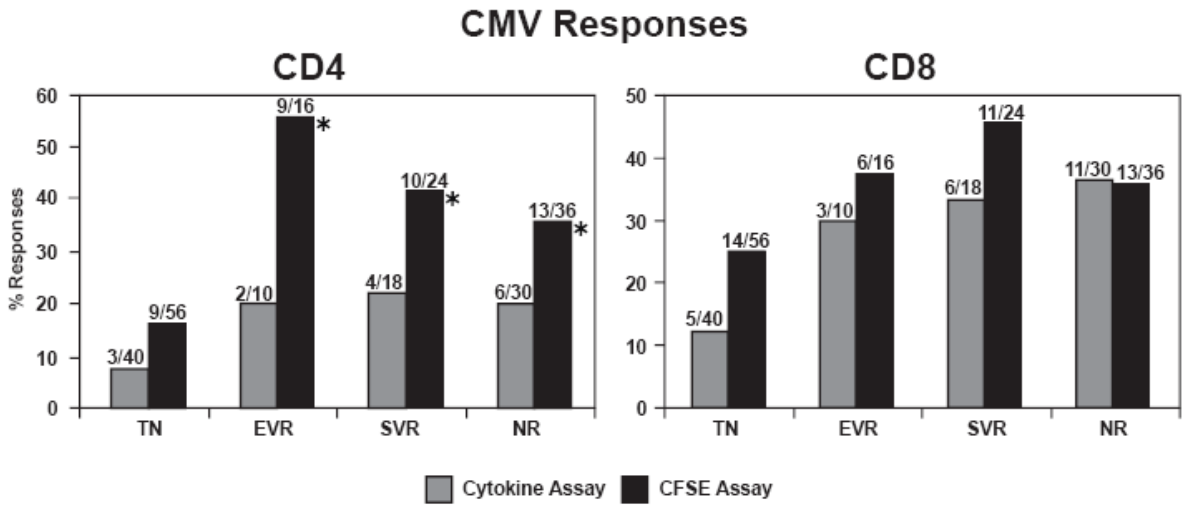


Figure 14. Patients treated with interferon/ribavirin therapy showed significantly higher CMV-specific CD4$^+$ T-cell responses, regardless of HCV status. This is a compilation of CMV-specific CD4$^+$ and CD8$^+$ T-cell responses detected by the cytokine and CFSE assays in the four cohorts of patients (layout similar to Figure 10). No statistical difference was found in the detection of responses by the two assays, despite a trend in the CD4 subset. However, as indicated by the asterisks, EVR's, SVR's and NR's showed significantly greater CMV-specific CD4$^+$ proliferative responses, compared to TN's.

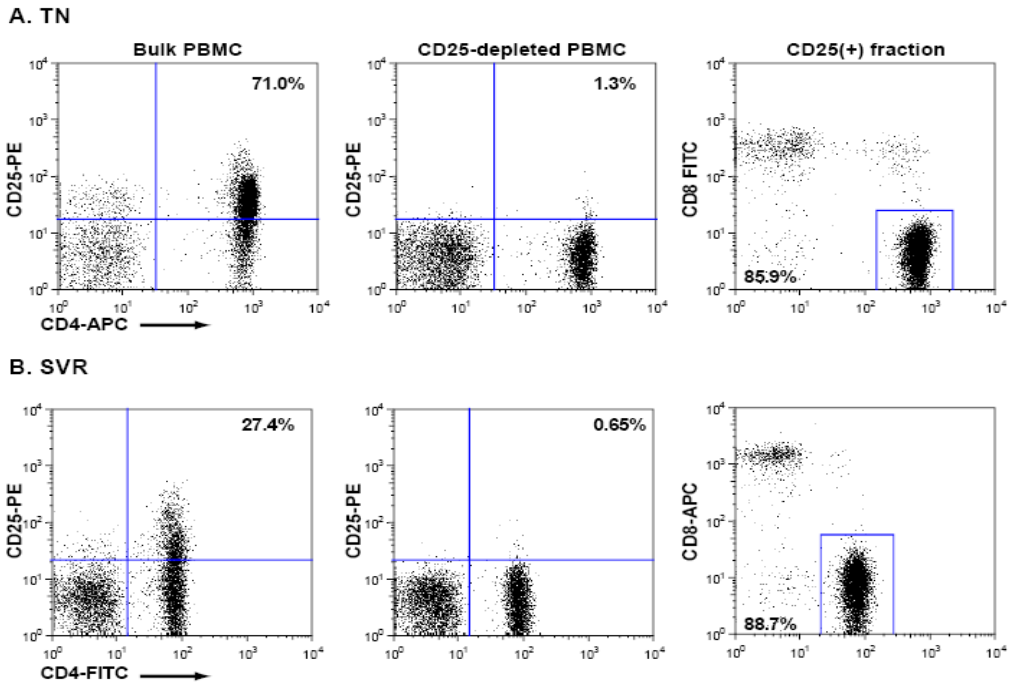


Figure 15. Treatment-naive patients show significantly higher frequency of $CD4^+CD25^+$ T cells compared to SVR's. This figure shows representative flow cytometric dot plots from one TN (Panel A) and one SVR (Panel B). The left panels demonstrate ex-vivo flow cytometric quantification of $CD4^+CD25^+$ T cells. The data represents gated $CD3^+$ T cells and the number denotes the percentage of $CD4^+$ T cells that are $CD25^+$. The middle panels show staining of CD25-depleted PBMC, confirming >95% depletion of $CD25^+$ cells. The right panels show the composition of $CD25^+$ fraction, with the numbers representing the proportion of $CD4^+$ T cells within this population (>70-90% in all cases).

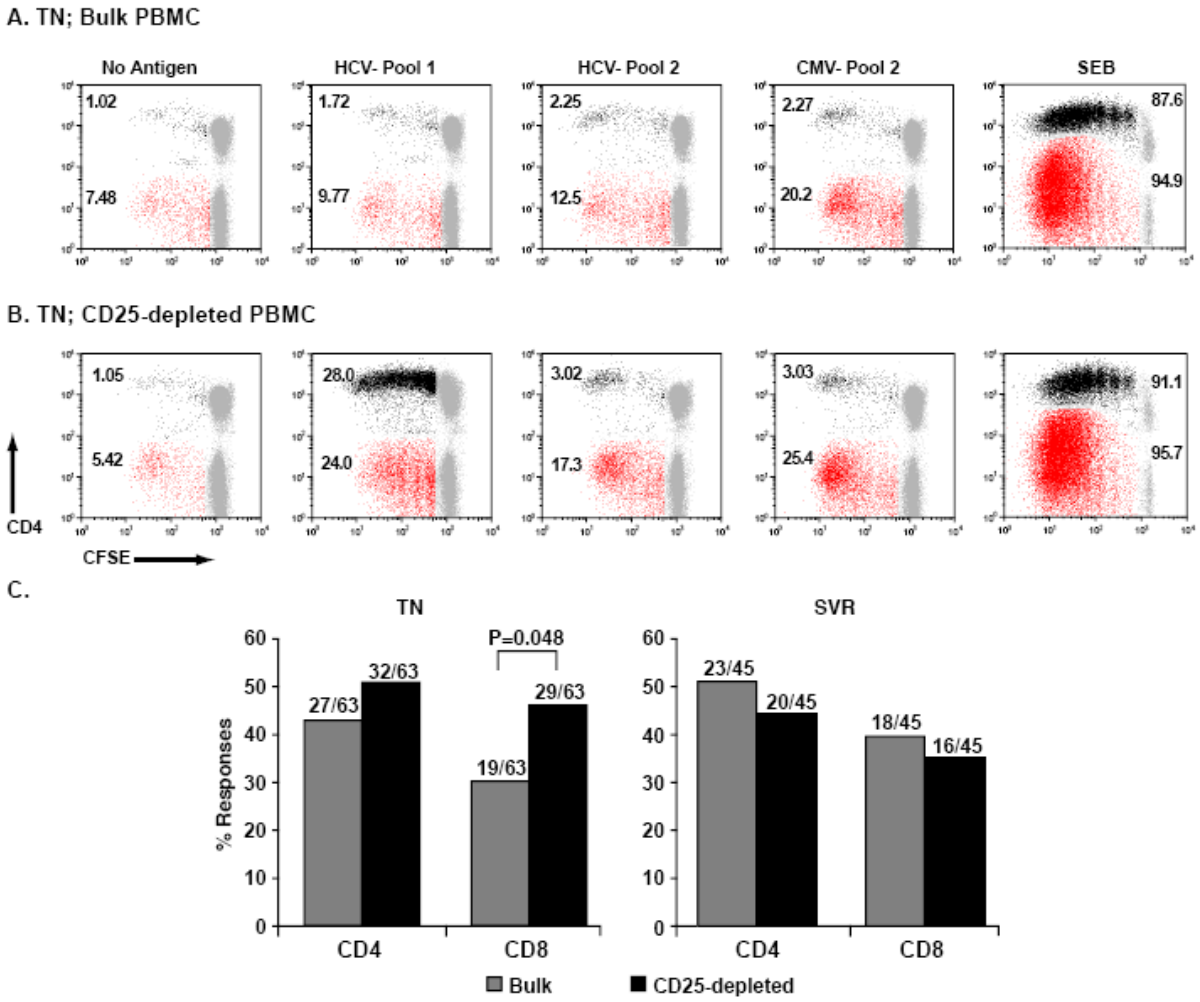


Figure 16. Depletion of CD25$^+$ cells increases HCV-specific CD8$^+$ T cell responses in TN's but not SVR's. Panels A and B show representative results from CFSE-based proliferation assays performed on bulk PBMC (Panel A) and CD25-depleted PBMC (Panel B) from a treatment-naïve patient. The numbers represent the proliferating fraction of CD4$^+$ T cells (black) and CD8$^+$ T cells (red) in response to the indicated stimuli.

Panel C shows a compilation of all depletion experiments, with each bar representing the proportion of detectable responses in bulk or CD25-depleted PBMC from TN's (left panel) and SVR's (right panel). Depletion of CD25$^+$ cells resulted in enhancement of HCV-specific CD8$^+$ T-cell responses in TN's. There was no significant effect on responses in SVR's.

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In Press, **Pillai, V.**, W.M. Lee, D.L. Thiele, and N.J. Karandikar. Clinical Responders to

Antiviral Therapy of Chronic HCV Infection show Elevated Anti-viral $CD4^+$ and $CD8^+$ T cell responses, Copyright (2006), with permission from Blackwell Publishing (140).

CHAPTER FOUR: TRANSIENT REGULATORY T CELLS: A STATE ATTAINED BY ALL ACTIVATED HUMAN T CELLS

INTRODUCTION

$CD4^+CD25^{+/high}$ regulatory T (T_{reg}) cells are an important arm of the immune system that down regulate potentially harmful effector immune responses (141). They have been shown to play a role in autoimmune disorders, infections, tumors, asthma, allergy and transplantation and hence their modulation in these diseases is thought to be of great potential benefit (142). Further elucidation of this subset has been hampered by the lack of a specific surface marker to isolate and study these cells. Markers identified as being expressed on T_{reg}s are also expressed by activated T cells. Hence, the discovery of the transcription factor FOXP3 as a marker that is expressed in T_{reg}s but not on activated T cells held major significance. Predominantly through murine studies it was shown that FOXP3 is necessary and sufficient for the development and function of T_{reg}s (18-21, 143). Transduction of FOXP3 in human $CD4^+CD25^-$ T cells appeared to confer regulatory properties to those cells (143). FOXP3 regulates T-cell activation by interacting with NFAT or NF-κB and consequently repressing IL-2 secretion (144, 145).

$CD4^+CD25^{+/high}$ T_{reg}s can be subdivided into natural T_{reg}s and induced T_{reg}s. Natural $CD4^+CD25^+FOXP3^+$ T_{reg}s are thought to arise in the thymus and suppress harmful immune responses in the periphery (141). While FOXP3 expression is thought to be a unique feature of natural T_{reg}s in mice (18-20), several human studies and some murine studies have suggested that $CD4^+CD25^-$ T cells may give rise to induced $CD4^+CD25^+FOXP3^+$ regulatory T cells following stimulation (38, 41, 146, 147). However, due to the lack of antibodies

against intranuclear FOXP3 at that time, it had been repeatedly suggested that such cells merely represent an expansion of contaminating populations of natural T_{reg}s (148-151). This continues to be a controversy in the field, with some studies reporting no up regulation of FOXP3 expression (143) and others proposing that induced $FOXP3^+$ T cells may not be regulatory in function (152). However, these studies did not directly evaluate suppressor function. Also, it is currently unclear if the induced and natural T_{reg}s represent separate lineages or if they belong to the same lineage but just differ in their location and timing of their origin. In either case, it is generally believed that FOXP3-expressing T cells, induced or natural, are a stable population of T cells with immune regulatory functions. Thus, the presence of FOXP3 and the ability to suppress effector T-cell responses in-vitro have been used as the hallmarks for the detection and quantification of this population (149). This approach has been widely used recently in human disease settings where presence or absence of $FOXP3^+$ T cells at the disease site or in the blood (with or without the presence of concomitant suppressive activity) is interpreted as evidence for involvement of T_{reg}s in the disease pathogenesis/modulation (67, 153-156).

My hypothesis was that FOXP3 is an activation marker and would be expressed by most activated T cells. I also hypothesized that antigen specific activation in the periphery is a means of generating $FOXP3^+$ regulatory T cells in humans. Hence to address the hypothesis, I evaluated the immune biology of adaptively induced $FOXP3^+$ T cells by using polychromatic flow cytometry and recently developed robust anti-FOXP3 antibodies including one that recognizes a specific spliced isoform of human FOXP3 (157). Using CFSE staining of highly purified T-cell populations, I tracked their dynamics and function following activation with different stimuli.

RESULTS

All $CD4^+CD25^-$ T cells up regulate FOXP3 transiently following activation

First, I negatively selected "untouched" human $CD3^+CD25^-$ (or $CD4^+CD25^-$) T cells and stained them with CFSE. These cells were stimulated either in a mixed lymphocyte reaction using allogeneic, T-cell-depleted antigen-presenting cells (APC) or with anti-CD3 and autologous APC to stimulate the formation of induced $FOXP3^+$ T cells, as previously described (146, 147). Following activation, these cells were monitored longitudinally for CFSE dilution and FOXP3/CD25 expression (to determine their proliferation and activation). In the absence of stimulation, the $CD4^+$ T cells did not show significant proliferation and remained $CD25^-$ and $FOXP3^-$ (Figs. 17a, 17b, 18 and 20). In contrast, stimulated cells showed distinct up regulation of FOXP3 and CD25 as early as 2 days post-stimulation with peak FOXP3 expression at 4-6 days, depending on the stimulus. Of note, virtually all dividing cells ($CFSE^{low}$) and $CD25^+$ (activated) cells expressed FOXP3 at earlier time points (Fig. 17a). This is clearly visible in allostimulated cells at the 6-day time point. A similar pattern was noted following stimulation of PBMC with nominal antigens, such as tetanus toxoid (TT) or cytomegalovirus (CMV) lysate (Fig. 17c). Importantly, FOXP3 expression by all activated cells was a transient phenomenon, lasting for 48-72 hours, followed by down regulation and plateauing around day 10-14 of culture. While the kinetics varied according to the stimulus and geometry of culture (flask versus tube), all stimuli invariably resulted in the same pattern of transient up regulation followed by down regulation.

The expression of FOXP3 was confirmed with RT-PCR assays. Ex-vivo purified or unstimulated $CD4^+CD25^-$ T cells showed no detectable FOXP3 (Fig. 17d). In contrast, cells

from activated cultures showed distinct up regulation of FOXP3, expressing both human isoforms (96). Real time quantification showed that the levels of FOXP3 in activated cells were higher than ex-vivo purified $CD4^+CD25^+$ T_{reg}s (Fig. 17e). As stated earlier and as shown in Fig. 17d, on day 6, FOXP3 expression had passed its peak in anti-CD3-stimulated cells, while it was at its peak in allostimulated cells. I also confirmed that FOXP3 message was down regulated at later time points (data not shown).

A significant portion of ex-vivo purified $CD25^-$ T cells is made up of antigen-experienced, memory T cells and it is possible that the induced $FOXP3^+$ T cells might predominantly arise from these antigen experienced populations. I next addressed whether $FOXP3^+$ T cells could be generated from naïve T cells. I depleted the initial $CD4^+CD25^-$ T cells of $CD45RO^+$ cells using microbeads, yielding >95% $CD45RA^+CD62L^{high}$ naïve T cells (158). Following anti-CD3 or allostimulation, I again observed a similar expression pattern of FOXP3 and CD25 over time (Fig.17f). Thus, virtually all activated/proliferating T cells express FOXP3, even when originating from naïve T cells.

It has been suggested that a minute population of contaminating $FOXP3^+$ or $CD25^+$ T cells may account for the $FOXP3^+CD25^+$ population following stimulation (148, 149). However, I did not observe appreciable FOXP3 expression in the initial or unstimulated population of $CD4^+CD25^-$ T cells, either by flow cytometry or by molecular assays. Even with liberal gating, $FOXP3^+$ cells were always less than 1%. Moreover, the use of CFSE in my system allowed us to "back-calculate" the percentage of cells from the initial population that responded to the stimulus and expressed FOXP3 (Figure 21). Following anti-CD3 stimulation, at least 40% of the original cells had divided and expressed FOXP3, making it impossible to explain this population merely as an expansion of contaminating $FOXP3^+$ cells.

Induced FOXP3$^+$ T cells show transient in-vitro suppressive ability

FOXP3$^+$ T_{reg}s suppress the in-vitro proliferation of autologous CD4$^+$CD25$^-$ effector T cells (146, 147). I asked whether the transient expression of FOXP3 in activated T cells also imparted a transient regulatory functionality. For this, I used two approaches. First, I confirmed previous results by utilizing CD25 magnetic microbeads to obtain CD25$^+$ T cells from early cell cultures (at this time point, CD25 expression correlated with high FOXP3 expression). I tested their ability to suppress the proliferation of freshly purified, autologous CD4$^+$CD25$^-$ T cells in standard ^{3}H-thymidine-based suppression assays (Fig. 22a). Similar to ex-vivo purified CD4$^+$CD25$^+$ T_{reg}s, the CD25$^+$ T cells from anti-CD3 or allostimulated cultures showed robust suppressive activity as well as a anergic phenotype (Fig. 22a). The induced T_{reg}s being highly enriched in FOXP3$^+$ T cells appeared to have higher suppressive activity than bead-sorted CD25$^+$ natural T_{reg}s. However, this could be explained by the fact that the natural T_{reg} population had contaminating CD25$^+$FOXP3$^-$ effector T cells (data not shown).

In the second approach, I addressed whether this suppressive potential diminished over time, corresponding with down regulation of FOXP3. For this, I could not use a CD25-based sorting technique as CD25 expression decreases over time. Thus, I flow-sorted the CFSE^{low} (dividing) and CFSE^{high} (non-dividing) cells from stimulated cultures either on day 5 or day 13, which correspond to peak and plateaued FOXP3 expression, respectively. In suppression assays, the dividing cells from day 5 cultures robustly inhibited in-vitro proliferation of CD4$^+$CD25$^-$ T cells (Fig. 19, Fig. 22b). Interestingly, suppressive ability was significantly diminished in dividing cells sorted on day 13, corresponding to down regulated FOXP3 expression. Non-dividing cells from the day 5 culture did not show either the anergic or the suppressive phenotype of 5-day dividing cells (Fig. 19).

$FOXP3^+$ induced T_{reg}s may serve to limit local immune responses and tissue damage (13, 149). In my culture system, activated T cells accumulate higher quantities of FOXP3 as they divide, with cells in later divisions showing preferential FOXP3 and CD25 expression, suggesting that these cells may inhibit the division of other cells in the same culture (especially, the non-dividing, $CFSE^{high}CD4^+$ cells). In order to test this hypothesis, I flow-sorted dividing and non-dividing cells from a 13-day anti-CD3-activated culture (Fig. 22c). On re-stimulation with anti-CD3 for an additional 6 days, the non-dividing cells robustly up regulated CD25, expressed FOXP3 and went through multiple rounds of division, suggesting that the non-dividing cells may have been suppressed in the prior culture. Re-stimulation of the $CFSE^{low}$ populations resulted in a re-up regulation of FOXP3 and CD25 (not shown), suggesting that T cells may potentially up regulate FOXP3 and CD25 during multiple activation cycles, attaining a transient regulatory phenotype each time.

Transiently induced T_{reg}s share immunophenotypic similarities with natural T_{reg}s

I used 6-10 color flow cytometric panels to evaluate the immunophenotype of the transiently induced T_{reg}s (Fig. 23a, Fig. 20, Fig. 24). The anti-CD3 or allo-induced $FOXP3^+$ T cells exhibited a CTLA-4 $(CD152)^{high}$, $CD25^{high}$, $CCR7^+$, $CD27^+$, $CD62L^+$, $CD28^+$ phenotype (Fig. 23a, Fig. 20, Fig. 24), similar to that of natural T_{reg}s in humans (159-161). As FOXP3 expression was down regulated, some of these markers were also down regulated among cells that had undergone multiple rounds of division (Fig. 24). Thus, transient FOXP3 expression by activated T cells corresponds to not only transient suppressor ("T_{reg}") functionality but also immunophenotypic features similar to natural T_{reg}s. These results suggest that, in humans, natural T_{reg}s, induced T_{reg}s or transient T_{reg}s may be similar or highly related populations with the major difference being the location or timing of their origin.

It has been recently shown that low CD127 (IL-7 receptor) expression may be a specific marker for natural FOXP3$^+$ T_{reg}s (151, 162). Hence, I evaluated CD127 status on transient T_{reg}s (Fig. 23b). I observed that the induced T_{reg}s down regulated CD127 and were CD127^{low} when they were FOXP3$^+$. They continued to remain CD127^{low} even as they became FOXP3$^-$. Hence, even CD127 does not appear to differentiate natural T_{reg}s from transiently induced FOXP3$^+$ T_{reg}s.

Relative expression levels of FOXP3 isoforms in human T-cell populations.

FOXP3 in humans exists as at least two isoforms, in contrast to mice where only one isoform has been described (96). The full-length isoform is suggested to be homologous to foxp3 in mice, while the isoform lacking exon 2 (Δ2) appears to be specific to human T cells. To see whether there were differences in isoform expression in natural vs. induced/transient T_{reg}s, I used commercially available antibodies to quantify the relative amounts of FOXP3 in human T-cell populations (Fig. 28a). Using CD25 microbeads, I purified two subsets of CD4$^+$ T cells ex-vivo: a subset enriched in CD25$^{high/intermediate}$ T cells and a CD25$^-$ subset. CD4$^+$CD25$^{high/intermediate}$ T cells expressed both isoforms ex-vivo, with a larger subset expressing the Δ2 isoform (Fig. 28b). Following anti-CD3 activation, CD4$^+$CD25$^-$ T cells induced predominantly the Δ2 isoform with a small subset expressing the full-length isoform (Fig. 28c). Activated CD4$^+$CD25$^{high/intermediate}$ T cells appeared to harbor a relatively larger subset expressing the full-length isoform. Allostimulation also produced a similar pattern of FOXP3 expression (not shown). In multiple experiments, the "back-calculated" frequency of cells giving rise to the full-length isoform matched the proportion of FOXP3-expressing cells in the initial populations. This suggested that the cells expressing the full-length isoform may have arisen from the CD25^{high} natural T_{reg} population. In contrast, the transiently

induced T_{reg}s may preferentially express the Δ2 isoform. $CD8^+CD25^-$ T cells also up regulated both the isoforms with the Δ2 isoform predominating (Fig. 28c).

These studies raise the possibility that the full-length isoform may represent the more long-lived natural isoform while the Δ2 isoform may represent the transient form. However, when I stimulated ex-vivo-purified $CD4^+CD25^{high/intermediate}$ T cells with anti-CD3, they expressed both isoforms and also down regulated both isoforms over time, suggesting that both isoforms could be expressed transiently (Fig. 29). Suffice it to say that both isoforms could be found in all populations of T_{reg}s, albeit at varying proportions.

Differential dynamics of effector functions and FOXP3 in transient T_{reg}s

As FOXP3 expression occurs during activation, I wanted to determine the relationship between transient T_{reg} function and T-cell effector functions. Thus, I quantified the longitudinal expression of specific effector cytokines following activation, in flow-sorted $CFSE^{low}CD25^+$ cells from allostimulated T-cell cultures (Fig. 25). Interestingly, IFN-γ message was observed in the initial stages of activation, whereas FOXP3 and IL-10 were co-expressed at later time points. IFN-γ expression was also lowest when FOXP3 and IL-10 expression were highest. Of note, TNF-α also showed high levels of expression at this time, suggesting that different effector functions were differentially regulated during activation. IL-2 and IL-4 messages were either low or undetectable at the peak of FOXP3 expression (not shown). Similar dynamics were observed even when bulk populations were used instead of flow-sorted, activated cells.

$CD8^+CD25^-$ T cells also show transient FOXP3 up regulation and suppressor activity following activation

While $CD4^+FOXP3^+$ T_{reg}s have been extensively evaluated, much less is known about the biology of $CD8^+FOXP3^+$ T cells. Human $CD8^+$ T cells have been found to express FOXP3 (48, 80, 152, 157, 163). I then wanted to determine if activated $CD8^+$ T cells also show kinetics of FOXP3 expression and suppressive activity similar to $CD4^+$ T cells. Ex-vivo, I did not detect appreciable FOXP3 expression on either $CD8^+CD25^-$ or $CD8^+CD25^+$ T cells. Starting with either $CD3^+CD25^-$ T cells or purified $CD8^+CD25^-$ T cells, I observed distinct FOXP3 up regulation in response to allo- (Fig. 26a) or anti-CD3 (Fig. 27) stimulation. Similar to $CD4^+$ T cells, virtually all the responding $CD8^+$ T cells expressed FOXP3, followed by eventual down regulation. Bead-sorted $CD8^+CD25^+$ T cells from early cultures showed robust in-vitro suppression of autologous $CD4^+CD25^-$ T cells, comparable to that of $CD4^+$ T_{reg}s (Fig. 26b). Similarly, flow-sorted dividing $CD8^+$ T cells from early cultures also showed robust in-vitro suppression, which was partially reversed at later time points (Fig. 26c). Activated $CD8^+$ T cells also expressed both isoforms of FOXP3 (Fig. 28c). Thus, the property of inducing transient $FOXP3^+$ T_{reg}s is not restricted to $CD4^+$ T cells, but may be a state attained by virtually all activated T cells.

DISCUSSION

It is known that $CD25^+FOXP3^+$ T cells with regulatory properties can be induced following activation (38, 41, 146, 147). For example, using PCR-based analysis, it has been demonstrated that $CD4^+CD25^-$ T cells can generate $FOXP3^+$ regulatory T cells (147). However, it was widely proposed that these cells arose from a contaminating population of natural T_{reg}s (148-150). Moreover, the proportion of activated T cells that become $FOXP3^+$ and the stability and significance of such expression are poorly understood. In general, $FOXP3^+$ T cells are thought to be a unique subset of stable, regulatory T cells.

In this study, I have demonstrated that virtually all activated $CD4^+CD25^-FOXP3^-$ T cells attain a transient phase of FOXP3 expression and regulatory activity. I also show that these $FOXP3^+$ cells must arise from $CD4^+CD25^-$ T cells and that this phenomenon occurs regardless of the stimulus used to activate these cells. While previous studies have shown that $FOXP3^+$ T cells can be induced from $CD4^+CD25^-FOXP3^-$ T cells, it is thought that only a small subset of approximately 10-20% of the activated T cells express FOXP3. Recent studies using anti-FOXP3 antibodies to detect FOXP3 expression have used either PBMC (152, 157) or PBMC depleted of $CD25^+$ T cells (152, 164) to study activation-induced FOXP3 expression. In those studies, cells evaluated for FOXP3 also included non-activated T cells in the culture. The use of CFSE in our assay system clearly shows that, even in anti-CD3 stimulated cultures, not all T cells undergo activation and proliferation. This allowed us to accurately gate on activated T-cell populations, demonstrating that all activated T cells, rather than a minor subset, undergo this change. Transient FOXP3 expression is better visualized following allostimulation compared to anti-CD3 stimulation as only a percentage of the responding cells are activated by the allostimulus. The use of the CFSE-based approach also allowed us to visualize the non-dividing (unstimulated) cells as an

internal control for CD25 and FOXP3 expression. Depending on the geometry of the culture (flask versus tubes) and the nature of the stimulus (anti-CD3 or antiCD3+antiCD28 versus MLR versus antigen), the kinetics of activation and consequently FOXP3 expression were different. Thus, if only a single time point is evaluated, one would reach a restricted conclusion about the number of cells that express FOXP3. Moreover, the actual day of peak expression is less important than the overall phenomenon of transient up regulation followed by down regulation. Our results support the conclusion that induction of a $FOXP3^+$ T_{reg} state is not a rare or unusual event, but rather the norm.

Importantly, most of the activated $FOXP3^+$ cells down regulate FOXP3 and by day 10-14, only a subset of that population remains $FOXP3^+$. I show that regulatory activity decreases at later time points when FOXP3 expression has plateaued. To our knowledge, these are the first studies to longitudinally follow regulatory function in activated T-cell populations. These results suggest that FOXP3 expression correlates with suppressive activity and that suppressive ability is a transient property of all activated $CD4^+$ T-cell populations. Even when starting with naïve $CD4^+$ T cells, the same pattern is observed, suggesting that activation might be a way of producing T_{reg}s in the periphery where every dividing T-cell has the potential to a become effector/memory T-cell and/or a T_{reg}. Presumably, with multiple rounds of activation, a higher proportion of cells may be sustained as T_{reg}s, as might happen in the setting of chronic infections, such as HCV and HIV (68-70, 165). More importantly, our results also suggest that mere depletion of circulating T_{reg}s may not be adequate therapy for tumors and infections. Not only would newer T_{reg}s be produced during sustained antigenic stimulation, but the therapeutic approach may also carry the risk of eliminating potential effector T cells. In these situations, tilting the balance from T_{reg} sustenance toward effector T-cell induction may be a plausible immunotherapeutic intervention. It is possible that FOXP3 expression could be coincident with suppressive

activity, rather than mechanistically involved in this function. Regardless of its involvement, FOXP3 expression and regulatory function appear to be a transient phenomenon in all T cells.

It has long been known that natural T_{reg}s show an activated T-cell phenotype and efforts are still underway to identify a specific marker for T_{reg}s that is not expressed by activated T cells. Every putative "specific" T_{reg} marker proposed thus far eventually turned out to be expressed by activated T cells as well. I show here that induced T_{reg}s also show an immunophenotype that is similar to that of natural T_{reg}s. I propose that the similarities between T_{reg}s and activated T cells may, in fact, not be a coincidence. A major subset of circulating human T_{reg}s may simply reflect the long-term sustenance of a subpopulation of activated cells. I found no obvious differences in the suppressive activity or phenotype between natural and induced T_{reg}s, suggesting that these may be related populations with the location and timing of origin being the main difference between them.

In this study, I also evaluated effector functions in the context of FOXP3 expression, by flow-sorting pure populations of activated cells at multiple time points post-stimulation. IFN-γ expression preceded and inversely correlated with FOXP3 expression. FOXP3 expression coincided with peak IL-10 production and TNF-α production in these cultures. Thus, I show that effector function and regulatory function can occur in the same T-cell population. These results suggest that therapeutic attempts to increase T_{reg}s may paradoxically increase potentially harmful effector responses. I speculate that this could be one of the plausible explanations for the results of the recent clinical trial using an anti-CD28 super agonist TGN1412, which attempted to expand T_{reg}s but resulted in severe multiorgan failure (77, 166, 167). Prior studies have shown increased T_{reg} induction by this agent (168, 169). However, based on our results, these may have potentially been transient T_{reg}s, rather

than a stable T_{reg} population, a notion supported by recent studies using this agent in a humanized mouse system (170). This underscores the importance of understanding the biology of induced T_{reg}s and its relationship with effector functionality.

$CD8^+$ T cells have been shown to possess regulatory activity distinct from that of natural $CD4^+CD25^+FOXP3^+T_{reg}$s (48, 171). Though a definitive $CD8^+$ counterpart of the natural $CD4^+CD25^+$ T_{reg}s has not been described, FOXP3 expression has been detected in $CD8^+$ T cells under various conditions (48, 80, 152, 157, 163). One of our key findings is that $CD8^+$ T cells also show similar dynamics, where all activated $CD8^+$ T cells (in the presence or absence of $CD4^+$ T cells) express FOXP3 and exhibit regulatory activity transiently. These findings strongly suggest that activation-induced FOXP3 expression and regulatory activity may be a broad T-cell phenomenon. Presumably, such a mechanism might allow T cells with the highest affinity to effectively suppress lower affinity responses in their milieu (172). It remains to be seen if the induced $CD8^+FOXP3^+$ T_{reg}s persist in the periphery for any significant length of time compared to the $CD4^+FOXP3^+$ T_{reg}s.

In summary, I have shown that virtually all activated $CD4^+$ and $CD8^+$ T cells transiently up regulate FOXP3 and acquire suppressive properties. Thus, during an active immune process, most of the $FOXP3^+$ cells could, in fact, represent effector T cells that can down regulate FOXP3 and lose regulatory activity. Thus, neither FOXP3 expression nor in-vitro suppressive ability is an indication of stable T_{reg} function and these readouts should not be used to designate a cell as a permanent T_{reg}. These observations warrant the re-evaluation of prior data relating to the role of T_{reg}s in various human diseases.

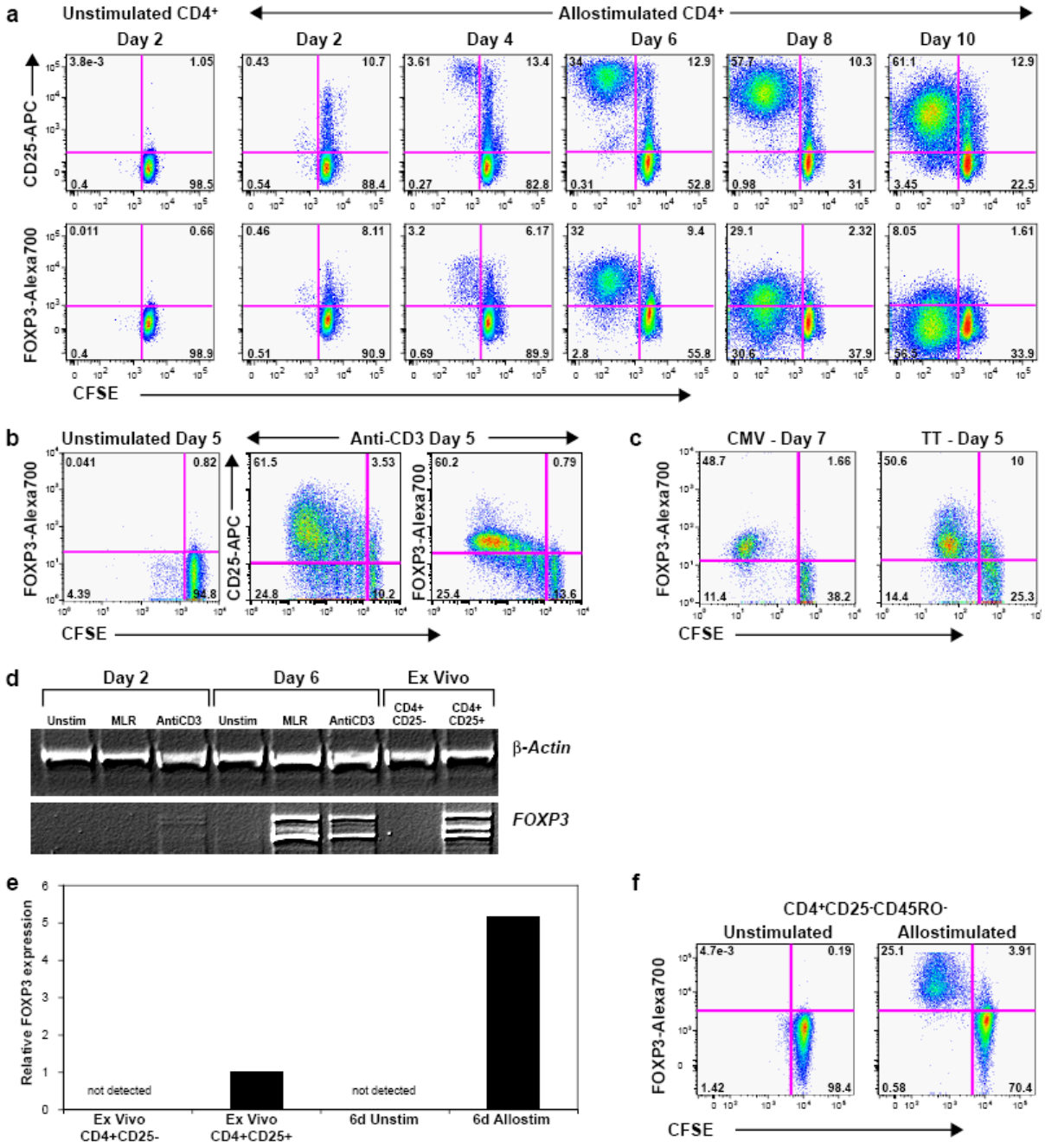


Figure 17. $CD4^+CD25^-$ T cells up regulate FOXP3 transiently following activation. Bead sorted 'untouched' $CD3^+CD25^-$ T cells (**a**) or $CD4^+CD25^-$ T cells (**b, d and e**) were stained with CFSE and activated with either allogeneic T-cell-depleted PBMC (**a**) or with anti-CD3 antibody and autologous APC (**b**). CD25 and FOXP3 expression and CFSE dilution were monitored over time. **a,** Color-coded contour plots of gated $CD4^+$ T cells from allostimulated cultures are shown, with CFSE on the X-axis and CD25 (top row) or FOXP3 (bottom row) on the Y-axis. The vertical lines separate dividing ($CFSE^{low}$) from non-

dividing ($CFSE^{high}$) cells. **b,** Anti-CD3 stimulated $CD4^+CD25^-$ T cells at five days of culture. **c,** CFSE-stained PBMC stimulated with either CMV lysate (day 7) or tetanus toxoid (day 5) are shown. Data are gated for $CD4^+$ T cells. **d,** RT-PCR analysis of $CD4^+$ T cells for FOXP3 expression is shown. Primers were picked such that full-length isoform and the shorter alternatively spliced form could be detected as different bands in agarose gels. Cells were obtained from 2-day or 6-day cultures or purified ex-vivo, as indicated. **e,** Total FOXP3 expression by quantitative real time PCR analysis. FOXP3 levels were first normalized to β-actin and then normalized to levels seen in ex-vivo-purified $CD4^+CD25^+$ T cells (assigned a value of 1.0). **f,** FOXP3 expression in allostimulated CD45RO-depleted $CD4^+CD25^-$ naïve T cells at 6 days of culture. All results are representative of at least 2 independent replicates performed on different donors (multiple replicates for **a** and **b**). Position of the positive gates for FOXP3 and CD25 in all graphs were determined based on the unstimulated control at the same time point and was also confirmed by either FMO (fluorescence minus one) staining (for polychromatic flow cytometry) or isotype control staining.

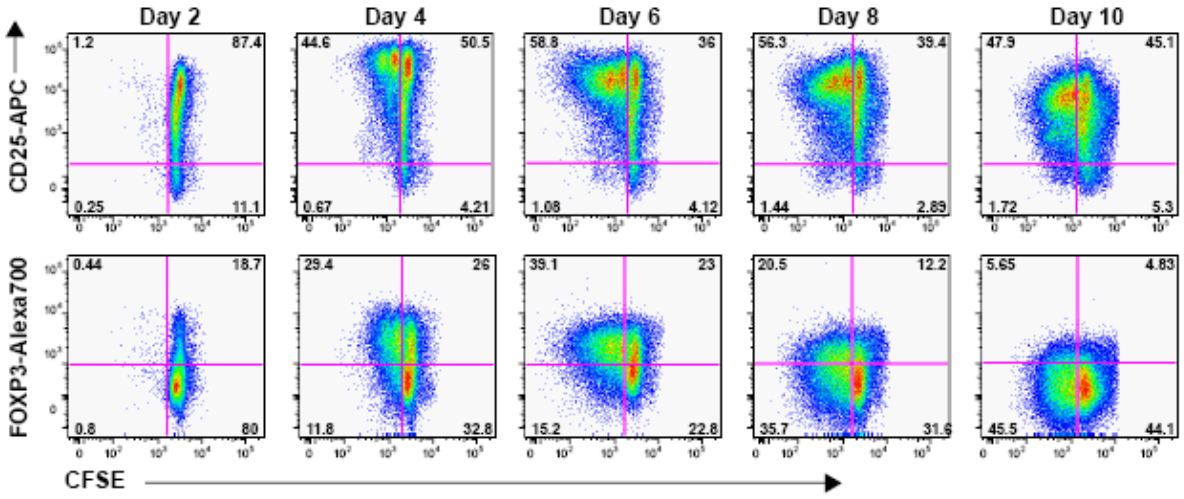


Figure 18. Anti-CD3 stimulated CD4$^+$T cells show transient expression of FOXP3. Bead sorted, untouched CD3$^+$CD25$^-$ T cells were activated with anti-CD3 in presence of autologous, irradiated APC's. Expression of FOXP3 and CD25 is shown days 2-10 post-stimulation in gated CD4$^+$ T cells.

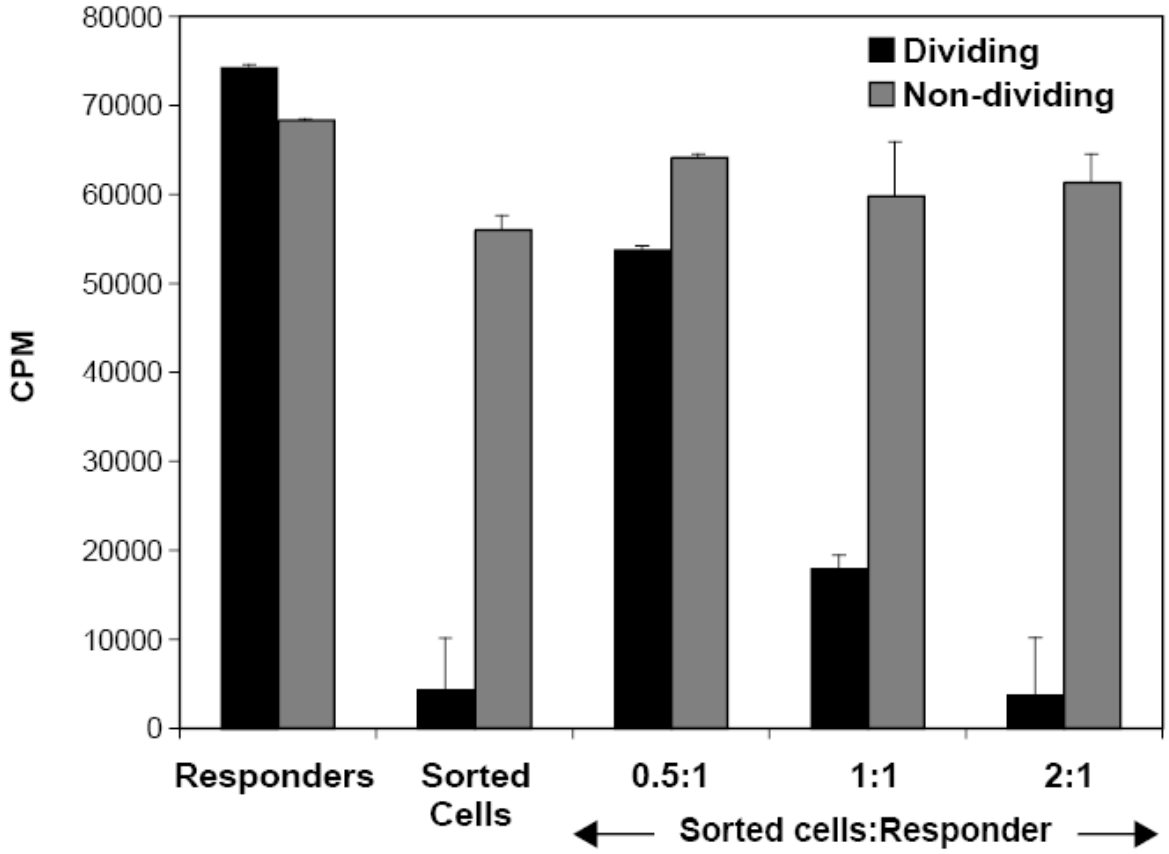


Figure 19. Non-dividing cells from day 5 cultures are not anergic and do not suppress. $CFSE^{low}$ dividing cells (black bars) and $CFSE^{high}$ non dividing cells (gray bars) were flow sorted on day 5 from an anti-CD3-activated $CD4^{+}CD25^{-}$ T-cell culture and evaluated for suppressive ability. The counts per minute (CPM) from a 5-day ^{3}H-thymidine based suppression assay are shown.

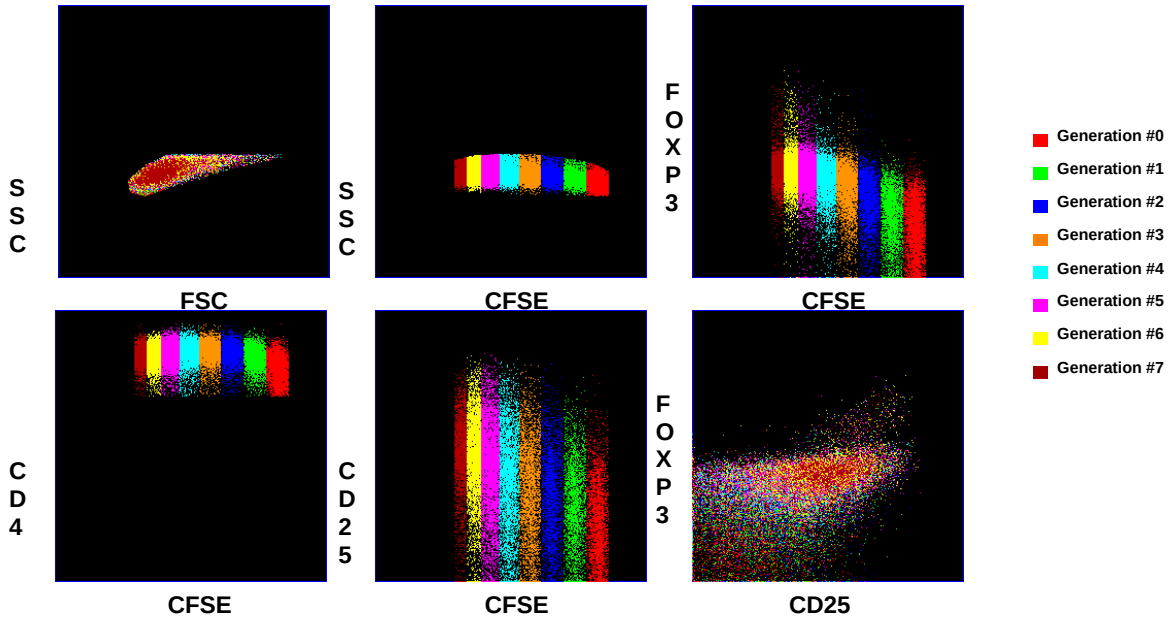


Figure 20. Phenotypic characteristics of every generation of transient T_{reg}s. $CD4^+CD25^-$ T cells were activated with an anti-CD3 stimulus. The cells undergo 7-8 divisions post culture. Every generation is painted in the color indicated to create the multicolor graphs shown. Phenotypic and other characteristics of every generation can be tracked in this manner. $CD4^+CD25^-$ T cells express increasing amounts of CD25 and FOXP3 as they divide.

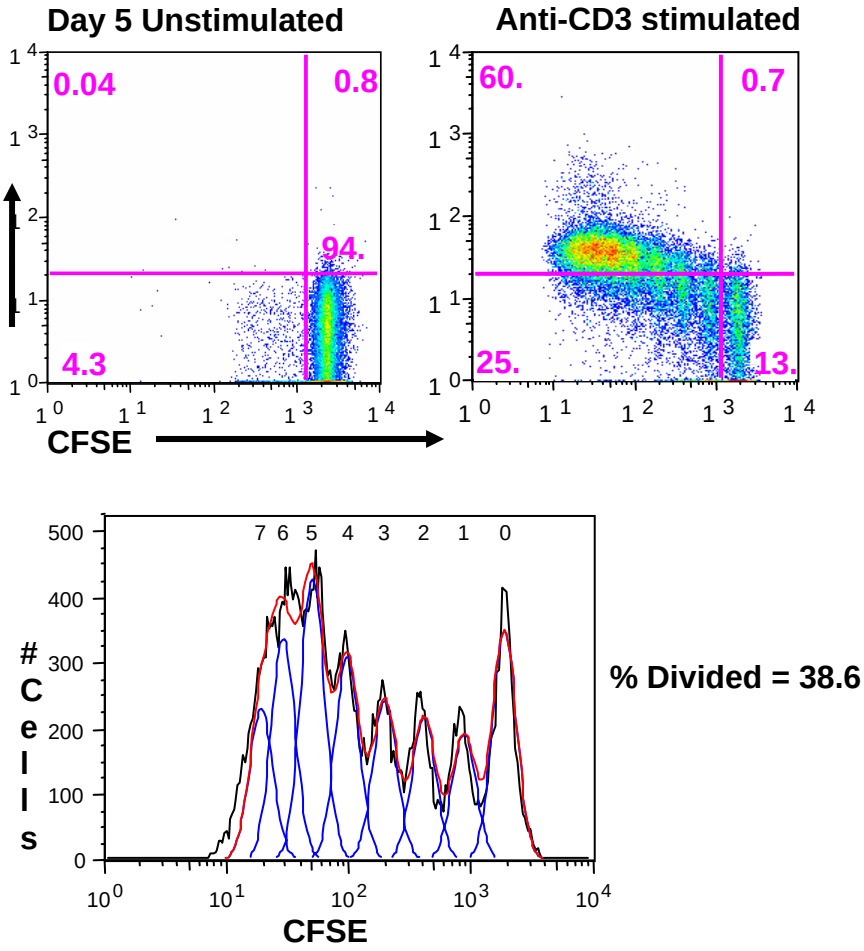
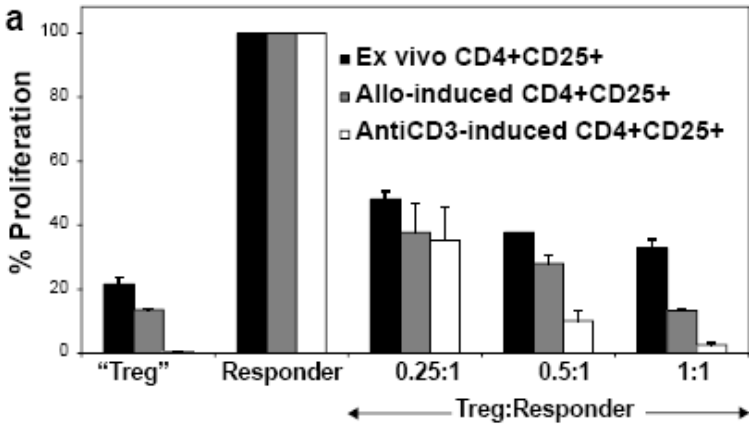
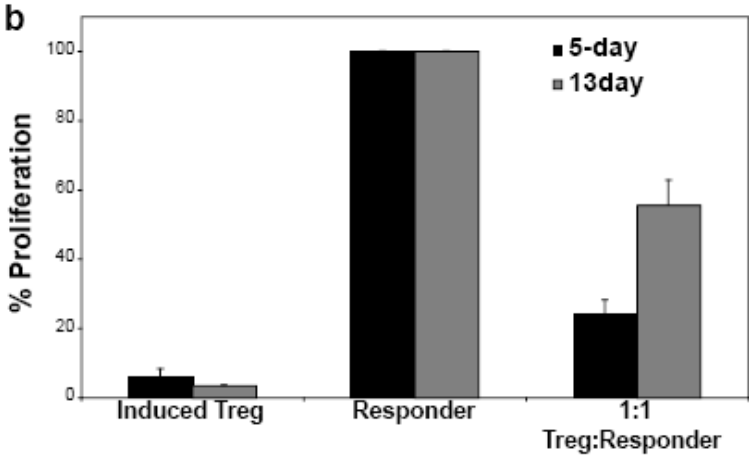


Figure 21. Calculation of precursor frequency of the dividing cells. The proliferation platform in FlowJo was used to calculate the precursor frequency of dividing and FOXP3 expressing cells. In this representative example shown about 85% of cells are in the dividing CFSE low region out of which around 80% express FOXP3. Back calculation of the percentage of the starting population these dividing cells represent indicated that they arise from 38.6% of the population. Hence, they cannot arise from the contaminating <1% of CD25 or FOXP3 expressing population in the starting population.


a
% Proliferation
100
80
60
40
20
0
Ex vivo CD4+CD25+
Allo-induced CD4+CD25+
AntiCD3-induced CD4+CD25+
"Treg"
Responder
0.25:1
0.5:1
1:1
Treg:Responder


b
% Proliferation
100
80
60
40
20
0
5-day
13day
Induced Treg
Responder
1:1
Treg:Responder

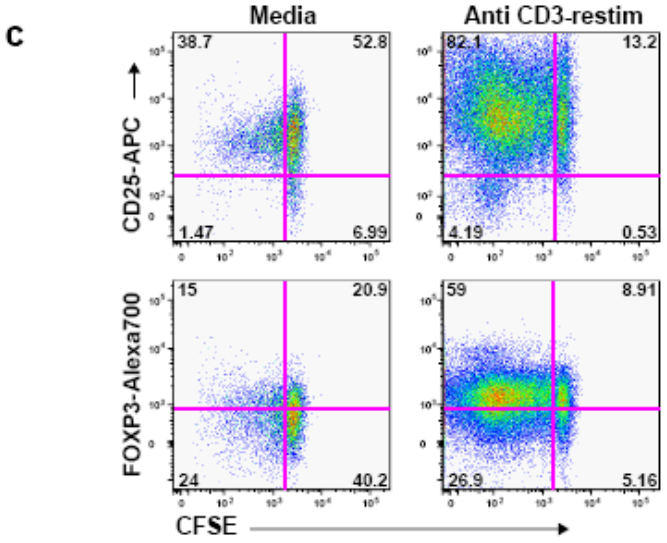
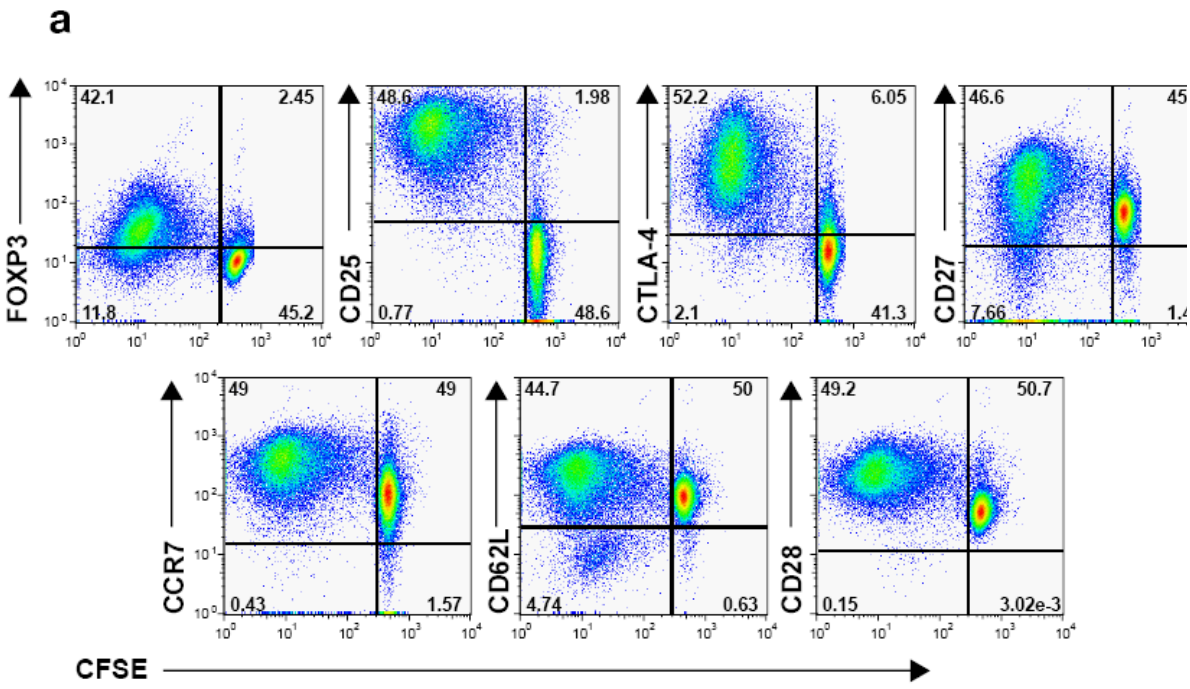

c
Media
Anti CD3-restim
CD25-APC
38.7
52.8
1.47
6.99
82.1
13.2
4.19
0.53
FOXP3-Alexa700
15
20.9
24
40.2
59
8.91
26.9
5.16
CFSE

Figure 22. Induced "T_{reg}s" show transient in-vitro suppressive ability. Bead-sorted, 'untouched' $CD4^+CD25^-$ T cells were activated with either anti-CD3 or allostimulation. **a,** $CD4^+CD25^+$ T cells were sorted from PBMC ex-vivo (black bars) or at 7 days of culture (as indicated) and tested for the ability to suppress the anti-CD3-mediated proliferation of freshly thawed, autologous responder $CD4^+CD25^-$ T cells in the presence of irradiated APC. The counts per minute (CPM) from a 5-day ^{3}H-thymidine based assay were obtained and then normalized to the anti-CD3-induced proliferation of responder cells (assigned a value of 100; CPM were in the 50,000 to 80,000 range in most experiments on various donors). The sorted $CD4^+CD25^+$ T cells were anergic and were able to suppress the responder cells at increasing ratios. In this experiment, the proportion of $FOXP3^+$ cells was highest in anti-CD3-activated cells followed by allostimulated cells (data not shown). **b,** $CFSE^{low}$ dividing cells were flow sorted on day 5 (black bars) or day 13 (gray bars) from an anti-CD3-activated $CD4^+CD25^-$ T-cell culture and evaluated for suppressive ability. **c,** $CFSE^{high}$ (non-dividing) cells from day 13 of an anti-CD3-activated culture were flow sorted and were re-cultured with fresh autologous APC in media alone or anti-CD3 for a further 6 days. Robust proliferation and up regulation of CD25 and FOXP3 are shown.


a
FOXP3
42.1
2.45
11.8
45.2
CD25
48.6
1.98
0.77
48.6
CTLA-4
52.2
6.05
2.1
41.3
CD27
46.6
7.66
CCR7
49
49
0.43
1.57
CD62L
44.7
50
4.74
0.63
CD28
49.2
50.7
0.15
3.02e-3
CFSE

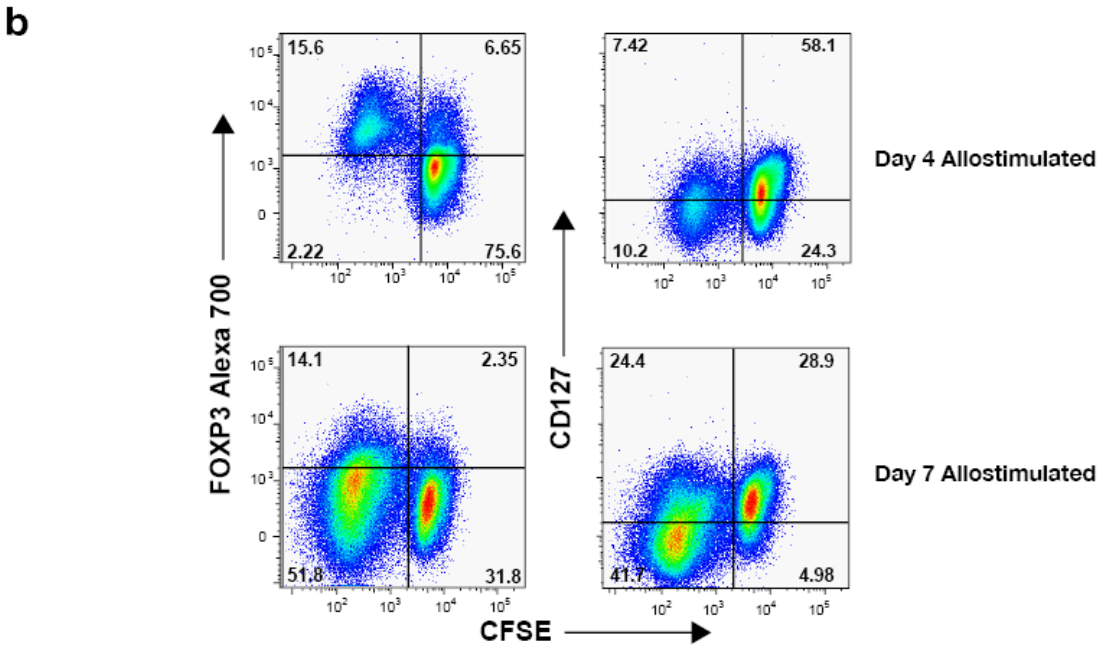

b
15.6
6.65
2.22
75.6
7.42
58.1
10.2
24.3
Day 4 Allostimulated
FOXP3 Alexa 700
CD127
14.1
2.35
51.8
31.8
24.4
28.9
41.7
4.98
Day 7 Allostimulated
CFSE

Figure 23. Phenotypic analysis of transient FOXP3^{+} regulatory T cells. a, Allostimulated, CFSE-stained CD4^{+}CD25^{-} T cells were phenotyped for FOXP3, CD25, CTLA-4, CD27, CD62L, CCR7 and CD28 expression at day 6 of culture. Dividing CFSE^{low} cells were positive for most of the markers shown, similar to the phenotype of ex-vivo purified T_{reg}s. **b,** CD127 (IL7-R) expression was monitored on allostimulated T cells, revealing CD127 down regulation with the induction of FOXP3. CD127 remained low at a point where FOXP3 was down regulated.

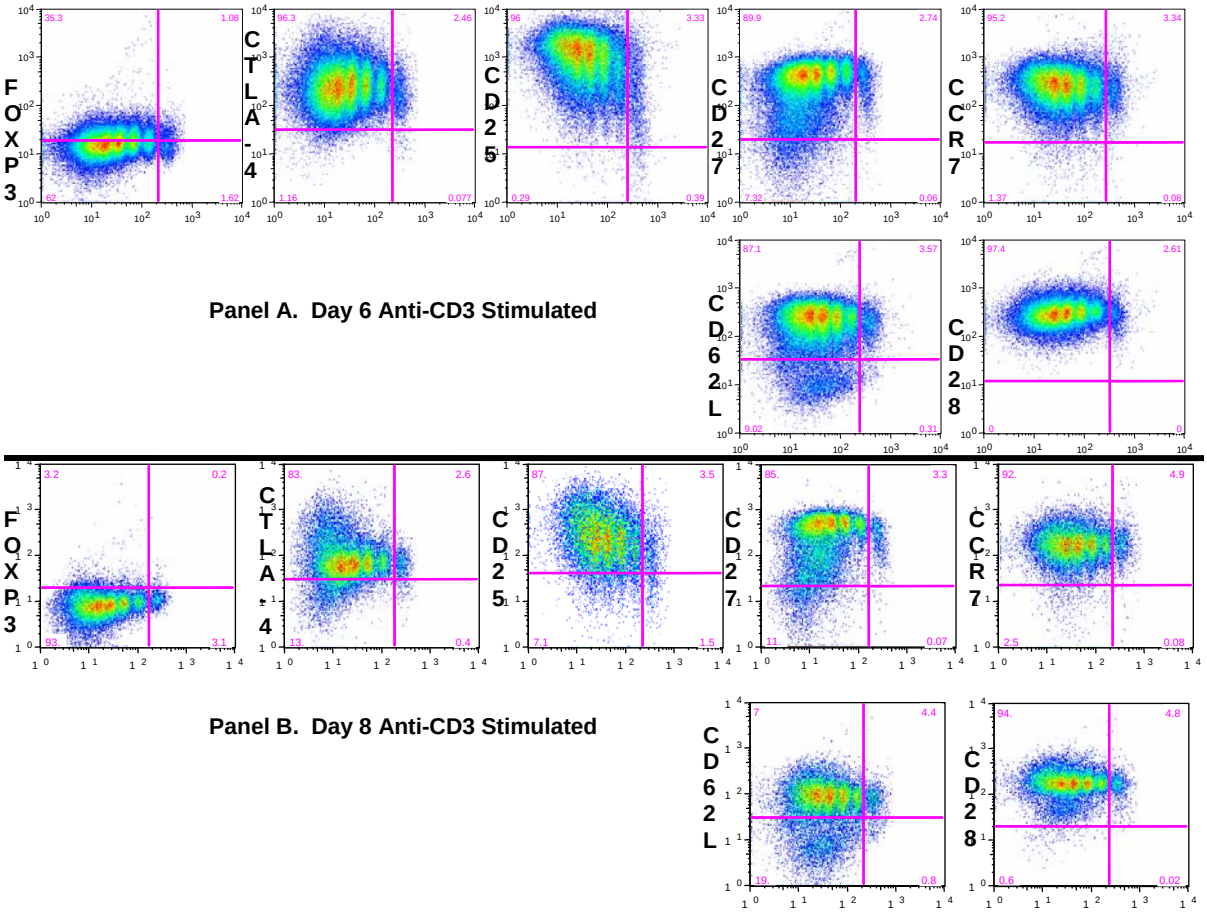


Figure 24. Down regulation of some phenotypic markers concurrent with a down regulation of FOXP3 expression. AntiCD3 activated $CD4^{+}CD25^{-}$ T cells were phenotyped using 8 color flow cytometry for common markers associated with a T_{reg} phenotype like CD25, CTLA-4, CD27, CCR7, CD62L and CD28. These markers play important roles in signaling, lymph node homing and migration. Activated T cells up regulate these markers along with up regulation of FOXP3 at day 6 (Panel A). Most of these markers are eventually down regulated after a few divisions concurrent with a down regulation of FOXP3 as seen in Panel B.

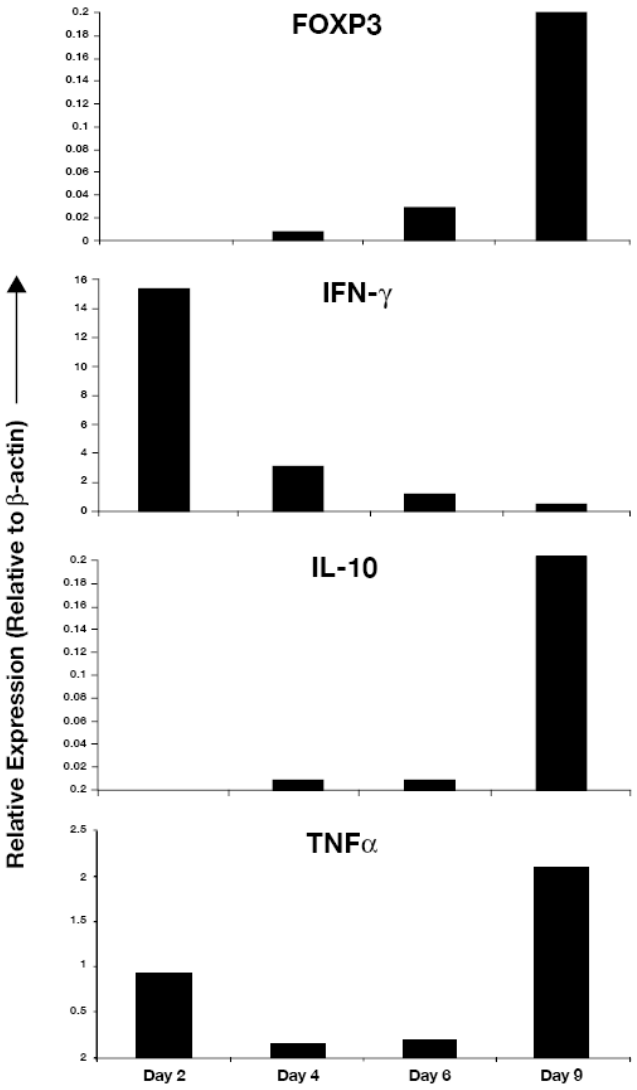


Figure 25. Effector functions are differentially regulated during T_{reg} induction. Allostimulated $CD4^+CD25^-$ T-cell cultures were flow sorted for $CD4^+$, $CFSE^{high}$, $CD25^+$ fraction (Day 2), $CD4^+$, $CFSE^{low}$, $CD25^+$, dividing fraction (Days 4, 6 and 9) to obtain highly pure induced T_{reg} populations at different stages of their formation. FOXP3, IFN-γ, TNF-α and IL-10 messages were quantified by real time PCR and normalized to β-actin. Presence of specific product was confirmed by dissociation curve and gel analysis.

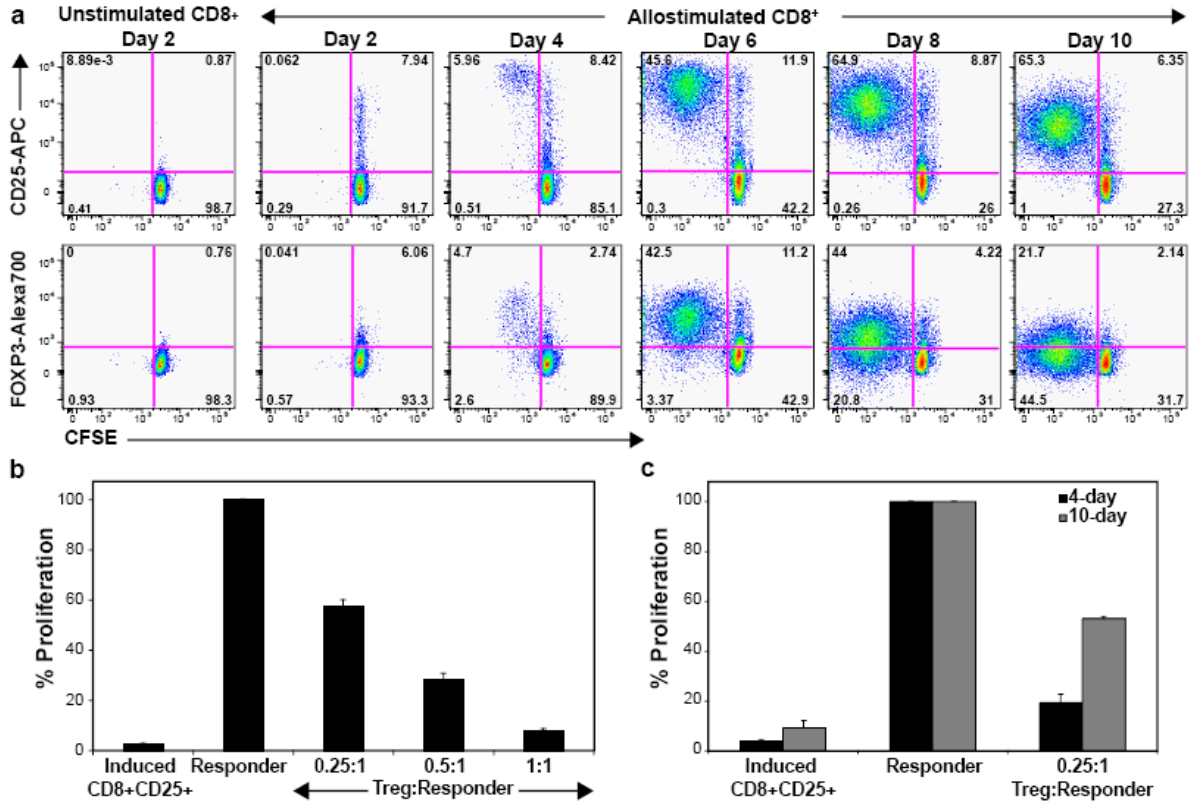


Figure 26. $CD8^{+}CD25^{-}$ T cells show transient FOXP3 up regulation and suppressor activity following activation. a, Gated $CD8^{+}$ T cells from an allostimulated $CD3^{+}CD25^{-}$ T-cell culture are shown. Transient up regulation of FOXP3 and CD25 on $CFSE^{low}$ (dividing) cells is depicted. **b,**$CD25^{+}$ T cells were sorted from 5-day cultures of anti-CD3-stimulated $CD8^{+}CD25^{-}$ T cells and tested for their ability to suppress autologous $CD4^{+}CD25^{-}$ T cells in ^{3}H-thymidine based suppression assays. Proliferation data was normalized to responder proliferation (100%). **c,** Suppressive ability of flow-sorted $CFSE^{low}$ (dividing) cells from day 4 and day 10 of anti-CD3-activated $CD8^{+}CD25^{-}$ T-cell cultures.

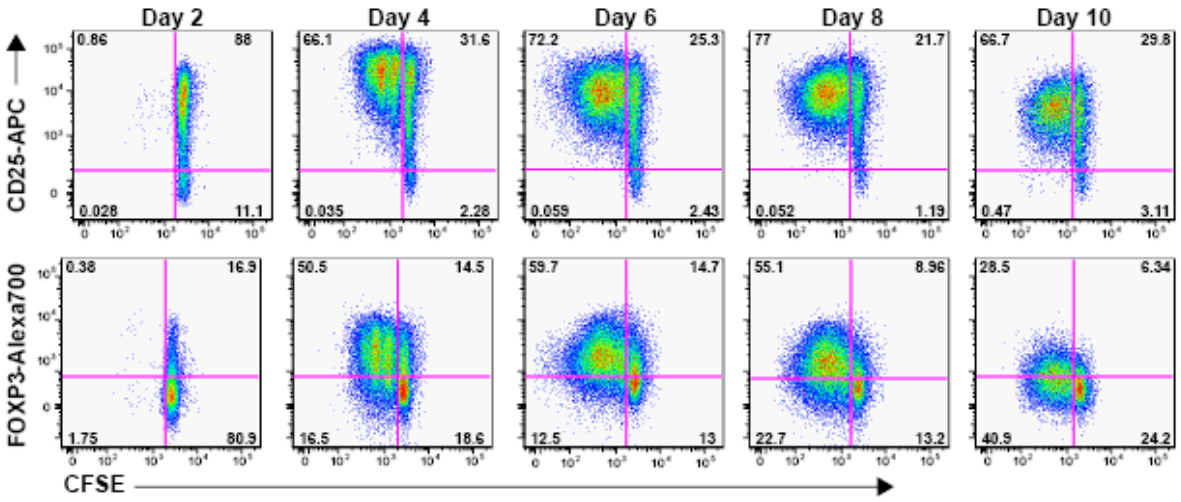


Figure 27. Anti-CD3 stimulated CD8$^+$ T cells show transient up regulation of FOXP3. Bead-sorted, untouched CD3$^+$CD25$^-$ T cells were activated with anti-CD3 in presence of autologous irradiated APC. Expression of FOXP3 and CD25 is shown days 2-10 post-stimulation in gated CD8$^+$ T cells.

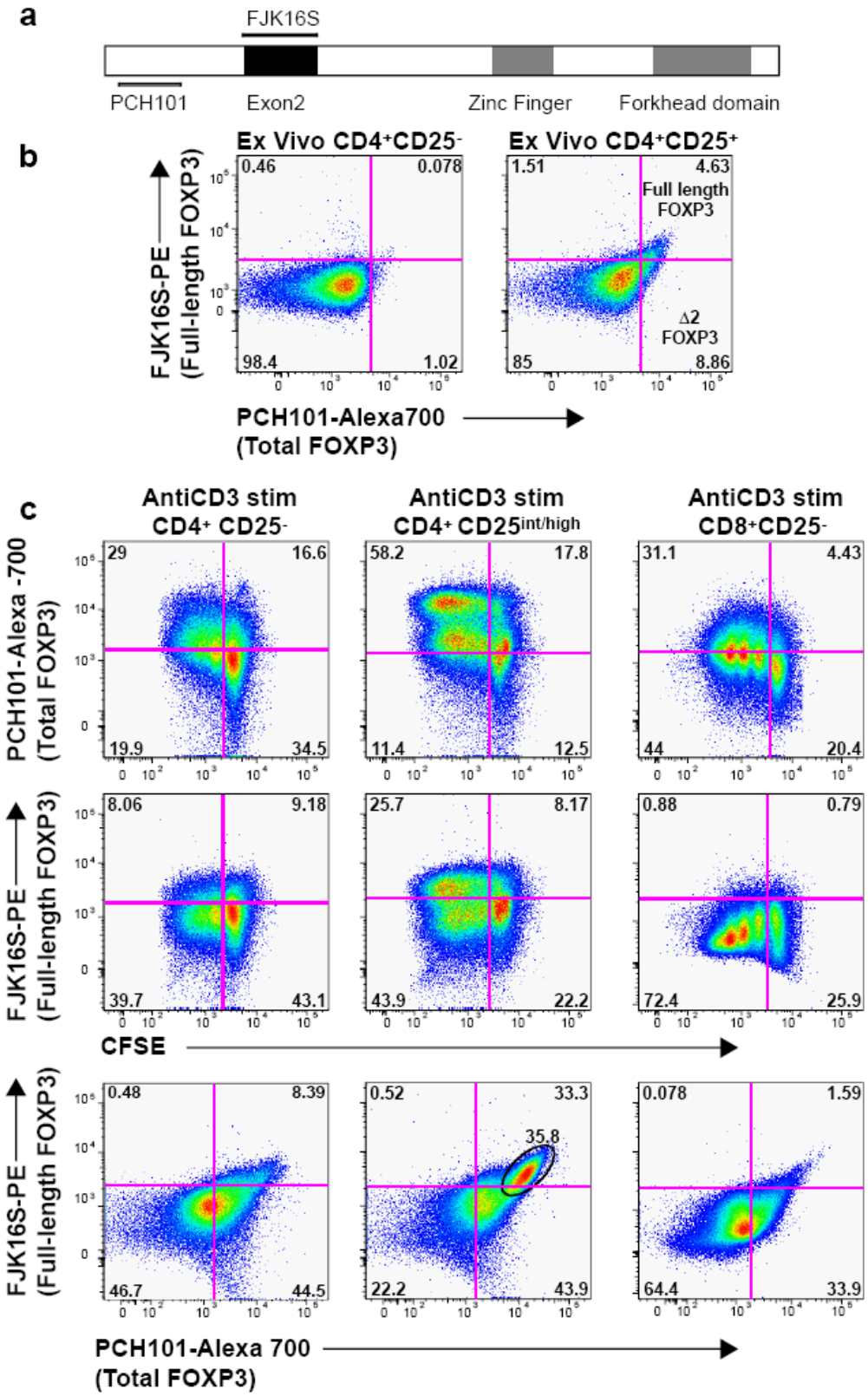

a
FJK16S
PCH101
Exon2
Zinc Finger
Forkhead domain
b
Ex Vivo CD4+CD25-
Ex Vivo CD4+CD25+
0.46
0.078
98.4
1.02
1.51
4.63
Full length FOXP3
Δ2 FOXP3
85
8.86
FJK16S-PE (Full-length FOXP3)
PCH101-Alexa700 (Total FOXP3)
c
AntiCD3 stim CD4+ CD25-
AntiCD3 stim CD4+ CD25int/high
AntiCD3 stim CD8+CD25-
PCH101-Alexa -700 (Total FOXP3)
29
16.6
19.9
34.5
58.2
17.8
11.4
12.5
31.1
4.43
44
20.4
8.06
9.18
39.7
43.1
25.7
8.17
43.9
22.2
0.88
0.79
72.4
25.9
FJK16S-PE (Full-length FOXP3)
CFSE
0.48
8.39
46.7
44.5
0.52
33.3
35.8
22.2
43.9
0.078
1.59
64.4
33.9
FJK16S-PE (Full-length FOXP3)
PCH101-Alexa 700 (Total FOXP3)

Figure 28. All sub-populations of T_{reg}s express both isoforms of FOXP3. Commercially available antibodies were used to quantify total (PCH101) and full-length FOXP3 (FJK16S) in various T_{reg} subpopulations. Spliced (Δ2) FOXP3 was detected by using a combination of both the antibodies ($PCH101^{+}/FJK16S^{-}$). **a,** Regions of FOXP3 molecule that are targeted by the two antibodies are diagrammed. **b,** Staining by the two antibodies in ex-vivo purified $CD4^{+}CD25^{-}$ T cells and $CD4^{+}CD25^{high/intermediate}$ T cells is shown. **c,** Bead-sorted $CD4^{+}CD25^{-}$ T cells, $CD4^{+}CD25^{high/intermediate}$ T cells or $CD8^{+}CD25^{-}$ T cells were CFSE-stained and activated with anti-CD3. Proportions of cells that expressed total and full length FOXP3 were quantified at day 4 of activation.

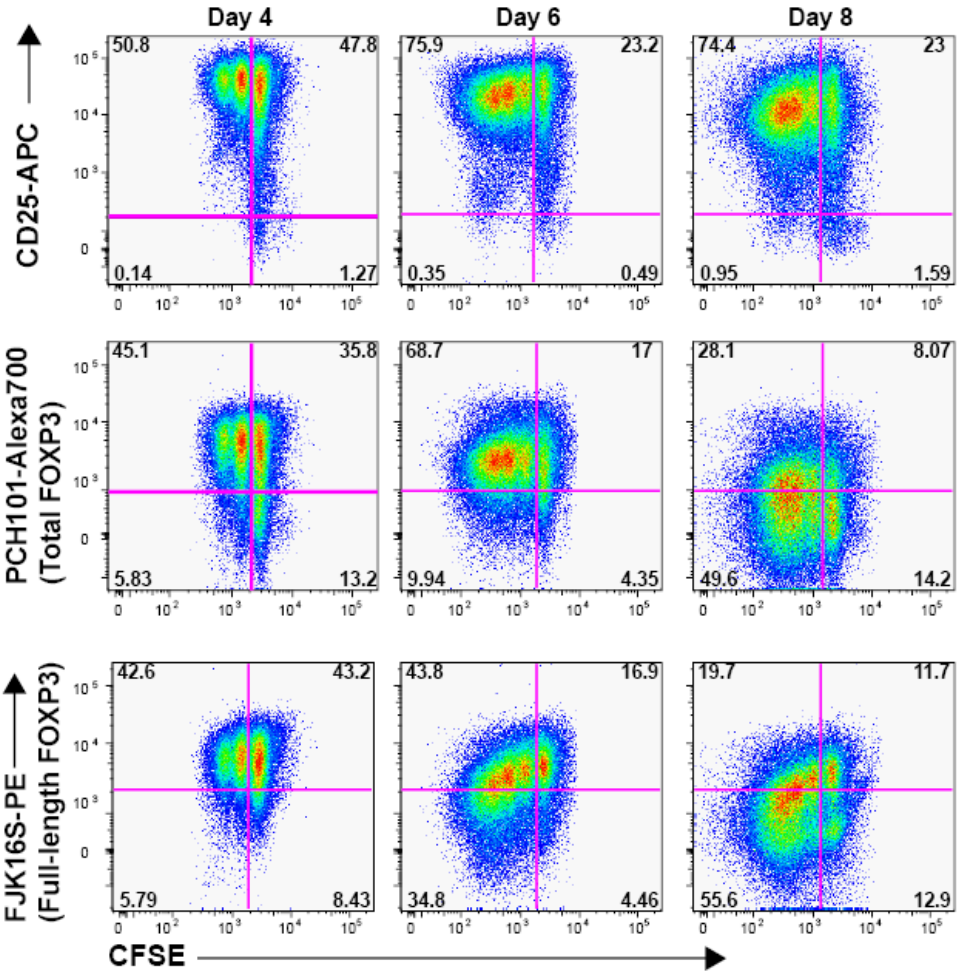


Figure 29. Full-length isoform of FOXP3 is also expressed transiently. Bead-sorted $CD4^{+}CD25^{intermediate/high}$ T cells were activated with anti-CD3 in the presence of autologous, irradiated APC. Expression of total, full-length FOXP3 and spliced Δ2 FOXP3 is shown at days 2, 4 and 6 post-stimulation in gated $CD4^{+}$ T cells.

Figures and text in this chapter were modified and reprinted from *Clinical Immunology,* In Press, **Pillai, V.**, S.B. Ortega, C.K. Wang and N.J. Karandikar. Transient regulatory T cells – A state attained by all activated T cells, Copyright (2007), with permission from Elsevier (173)

CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS

INTRODUCTION

In recent times, regulation of the immune system has emerged as a key concept in immunology with many cells in the immune system possessing the capacity to regulate immune responses. Subsets of cells shown to possess this capacity include the $CD4^+$ ($CD25^{+/high}$, Tr1 and Th3), $CD8^+CD28^-$, $CD4^-CD8^-$, γδ T cells and NKT cells (32). The mechanisms of suppression are not clear and seem to vary considerably between the various subsets and also between in-vivo and in-vitro conditions (28).

The best studied among the various populations with regulatory capability are the $CD4^+CD25^{+/high}$ T cells. In mice, FOXP3 has emerged as the definitive marker for regulatory T cells (149). Recent papers have also elucidated the molecular mechanisms underlying the suppression by FOXP3. FOXP3 acts by repressing NFAT and NFkB and consequently IL-2 secretion (174, 175). Continued presence of FOXP3 is essential throughout the lifespan of mice to prevent autoimmunity (22, 176). Recent studies altering the dose of FOXP3 in-vivo have indicated that FOXP3 is important for the anergic properties of T cells and absence of FOXP3 in a cell leads to the formation of Th2 type T cells (177, 178).

However in humans, the role of FOXP3 is complicated by the knowledge that $CD4^+CD25^-FOXP3^-$ T cells can be induced to express FOXP3 simply on activation (13). This does not happen in mice under similar activation conditions. However in mice, special conditions like presence of TGF-β, low dose antigenic stimulation can result in the de-novo induction of $FOXP3^+$ cells with regulatory activity (38, 41). This phenomenon raised the

important question of whether T_{reg}s are formed in the thymus and then persist/expand in the periphery or whether they can be induced de-novo in periphery during antigen specific stimulation. While in mice the former mechanism has been shown to be important (179), I believe that in humans the latter mechanism is more likely for reasons outlined in this review. It is crucial to know this difference since attempts are being made to induce/expand regulatory T cells in humans to be used for therapeutic purposes (180), sometimes with disastrous consequences (181, 182). Lack of understanding of this crucial phenomenon of peripheral induction of FOXP3 on activation has also led many to misinterpret presence of $FOXP3^+CD25^+$ T cells and/or suppression in the periphery/disease site to mean involvement of T_{reg}s in the disease process. This has led to the current situation wherein T_{reg}s are hypothesized to be involved in various human diseases from cancer, infections, autoimmunity to allergy and asthma (51, 52, 62, 183, 184). I believe that most of the evidence can simply be explained by the fact that all of these diseases involve immune activation during their pathogenesis and as shown by numerous studies now, $FOXP3^+$ T cells with regulatory activity can be formed during immune activation.

INDUCTION OF FOXP3 EXPRESSION BY ALL T CELLS ON ACTIVATION

Induction of FOXP3 on activation of $CD4^+CD25^-$ T cells was first noted in studies from the laboratory of Ziegler SF et al as early as in 2003 (146, 147). However, due to lack of antibodies at that time it was widely thought that the induced $FOXP3^+$ T cells are expanded from contaminating regulatory T cells in the starting population (148-151). Development of flow cytometric antibodies against FOXP3 has allowed us to exclude that

possibility and confirm earlier findings that FOXP3 can indeed be expressed on activated $CD4^+$ T cells (152). Most importantly, it is not just a subset of activated cells that express FOXP3 but every T cell, which gets activated goes through a phase of FOXP3 expression raising the possibility that expression of FOXP3 and regulatory activity might just be a step in the activation of all T cells. Transient acquisition of suppressive activity during activation might be a mechanism of limiting excessive immune activation and damage at the site of inflammation.

The majority of activated cells, which express FOXP3, however down regulate their FOXP3 expression eventually. However, a small percentage of cells continue to express FOXP3 even when all the other activated cells have down regulated FOXP3. Residual expression of FOXP3 was seen under all activation conditions even 15-20 days post stimulation. This residual T_{reg} population was responsible for some suppression even in 14-day-old cultures. Hence, it is possible that this might be a way of generating T_{reg}s in the periphery following antigen stimulation. This mechanism would also ensure that all the T_{reg}s produced have a very broad reactivity against self and foreign antigens and could be responsible for the similar reactivity found in natural T_{reg}s. Thymic generation of T_{reg}s alone cannot explain these findings.

Rapid down regulation of FOXP3 on activated T cells have led some to question the suppressive capability of these induced FOXP3 expressing cells without even performing any suppression assays on these populations (32, 152). However, transient induction of FOXP3 by activation in humans has now been shown to confer regulatory properties to those cells in multiple studies (13, 80, 146, 147, 185). I have shown that this transient FOXP3 expression

is also coincident with suppressive activity in both activated $CD4^+$ and $CD8^+$ T cells and more importantly down regulation of FOXP3 coincides with a decrease in suppressive activity. Transient expression of FOXP3 is similar to the transient expression of CD25 in activated T cells where they play a role in T cell activation by receiving IL-2 signals from the environment and other T cells. Transient expression of CD25 is also a way of generating the $CD25^+$ T cells found in the peripheral blood. It is conceivable that a similar mechanism might be operating in case of FOXP3 too. Hence it is more probable that transient FOXP3 expression has functionality instead of not having any function as suggested (32). A recent report showed that presence of FOXP3 during activated is associated with acquisition of a hyporesponsive phenotype in all the donors (186). However, activated T cells from only three out of nine donors acquired a suppressive phenotype in their hands. Moreover, only the donors whose T cells expressed FOXP3 stably acquired regulatory function. Hence, the actual dynamics and levels of FOXP3 expression might dictate when and where peak suppression occurs.

In the mouse thymus it has been noted that cells with the highest affinity go on to become $FOXP3^+$ T_{reg}s (187). Similar mechanisms might operate in the periphery during activation wherein responding cells with the highest affinity/avidity would go on to become regulatory T cells. Supporting this idea, a recent study using MHC class II tetramers and activation analysis to detect auto antigen specific T cells in the peripheral blood of type I diabetes mellitus patients found that the high avidity T cells preferentially acquired regulatory features compared to the low avidity T cells (172).

One potential caveat is that transient FOXP3 expression may be coincident with suppression rather than the cause of suppression. However, it remains to be seen if FOXP3 expression is the cause of the suppressive activity or if other mechanisms of suppression might be involved too as suggested (185). This important question will have to be conclusively addressed by SiRNA knockdown of transient FOXP3 expression to determine if the suppressive activity is completely due to FOXP3 expression.

Natural T_{reg}s have been shown to be anergic in proliferation and cytokine secretion in vitro, but the same may not be true in-vivo (141). An important observation is that suppressive activity of induced $FOXP3^+$ T cells is coincident with effector activity. I have shown that induced $FOXP3^+$ cells have the ability to secrete effector cytokines. The ability to secrete cytokines has been interpreted previously to mean that they do not have regulatory properties (152). However, I believe that regulatory activity and effector function can co-exist in the same cell at the same time and mere presence of some effector function does not automatically exclude any regulatory function. Such a cell possessing dual function of suppressor and helper activity, called the "Hermaphrocyte", has been described in the past (188).

PERIPHERAL GENERATION/PERSISTENCE OF T_{REG}S

While natural T_{reg}s might be more important during the short lifespan of naive mice, it is highly unlikely that a small subset of thymically generated regulatory T cells would persist throughout the lifespan of humans without any peripheral induction or expansion of naturally derived T_{reg}s. Key evidence for the peripheral generation of T_{reg}s comes from an elegant study from the laboratory of Akbar AN., where they used a novel deuterium labelling

method to determine how human T_{reg}s persist in the periphery (189). Human T_{reg}s were found to be a rapidly proliferating population compared to naive and memory T cells and was short lived and underwent apoptosis at a far higher rate than the naive and memory cells. Hence, they conclude that thymically generated T_{reg}s or expansion of natural T_{reg}s cannot explain lifelong persistence of human T_{reg}s and that most of the T_{reg}s must be constantly produced in the periphery from another source. When they compared the TCR repertoires of $CD4^+CD45RO^+CD25^{hi}$ and $CD4^+CD45RO^+CD25^-$ populations, they found striking similarity in the breadth of Vβ usage between the two populations. Furthermore, it was also determined that there was close clonal homology between the two populations suggesting that they are derived from the same precursor population. Vβ usage in CMV specific T cell populations was similar between memory T cells and $FOXP3^+$ regulatory T cell populations suggesting that they were formed from the same precursor population at the same time during an anti-CMV immune response in the body. I found generation of transient T_{reg}s from $CD4^+CD25^-FOXP3^-$ T cells when they were stimulated with antigens such as TT, CMV and during allostimulation. I have seen that formation of both transient and long lived FOXP3 expressing cells is even greater from $CD25^+$ T cells compared to $CD25^-$ T cells potentially explaining the conclusion that T_{reg}s seem to arise more from memory T cells.

Presence of $FOXP3^+$ T cells at inflammatory sites and chronic infections suggest the in-vivo relevance of these mechanisms (discussed later). Various immunomodulatory therapies such as glatiramer acetate therapy for multiple sclerosis and allergen injections for allergic rhinitis and venom anaphylaxis have also been known to induce various forms of regulatory T cells suggesting potential peripheral de-novo origin of these cells (74, 75, 190)

These data provide clear evidence for the hypothesis that most if not all of the regulatory T cells in the periphery are derived during antigen specific activation in the periphery rather than an expansion and persistence of the thymically derived population of natural T_{reg}s. It remains to be seen what factors will result in the persistence of these activated T cells/T_{reg}s in the body. I have shown that removal of induced T_{reg}s allows previously undivided T cells to proliferate further suggesting that they might have been suppressed in the first place during antigen stimulation. This was observed even during antigen specific stimulation of PBMC's.

The hygiene hypothesis attributes the increase in asthma and allergy in industrialized nations to increased hygiene and vaccinations resulting in decreased childhood infections (191). Infections would produce regulatory T cells every time they cause an immune activation and might result in accumulation of T_{reg}s against a multitude of antigens. These T_{reg}s might then non-specifically suppress allergen induced Th2 responses. Lack of infections in a relatively infection free environment would result in the lack of peripherally produced T_{reg}s. Lack of T_{reg}s could lead to allergy and asthma inducing Th2 responses in those individuals. In fact, many infections have been known to have a protective effect against allergy and asthma (192-194). Hence, evidence for the hygiene hypothesis provides further evidence for the in-vivo induction and importance of peripheral T_{reg}s.

TRANSIENT FORMATION OF $CD8^+FOXP3^+T_{REG}$S DURING ACTIVATION

Though FOXP3 expression is associated with $CD4^+$ T_{reg}s, it is very interesting to note that $CD8^+$ T cells have also been shown to express FOXP3 (48, 80, 152, 157, 163). $CD8^+FOXP3^+$ T cells with contact dependent suppressive activity were induced in type 1

diabetes patients treated with a modified anti-CD3 monoclonal antibody (80). The $CD8^+$ T_{reg}s were able to mediate contact dependent suppression of $CD4^+$ T cells in-vitro to both antigen specific and non specific stimulation. A longitudinal study revealed increased numbers of $CD8^+FOXP3^+$ T cells in treated patients compared to untreated patient controls after 12 days of anti-CD3 mab treatment. Some $CD8^+FOXP3^+$T cells were found to persist even 12 weeks after treatment. The presence of these cells is direct evidence for the induction and persistence of $FOXP3^+$ T cells on activation in-vivo. It remains to be seen if the $CD8^+FOXP3^+$ T cells possess any cytotoxic functions besides the direct suppressive function. $CD8^+CD28^-FOXP3^+$ T cells are known to be formed under conditions of allostimulation in human transplant patients (48). However, they seem to suppress by up-regulation of immunoglobulin like transcripts-ILT-3 and 4 on APC's (49, 195). However, an important question that remains is why don't $CD8^+$ T cells in the PBMC of healthy volunteers express much FOXP3 or even CD25 for that matter compared to $CD4^+$ T cells in the periphery? One would expect them to show similar levels of FOXP3 and CD25 expression since their kinetics of CD25 and FOXP3 expression after stimulation are similar. It is possible that most of the FOXP3 expressing T cells are short lived and undergo apoptosis or they may persist at the site of activation rather than the peripheral blood.

SIMILARITIES BETWEEN ACTIVATED T CELLS AND T_{REG}S.

T_{reg}s have been associated with multiple markers including CD25, CTLA-4, GITR, CD69, CD44, FOXP3 and more recently CD127. Despite intensive research over the last few years, there are still no surface or intracellular markers, which clearly distinguish T_{reg}s from activated T cells in humans. Even the most promising markers in recent times like

FOXP3 and CD127 have turned out to be expressed (or down regulated) by activated T cells (151, 162). Hence, we should start giving serious thought to accumulating evidence that T_{reg}s might be a form of activated T cell and there may never be a clear surface marker differentiating the two populations.

Furthermore, a recent study looking at micro RNA's expressed by regulatory T cells determined that both T_{reg}s and activated T cells have very similar micro RNA expression profiles suggesting that even their regulation at the mRNA level might be similar (196). Activation through TCR is closely linked to the suppressive activity. It is known that specific or non-specific TCR stimulation is necessary for suppression by T_{reg}s in in-vitro cultures (197). Activation through TCR also seems to be necessary for FOXP3 binding and repression by histone deacetylation of the IL-2 and IFN-γ locus in T cells (198) .

UPDATED ROLE OF T_{REG}S IN HUMAN DISEASES

In light of the new data regarding the origin of human T_{reg}s it might be prudent to re-evaluate existing data regarding the involvement of regulatory T cells in human diseases. T_{reg}s are hypothesized to be involved in the pathogenesis of autoimmune disorders. Many studies have now shown that T_{reg}s from multiple sclerosis patients have a reduced capacity to suppress compared to T_{reg}s from healthy individuals while the total T_{reg} numbers are comparable (199, 200). A similar combination of functional defects and normal T_{reg} numbers were also found in other autoimmune disorders like myasthenia gravis (201), psoriasis (202) and autoimmune polyglandular syndrome type II (203). There was a decrease in regulatory function in rheumatoid arthritis patients which was reversed by anti-TNF therapy (204). However, at the site of inflammation increased numbers of $FOXP3^+$ T cells were found,

which in light of my work can be attributed to immune activation at the site of inflammation (156, 205). The defect in the suppressive capability of T_{reg}s in patients with autoimmune disorders compared to healthy patients could also be the result of the differences in their origin rather than an intrinsic defect in their function.

Increased T_{reg}s have been found in patients with breast, ovarian, pancreatic, skin, liver and lung cancers (55, 206-210). Results from such studies have led most to conclude that presence of 'Regulatory T cells' in tumors means that an anti-tumor immune response is being inhibited by regulatory T cells and that depletion of regulatory T cells would strengthen and allow the immune response to eventually clear the tumor. However, presence of regulatory T cells in tumors would be expected because it is the natural outcome of the presence of an anti-tumor immune response and activated T cells. The tenuous evidence for T_{reg}s in cancer clearly shows why merely testing the function of T_{reg}s or suppressive activity is tumors is not conclusive of a role for T_{reg}s in the disease pathogenesis and therapeutic attempts to modulate T_{reg}s in those diseases should not be based on such evidence.

Evidence for T_{reg}s in chronic infections such as hepatitis B and C, HIV and CMV is based on data wherein increased frequencies of T_{reg}s are detected in the peripheral blood of such patients and depletion of T_{reg}s in-vitro increases antiviral immune responses (65, 66, 119, 211). Presence of increased numbers of T_{reg}s is attributable to increased T cell activation due to persistent viral antigens in chronic viral infections. It is also not surprising that since regulatory T cells are involved in suppression of immune responses, their depletion would result in increased immune responses to viral peptides in-vitro. Hence, it would be

premature to conclude that T_{reg}s are responsible for actively suppressing the anti-viral immune response and causing persistence of the viral infection.

Allergy and asthma are thought be due to increased Th2 responses and T_{reg}s are thought to suppress these responses in the periphery (183). In individuals with atopic dermatitis, there was a higher proportion of $CD4^+CD25^+$ T cells expressing FOXP3 (by PCR) in peripheral blood and skin, which is in agreement with an activated immune state. However in an another elegant study, it was found that suppressive function of $CD4^+CD25^+$ T cells from atopic donors was defective compared to healthy volunteers during the non allergy season and were even more defective in their suppressor function during the allergy season suggesting some involvement of regulatory T cells in the pathogenesis as in autoimmune disorders (212).

CAVEATS OF INDUCING/EXPANDING/DEPLETING T_{REG}S IN HUMANS.

Regulatory T cells are actively being targeted for therapeutic purposes. My work has deep implications for these efforts. Some of the common approaches being tried are in-vitro/in-vivo expansion of T_{reg}s for use in autoimmune diseases, allergy, asthma and atopy and depletion/blocking of T_{reg}s for cancer and infectious diseases. However, as I have argued above, the evidence for the involvement in these diseases is weak at best. Hence, I suggest great caution in developing and testing these therapies in humans. As I argue further, manipulation of T_{reg}s in humans might be dangerous besides not resulting in the desired or expected effects.

There are reports in the literature of protocols to expand/induce T_{reg}s using antiCD3, anti-CD28, IL-2 or modified DC (76-80). Again, all of these are T cell activating protocols, which can lead to a transient formation of $FOXP3^+$ T cells. Most of these $FOXP3^+$ T cells will down regulate FOXP3 expression and lose regulatory activity. Even if we somehow manage to achieve long lasting expression of FOXP3 in expanded T cells, most of them would undergo apoptosis in-vivo leading to the loss of regulatory effects. More importantly, these induced $FOXP3^+$ T cells can also have effector function making them potentially dangerous when induced therapeutically in humans. Such an unfortunate sequence of events is exemplified by the recent anti-CD28 super agonist (TGN 1412) trial where attempts to expand T_{reg}s in humans ended in disaster (166, 182). Even though their in-vitro experiments showed increased T_{reg} induction based on a FOXP3 readout (213), most of their induced T_{reg}s would have down regulated their regulatory activity and FOXP3 post stimulation and would have had effector function at the same time. It was clear from the case report on the trial that the massive cytokine storm from unrestrained T cell activation was responsible for all their symptoms (214). Even though actual data on the effects of anti-CD28 super agonist on human PBMC's have not been released by the investigators, my hypothesis is strongly supported by my data using anti-CD28 and other data in humanized mice and rats using the same super agonist (170, 215).

The anti-CD28 super agonist was claimed to be different from other antibodies like the OKT3 anti-CD3 antibody because it bypasses TCR stimulation to trigger T cell activation and proliferation through the CD28 receptor (216). However, it is not essential to activate T cells through their TCR to induce transient FOXP3 expression and suppressive activity. I

have found that even activation of T cells using stimuli that bypass the classical TCR pathway like PMA/ionomycin, concavalin A and SEB can result in total expression of FOXP3 suggesting that triggering of the T cell activation program is sufficient for transient FOXP3 expression and acquisition of regulatory capacity (unpublished data from our lab). In light of these findings, I suggest a moratorium on human trials modulating T_{reg}s until we have a better understanding of regulatory T cells in humans and the controversies in this field have been resolved.

In cancer and infectious diseases, it has been suggested that regulatory T cell depleting therapies might help in removing the suppression to allow the natural antiviral response to come up. However, this approach is not likely to work since newer $FOXP3^{+}T_{reg}$s will be produced during antigenic stimulation every time. This situation is similar to the situation where removal of CD25 expressing cells is not likely to work long-term because $CD25^{+}$ T cells can eventually arise. Blocking the function of regulatory T cells might have a better chance of success in this situation.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Present day T_{reg}s are the re-incarnation of suppressor T cells of the 70's and 80's**.** Suppressor T cells fell into disfavor because their existence could not be confirmed by technology prevailing at that time. "Suppressor T cells" became the dirty "S word" in immunology and became synonymous with over enthusiastic interpretation and extrapolation of weak data (8). The idea of specialized T cell subset for suppression was slowly abandoned in the 80's after the discovery of Th1/Th2 T cell subsets and suppression was thought to be a natural outcome of regulation during the formation of other T cell subsets

(123). However the concept of a specialized T cell subset for suppression was revived and revitalized in the late in the last ten years (123). We seem to have come full circle with the concept of suppression. It is interesting to note that current day knowledge about T_{reg}s also suffers from same old flaws in interpretation. Current evidence in the field of human T_{reg}s are more suggestive of model where peripheral generation of T_{reg}s is likely to be far more important than a thymic T_{reg} generation model and multiple subsets seem to be capable of suppression. However, a few crucial questions remain to be answered to conclusively prove that hypothesis. It remains to be conclusively proven that FOXP3 expression occurs in-vivo in humans after activation under natural conditions and if it persists for a significant length of time. Presence of FOXP3 expressing T cells at inflammatory sites or in chronic activation states such as chronic infections and cancer are an indirect evidence of formation and persistence of these cells in humans. Presence of increased numbers of $FOXP3^+$ T_{reg}s during acute viral infections would also be an indirect evidence of their formation during antigen specific activation. One potential experiment which could be performed to directly show their induction under defined conditions would be to look for presence of influenza $tetramer^+$ $FOXP3^+$ antigen specific T_{reg}s following influenza vaccination. However, it is possible that an appreciable increase in T_{reg}s may not be obvious in peripheral blood and most of induced T_{reg}s might be sequestered at the site of activation.

If the hypothesis that T_{reg}s are formed during every antigen specific activation is true then the obvious question that would arise is how any effector activity or proliferation ever takes place. The answer to that is total FOXP3 expression and peak suppression is very transient and may be limited to suppressing the high affinity/high avidity type T cells, which

would be more prone to causing damage at the site of activation. Hence, proliferation and effector activity may not be impeded to a great extent.

Another purpose of FOXP3 expression in activated T cell might be to limit its own proliferation (217). All activated T cells get activated through activation of the NFAT transcription factor and IL-2 secretion. FOXP3 suppresses T cell activation by binding to sites on the NFAT reporter and repressing it. FOXP3's actions on the NFAT provides an elegant molecular mechanism for its function in activated T cells.

Another important question that arises is how does autoimmunity happen if T_{reg}s produced during activation of auto reactive T cells can limit the damage? Evidence from multiple sclerosis and rheumatoid arthritis in humans indicate that even though T_{reg} numbers in the periphery in these patients are similar to that found in healthy individuals, the T_{reg}s are functionally deficient. Repeated infections from childhood would produce a population of normal T_{reg}s in healthy individuals, which would protect them from autoimmune disease. People prone to autoimmunity would be deficient in the function of induced T_{reg}s. The induced T_{reg}s in these patients could be functionally deficient because they are intrinsically different from the T_{reg}s in healthy individuals. The answer could also lie in a defect in the dendritic cell populations that produce T_{reg}s.

If T_{reg}s are being produced every time T cells get stimulated then why does the total number of T_{reg}s remain the same in older individuals (189)? This could be explained by the fact that T_{reg}s undergo proliferation and apoptosis at a far higher rate than naive or memory T cells (189). Hence, any T_{reg} that would be produced may not persist for the lifetime of the

individual. T_{reg}s could also be mostly sequestered in the lymph nodes or at the site of activation.

Even though a clear distinction is commonly made in the literature between natural thymically generated T_{reg}s and induced T_{reg}s, so far there is no clear marker that can distinguish them in-vivo. In fact, it is not even clear if they are functionally distinct subsets. In mice, it is conceivable that natural T_{reg}s might play an important role in controlling immune responses because mice have short life spans and are housed in an infection free controlled environment. However, the same cannot be extrapolated to humans with their long life spans and early thymic involution. Humans are exposed to a multitude of self and foreign antigens every day, each of which could potentially lead to the formation of T_{reg}s every time.

There is no doubt that FOXP3 is currently the best marker for T_{reg}s in mice and in humans. However, a little understood and appreciated fact is that transient expression of FOXP3 occurs on activation and does indeed confer regulatory properties to activated cells. Furthermore, human T_{reg}s are activated T cells stuck in that phase. However, the implications of these findings have been inadequately understood due to apparent biases and confusion arising from mouse T_{reg} literature. It is now becoming clear that FOXP3 expression and regulation differs greatly between mice and humans. This is a major difference to be considered when the presence of FOXP3 expression in peripheral tissues is widely being interpreted to mean involvement of regulatory T cells in a human disease. More importantly, potentially dangerous attempts are being made to expand/induce T_{reg}s by basically activating T cells with super agonists or by other means in humans for therapeutic

purposes. Stability of these induced/expanded T_{reg}s and their potential interconversion to effector T cells needs to be taken into account while performing these experiments to prevent further disasters.

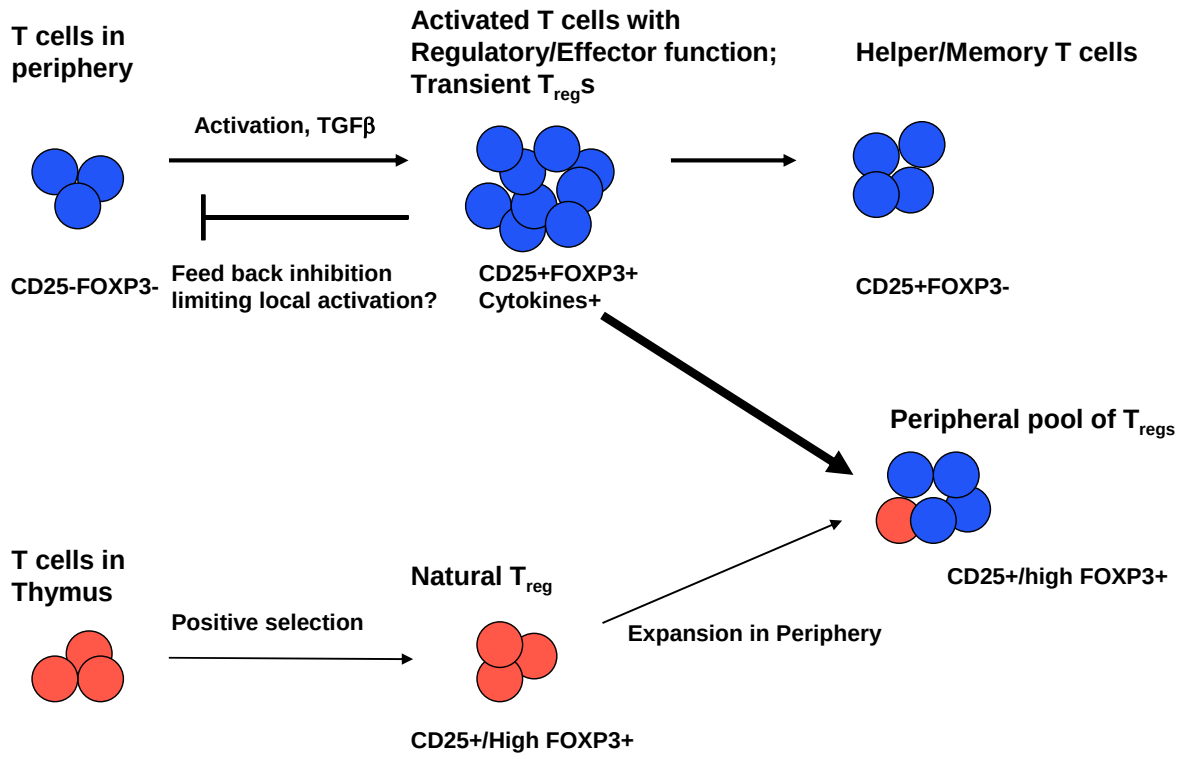


Figure 30. Peripheral generation of FOXP3$^+$ Regulatory T cells in humans.
My model for T_{reg} generation suggests that every time there is an activation of T cells in the periphery, all responding T cells would upregulate FOXP3 and CD25 transiently. There would also be an increase in suppressive activity, which would limit local activation. At this stage of activation, they are capable of both regulatory and effector functions. However, only a subset of the activated T cells would retain FOXP3 post stimulation. These cells would go on to add to the peripheral pool of FOXP3$^+$ T_{reg}s and persist for a longer period. The majority of the activated cells would down regulate FOXP3$^-$ and go on to become helper and memory T cells. Positive selection of T cells with intermediate affinity for self antigens in the thymus in early life would result in the formation of a population of natural FOXP3$^+$ T_{reg}s. Most of the natural T_{reg}s would not be able to persist for long in the periphery due to a high rate of proliferation and apoptosis and hence would have minimal contribution to the peripheral pool of FOXP3$^+$ T_{reg}s.

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VITAE

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