CD4+ Foxp3+ Regulatory T cell Homing & Homeostasis

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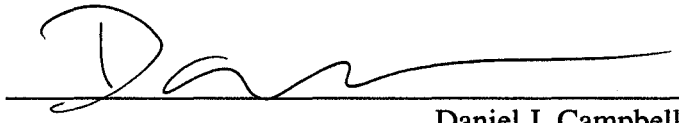

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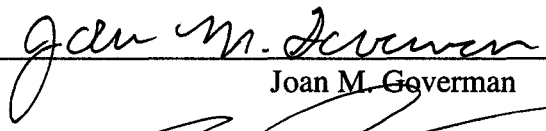

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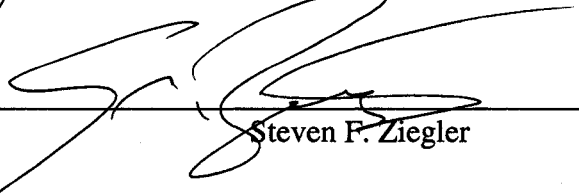

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Abstract

CD4+ Foxp3+ Regulatory T cell Homing & Homeostasis

Blythe Duke Sather

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$CD4^+Foxp3^+$ T cells (T_R) are essential for maintaining self-tolerance, but their sites of action *in vivo* and homeostatic mechanisms are poorly defined. I examined homing receptor (HR) expression by T_R in the steady state and determined whether altering T_R distribution by removal of CCR4 impairs their ability to maintain tissue-specific tolerance. Additionally, I examined signals that alter T_R HR expression and what impact this has on T_R homeostasis.

I found T_R in all non-lymphoid tissues tested, particularly in skin, where they express a unique $CCR4^+CD103^{hi}$ phenotype. T_R expression of CCR4 and CD103 is induced by antigen-driven activation within sub-cutaneous lymph nodes, and accumulation of T_R in skin and lung airways is impaired in the absence of CCR4 expression. Mice without CCR4 expression in T_R develop inflammatory disease in skin and lungs, accompanied by lymphadenopathy and an increase in skin-tropic $CD4^+Foxp3^-$ T cells. Additionally, CCR4-ligand interactions mediate efficient homeostasis of T_R. CCR4-deficient T_R undergo faster homeostatic expansion than WT T_R, but cannot sustain themselves during homeostatic maintenance. Together, these data highlight the importance of CCR4 expression by T_R in meditating their localization. This is crucial for their activity within non-lymphoid tissues to protect against tissue-specific disease, as well as within lymphoid tissues to receive homeostatic signals.

I also explored the contribution of signals mediated by Wiskott-Aldrich syndrome protein (WASp) to T_R homeostasis. WASp is essential for optimal T cell activation and patients with WAS exhibited both immunodeficiency and autoimmunity. We investigated whether impaired T_R function explained these paradoxical observations. WASp-deficient ($WASp^{-/-}$) mice exhibited normal thymic T_R generation, but the competitive fitness of peripheral T_R was compromised. The percentage of $Foxp3^+$ T_R was reduced, and $WASp^{-/-}$ T_R were outcompeted by $WASp^+$ T_R *in vivo*. These findings correlated with reduced expression of HR associated with self-antigen–driven T_R activation and homing to inflamed tissue. Furthermore, $WASp^{-/-}$ T_R were unable to control lymphocyte activation and autoimmune pathology in *Foxp3*$^{-/-}$ *sf* mice. Finally, $WASp^+$ T_R exhibited a selective advantage in a WAS patient with a revertant mutation, indicating that altered T_R fitness likely explains the autoimmune features in human WAS.

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Dedication

For my husband Chris, who has been my inspiration, my rock and my biggest supporter throughout this entire endeavor. He taught me the importance of breathing deeply, smiling genuinely and embracing life in all its glory. Without him I might have lost myself in the madness of graduate school and his love and compassion was my center and light when all seemed lost and dark.

Chapter 1: Introduction and Background

The Critical Role of Self-Tolerance

One of the most extensively studied aspects of immunology is the development of self-tolerance, or how the immune system is able to discriminate between self-proteins and non-self, pathogenic proteins. This stage in immune system development is critical in maintaining a healthy balance between eliminating pathogens and eliciting immune-mediated tissue damage due to an overly robust immune response. As early as 1905, investigators showed that individuals could not easily be immunized against their own tissues suggesting there was some recognition of "self" by the immune system(1). Studies of dizygotic twins by Owen et. al. in 1945(2) showed that this tolerance to self-tissues is established during the early development of the immune system. In 1953, Billingham, Brent and Medawar (3) conducted the first experiments to directly address the cellular mechanism of immunological tolerance. They injected allogenic tissues into fetal mice *in utero* and showed that the resulting mice reached maturity with a lesser ability to reject skin grafts from the same allogenic mouse, when compared to a third party graft from a different allogenic strain. The mechanism proposed to explain this "acquired" tolerance process was selective clonal deletion of lymphocytes specific for the injected alloantigens(4). However, additional experiments attempting to reconstitute these tolerant animals with normal lymph node populations were much less effective, suggesting that there must be other, more complicated mechanisms involved in self-tolerance(5). Since

these studies, many groups have shown that self-tolerance is established through a complex combination of selection and deletion of lymphocytes (based on their self-antigen specificity and affinity) during their maturation process(6-8), as well as intricate control of the lymphocytes that survive this process and are circulating in the periphery(9-11). Without this tolerance to self-proteins, the developing immune system would be unable to distinguish between foreign pathogens and its own tissues, resulting in widespread immune-mediated damage and death. Indeed, it is widely accepted that autoimmune diseases result from the dysregulation of the basic processes designed to maintain self-tolerance. One major goal of immunologists in the field today is to discover and understand the key components that control self-tolerance, to one day thwart ongoing disease in patients.

The immune system has evolved several mechanisms to establish and sustain unresponsiveness of lymphocytes to self-antigens including physical elimination(6) and functional inactivation(12) of self-reactive lymphocytes (clonal deletion and anergy respectively). The deletion of self-reactive T and B cells exposed to their antigens at immature stages of their development in the thymus is the initial and primary mechanism of tolerance, described as central tolerance, but ample evidence has shown that this mechanism is not complete (13). Many lymphocytes carrying self-reactive receptors survive this process and can be found circulating in the periphery of normal individuals. As a result, several other mechanisms have evolved, collectively called peripheral tolerance, to maintain control of these potentially self-reactive cells. Their activation is prevented by

rendering them unresponsive, inducing their death due to the weak or incomplete signals and/or sequestration from antigen. One critical mechanism utilized to render T cells unresponsive is active suppression by $CD4^+$ regulatory T cells (T_R)(8,14). The existence and contribution of T_R to peripheral tolerance has been very controversial. Initially, researchers were unable to precisely identify them due to a lack of reliable markers and the ambiguity of their functions at a molecular level. However, in recent years there has been a windfall of data identifying several subsets of T_R (15)and firmly establishing their role in the maintenance of peripheral tolerance.

Suppressor T cells Evolve into Regulatory T cells (T_R)

Over three decades ago, two major observations became the foundation of the current dogma that T-cell mediated control of self-reactive T cells is a key mechanism of self-tolerance. In 1969, it was shown by Nishizuka and Sakakura that neonatal (2-5 days of age) thymectomy of normal mice lead to autoimmune-mediated destruction of the ovaries(16). A few years later in 1973, Penhale and colleagues(17) showed that thymectomy in conjunction with sub-lethal irradiation of adult rats resulted in autoimmune thyroiditis. Additional data showing that these disease processes could be prevented by the transfer of normal T cells led many to hypothesize that a "suppressor" population of lymphocytes was important for the protection against autoimmune pathology(18,19). These early studies attempted to identify the mechanism of suppression, but were complicated by the unsuccessful search for multiple suppressor factors, anti-idiotypic T cell networks and

"suppressor-inducer" or "contra-suppressor" cells(19). The inability to identify any of these as a mechanisms as driving the activity of suppressor T cells led this field to be largely discredited(20). But several key experiments in the 1980's prompted a resurgence in the study of suppressor T cells. In 1985, Sakaguchi et.al. (21) published a critical experiment showing that when $CD4^+$ splenic cells from normal BALB/c mice were depleted of $CD5^{high}CD4^+$ T cells and were transferred into congenitally T-cell deficient BALB/c nude mice, the recipient mice developed multi-organ autoimmune disease a few months after transfer. A few years later, Powrie et.al. (22) showed that athymic nude rates reconstituted with $CD45RC^{low}CD4^+$-depleted T cells developed a graft-versus-host disease, as well as autoimmune tissue damage in multiple organs, mediated by $CD45RC^{high}CD4^+$ cells. Additionally, they (and others) showed that transfer of CD4+ T cells enriched for $CD45RB^{high}$ into T/B-deficient BALB/c SCID mice induced severe inflammatory bowel disease (IBD) and this disease could be prevented with the co-transfer of purified total CD4+ T cells (23,24). Though all of these studies indicated a sub-population of T cells was necessary to protect mice from autoimmune-mediated damage, the prevalence of the markers CD5 or CD45RB on T cell populations (75% and 25% respectively) led researchers to search for a more specific marker for T cells that could mediate suppression. In 1995, a crucial study by Sakaguchi et.al. (25) showed that among $CD5^{high}$ and $CD45RB^{low}$ T cells, was a subset (about 5-10% of CD4+ T cells) continuously expressing the IL-2 receptor α-chain or CD25. When BALB/c splenic suspensions were depleted of $CD25^+CD4^+$ T cells

and transferred into athymic nude mice, the recipient mice developed autoimmune-mediated damage at even higher incidences, involving an even wider range of tissues than seen in any of the previous experiments (Fig. 1). They went on to show that the co-transfer of a small number of $CD25^+CD4^+$ cells with their CD25-depleted T cells completely abrogated the autoimmune damage in all tissues. Thus, these experiments were to first to show that $CD25^+CD4^+$ T cells, or regulatory T cells (T_R), were critical for maintaining peripheral tolerance to self and the loss of these cells resulted in multi-organ autoimmune disease.

T_R activity: In vitro vs. in vivo

Once it was recognized that $CD4^+CD25^+$ T_R were the cell subset mediating suppression *in vivo*, an *in vitro* experimental system was established that many researchers have used to tease apart the mechanisms of T_R-mediated suppression (26-28). In the typical set-up, $CD4^+CD25^-$ effector T cells were cultured with irradiated T cell-depleted antigen presenting cells (APCs) and stimulated alone or with the addition of $CD4^+CD25^+$ T_R. After several days, [H^3]thymidine is added to each culture, which can be incorporated into proliferating cells and measured by a β scintillation counter. With these assays, researchers were able to establish several key features of T_R development and activity.

One of the earliest *in vitro* observations of T_R was that they were anergic, meaning they do not proliferate in response to stimulus through their TCR(26,27). However, T_R can proliferate if they are stimulated with high amounts of exogenous IL-2 and given a strong CD28 signal. The suppressive ability of T_R is closely linked

to this anergic state. The abrogation of their anergic state by TCR stimulation in the presence of high levels of IL-2 results in the ability of T_R to proliferate and a simultaneous loss of suppressive activity. Interestingly, the primarily anergic state of T_R seen *in vitro* does not hold true *in vivo*. Polyclonal $CD4^+CD25^+$ T cells transferred into lymphopenic $Rag^{-/-}$ mice undergo homeostatic expansion at a similar rate as $CD4^+CD25^-$ cells as measured by CFSE dilution(29,30). Additionally, naïve antigen-specific T_R proliferated as extensively as naive $CD4^+$ T cells after immunization without losing their suppressive function *in vivo* and *in vitro*(31,32). Finally, we (BDS unpublished data) and others (33,34) observed that T_R actually proliferate at a higher rate during ongoing homeostasis compared to T_{eff}, as measured by BrdU incorporation by $CD4^+CD25^+$ and $CD4^+CD25^-$ cells in wild type (WT). Together, these data suggested that T_R have a very specific activation and homeostatic program whose default position seems to be anergy and can be triggered to proliferate with the specific signals given by the *in vivo* environment.

The second major observation was that stimulated T_R are able to suppress T_{eff} proliferation, both CD4 or CD8 T cells (26,27). *In vitro* studies demonstrated that suppressive activity required antigen-specific activation of T_R through the TCR, but once activated, T_R were capable of suppressing T cells with other specificities (35). Analysis of T-cell receptors from T_R demonstrated that a significant proportion of these T cells recognize constitutively presented peripheral self-antigens (36). Consistent with these data, it was hypothesized that T_R

chronically stimulated by ubiquitously expressed peripheral autoantigens develop a stimulation threshold that must be overcome to initiate an antigen-specific immune response. However, there is increasing evidence that suggests that T_R function and organ-specific tolerance is critically dependent on the antigen specificity of T_R(37). Recent studies utilizing non-lymphopenic mouse models of autoimmune diabetes showed that organ-specific T_R were superior in disease protection compared to polyclonal T_R(38-40). These data suggest that antigen specificity is important for T_R function and if they were to be useful therapeutically, more research would be needed to clearly understand any functional differences between organ-specific populations of T_R.

The third observation from these *in vitro* suppression studies was that the balance between the number of T_R and T_{eff} is critical to their activity. The most complete *in vitro* suppression of T_{eff} is usually observed when the ratio of T_R to T_{eff} was 1:1(26-28). Less suppression was observed as the ratio of T_R to T_{eff} was reduced to 1:4, 1:8 and below, where very little suppression was observed. Since the typical ratio of T_R to T_{eff} *in vivo* is from 1:5 to 1:10, depending on the lymphoid organ examined, this implies that the microlocalization of T_R to T_{eff} is critical to their function. Indeed, this ratio is under tight control *in vivo* since T_R transferred into lymphopenic mice only expand to fill about 10% of the $CD4^+$ compartment. Together, these data imply that there is a "T_R niche" that is occupied by a specific subset of CD4+ T cells and is held under tight control *in vivo*.

Control of T_R Homeostasis

Clearly there is a precise homeostatic program to control the numbers of T_R *in vivo* and there is substantial evidence that a number of signaling pathways are involved in this process. There are several molecules whose deficiency or functional alteration affects the generation or homeostasis of natural T_R, resulting in autoimmune disease. Of particular importance are cytokines such as IL-2 (33,41-44)and TGFβ(45,46), as well as co-stimulatory molecules such as CD28 and CTLA-4 (cytotoxic T-lymphocyte antigen 4) (47,48). The mechanism by which each of these mediate T_R homeostasis is not completely understood, but recent work has shown that they may all be important contributors to this process.

Recent work has clearly shown that IL-2-IL-2R-dependant events contribute to T_R homeostatic maintenance(33,41-44). IL-2, IL-2Rα and IL-2β-deficient mice exhibit a lethal lymphoproliferative disorder accompanied by severe autoimmunity(49-51). Though it was initially thought that this was due to a lack of T_R production, recent work has shown IL-2 signaling to be dispensable for the thymic development of T_R. On the contrary, IL-2 signaling has been shown to be most important in maintaining T_R numbers in the periphery. Without IL-2 produced by non-T_R $CD4^+$ T cells, T_R numbers in the periphery are very low, though these cells still maintain the ability to proliferate, as well as suppress CD4+ effector T cells (T_{eff}). Therefore, it is important to understand what signals and transcriptional programs are involved in this homeostatic mechanism to fully understand how T_R are maintained in the periphery throughout life.

In addition to IL-2, the role of TGFβ1 in the production and activity of T_R has been hotly debated. TGFβ1 has pronounced immunosuppressive effects (52) and its deficiency results in a lethal autoimmunity in mice(53,54), a disease phenotypically similar to what is observed for IL-2, IL-2Rα and IL-2Rβ-deficient mice. *In vitro* suppression assays (55), as well as an IBD model (56), showed that α–TFGβ antibody treatment resulted in abrogation of T_R mediated suppression, although contradictory data (57) has made this observation quite controversial. One report hypothesized that TGFβ1 bound to T_R TGFβ1 receptors may be a mechanism by which they exert their suppression (58). However, studies of young TGFβ1-deficient mice showed T_R-bound TGFβ does not act directly as a suppressor cytokine, nor is it necessary for the development and selection of T_R(45). Instead, they showed TGFβ1 is critical for T_R peripheral homeostasis and the TGFβ1 in the previous studies was coming from the APCs and not the T_R themselves. Therefore, TGFβ1 producing APCs play an active role in maintaining T_R numbers in the periphery and it will be important to understand how T_R are interacting with these particular subsets of APCs and what signals mediate their production of TGFβ1.

Aside from cytokine-mediated signaling, signals through several other molecules expressed by T_R are critical for their homeostasis. T_R express high levels of CTLA-4, a negative regulator of T cell activation (59-61). Treatment of mice with antibodies to CTLA-4 abolishes the protective capacity of T_R by reducing their numbers and CTLA-4-deficient mice develop lymphoproliferative disease due

to a low number of T_R(62). Additionally, CTLA-4-deficient T_{eff} were efficiently suppressed by T_R expressing normal levels of CTLA-4, demonstrating that the requirement for CTLA-4 was T_R specific, though this remains controversial since CTLA-4 knock-out T_R have been shown to be functional *in vitro* (48). Finally, the interaction of CTLA-4 on T_R with CD80/CD86 on APC triggers the induction of the enzyme indolamine 2,3-dioxgenase(63-65). This enzyme catalyzes the conversion of tryptophan to kynurenine and other metabolites, which have potent immunosuppressive effects on the local environment of the APC. Together, these data suggest that the CTLA-4 signal on T_R may induce APC to have more suppressive qualities and reduce their capacity to drive T_{eff} proliferation.

In addition to CTLA-4, its counterpart CD28 was shown to be necessary for the thymic generation of T_R and importantly, for their self-renewal and survival in the periphery (47,59). The number of T_R is substantially reduced in the thymus and periphery of CD28, B7-1 or B7-2 (CD80 or CD86)-deficient mice and the abrogation of signals mediated by these molecules with blocking antibodies reduced T_R numbers in a similar manner. It is possible that a CD28/B7 signaling blockade may hamper the activation of conventional T cells and consequently their IL-2 production, leading to a reduction in the homeostasis of T_R due to IL-2 deficiency. Conversely, the strength of the CD28 signal may be a primary mediator of T_R expansion and homeostasis. There are profound differences in T_R mediated suppression depending on the strength of co-stimulatory signal sent by the APC it interacts with (27). Highly activated $CD86^{high}$ APC induce the proliferation of T_R

and down-modulate their suppressive capabilities, while increasing their proliferation(66). Conversely, immature APC, expressing low levels of CD80 and CD86, seem to be more efficient in initiating T_R suppressive activity. Since CTLA-4 has a higher avidity for CD80/CD86 when compared to CD28 (67,68), APC expressing lower levels of these co-stimulatory molecules may induce a suppressive signal sent by CTLA-4 and activate T_R-mediated suppression. On the contrary, higher expression of CD80/86 on activated APC may send a more efficient CD28 signal and in turn reduce the T_R-suppressive capacity and increase proliferation. Understanding the balance between these co-stimulatory signals is critical for understanding how T_R are homeostatically maintained.

Foxp3: A Crucial Regulator of T_R Development and Function

The discovery that $CD25^+CD4^+$ T_R are a critical T cell subset responsible for maintaining peripheral tolerance to self-antigens led to an explosion in the study of T cell-mediated suppression. Although the CD25 expression on T_R was useful in their isolation, the fact that CD25 expression was well documented on recently activated effector T cells lead researchers to search for a more specific marker expressed exclusively by T_R. The expression of other markers were proposed as identifiers of T_R, such as GITR (glucocorticoid-induced tumour-necrosis-factor-receptor-related protein) and CTLA-4, but again, these markers are also expressed on some activated non-T_R effector cells. Some groups proposed that T_R were a just a subset of effector T cells that arise during an immune response to control the reaction and that T_R are not a independently differentiated lineage (69). This was

based on studies showing that $CD4^+CD25^-$ naïve antigen-specific T cells repeatedly stimulated with antigen could become a T cells subset distinct from Th1 or Th2, that made suppressive cytokines such as IL-10 and had immunosuppressive activity *in vitro* (70). On the contrary, others felt that there must be a specific factor that, when expressed in $CD4^+$ T cells, drove them into the dedicated lineage of T_R. Thus, it was hypothesized that both the adaptive and naturally selected T_R (Fig. 2) were likely to exist, but a specific marker remained elusive.

The breakthrough came when several groups discovered that a mutation in the gene encoding a forkhead-winged-helix family transcription factor called Foxp3, was the cause of the fatal human autoimmune disorder "Immune Dysregulation Polyendocrinopathy, Enteropathy, X-linked" (IPEX) and the analogous disease in a spontaneous mouse mutant, scurfy (*sf*)(71-74). IPEX was first described in 1982 as an X-linked immunodeficiency syndrome seen in young male patients characterized by neonatal diabetes mellitus, enteropathy and endocrinopathy, IBD, atopic dermatitis, food allergies and fatal infections(75). This disease can be caused several types of mutations in the X chromosome gene FOXP3, both mis-sense and frame-shift mutations, some of which result in a reduction or loss of FOXP3 protein production. These patients can be treated with immunosuppressive therapies or, in more severe cases, hematopoietic bone marrow transplant. In the later case, even when there is a low engraftment of donor bone marrow or other complications, patients have clinical improvements, indicating that expression of FOXP3, particularly in T cells, mediates a selective advantage(76).

In mice, a 2-bp frameshift insertion in the X chromosome gene Foxp3 results in a truncated gene product lacking the C-terminal forkhead domain. Male *sf* mice hemizygous for the Foxp3^{sf} mutation completely lack Foxp3 protein expression and succumb to a CD4$^+$ T cell-mediated multi-organ lymphoproliferative disease characterized by pronounced lymphadenopathy and splenomegaly, lymphocytic infiltration of the skin, intestines, liver and other non-lymphoid tissues, dermatitis and runting, resulting in death by 3-4 weeks of age(77). The *sf* disease is driven by the polyclonal activation of CD4$^+$ T cells that appear in the periphery of *sf* mice as early as 3 days of age. These cells mediate disease by initiating the production of a broad spectrum of pro-inflammatory cytokines, as well as autoantibodies(78,79). Several groups have shown that this phenotype can be replicated by actively removing Foxp3$^+$ cells, either by completely blocking the expression of Foxp3 during development (targeted KO mice(80,81)) or by removing Foxp3$^+$ T cells in adult mice (Foxp3-DT(82)). Additionally, constitutive over-expression of Foxp3 in T cells results in a reduced number of peripheral T cells and the remaining T cells show impaired responses to TCR ligation and increased apoptosis(83,84). Together these data suggested that Foxp3$^+$ T cells are important mediators in regulating systemic lymphocyte activation.

Foxp3 belongs to a family of transcription factors identified by their C-terminal winged helix-forkhead DNA-binding domain and exclusive expression in the nucleus. In addition to the forkhead domain, Foxp3 contains a Cys_2His_2 zinc

finger domain and a coiled-coil-leucine zipper motif (Fig. 3). Homology among full-length human, mouse and rat Foxp3 is very high, suggesting a highly conserved function. The data showing that mutations in Foxp3 are the causative factor in both IPEX and *sf* disease, and that the lack of Foxp3 resulted in hyper-responsive T cells prompted researchers to evaluate Foxp3 expression in T_R. Comparison of both mRNA and protein levels of Foxp3 in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells showed very high, specific expression in the $CD4^+CD25^+$ T_R(80). These results lead to the hypothesis that Foxp3 is a specific molecular marker for these cells and that its transcription was regulating the activity and/or development of T_R.

Additional evidence has now shown Foxp3 to be necessary and sufficient for the development of $CD4^+$ T_R and that these cells are responsible for controlling the multi-organ autoimmune disease in *sf* mice and IPEX patients. First, in mixed bone marrow chimeras made with a 1:1 mixture of WT and $Foxp3^{null}$ bone marrow, the development of $CD4^+CD25^+$ T_R was exclusively from the WT donor (80). This data suggests that the expression of Foxp3 is required for T_R during development and selection. Second, the transfer of $CD4^+CD25^+$ T cells into neonatal (before three days of age) male *sf* mice completely rescues these mice from their autoimmune phenotype. Though this rescue does not last indefinitely, disease is prevented until at least 100 days post transfer, at which point the loss of protection correlates with a progressive inability of these cells to continue their self-replenishment (BDS unpublished data). This suggests that the presence of $Foxp3^+$

cells alone is enough to control the aberrant T cell responses in these mice. Third, retroviral transduction of $CD4^+CD25^-$ T cells with Foxp3 results in the acquisition of regulatory properties, suggesting that Foxp3 is sufficient for instilling a regulatory program in $CD4^+$ T cells. Finally, transgene over-expression of Foxp3 in mice resulted in an increase in T_R cells and the acquisition of regulatory properties by some $CD4^+CD25^-$ and $CD8^+$ cells(83). Together, these data show that expression of Foxp3 alone is enough to drive the development of $CD4^+$ T cells with the regulatory properties required to control autoimmune-mediated T cell responses.

Recently published data has begun to further define the molecular activity of Foxp3 through site-directed mutagensis(85-87) of the protein itself and ChIP-on-chip (chromatin immunoprecipitation-on-chip) technology to identify proteins interacting with Foxp3. The mutagenesis studies showed Foxp3 can bind directly to the ARRE2 site in the promoter for IL-2 and thereby inhibit transcriptional activation. The ability of Foxp3 to inhibit transcription was abolished by mutation of the residues in its forkhead domain that are predicted to interact with NFAT. These data indicate that at least one mechanism of Foxp3-mediated transcriptional repression involves direct contact with NFAT and its subsequent inhibition. In addition to IL-2, Foxp3 can target genes other than cytokine genes or genes that are regulated by NFAT. Several ChIP-on-chip (chromatin immunoprecipitation-on-chip) studies recently reported between 700 and 1,100 genes may be regulated by Foxp3(88-90). The differences in the results of these studies may be attributed to

the different systems used. All studies found that most of the Foxp3-target genes identified were differentially regulated in naturally occurring T_R cells; however, Zheng et al(88). found that the Foxp3-target genes accounted for only 6% of FOXP3-regulated genes, suggesting that the indirect regulation of gene expression by FOXP3 is also a crucial aspect of its function. All groups agreed that Foxp3 could function as both a transcriptional activator and a transcriptional repressor. Consistent with this dual role of Foxp3, a recent report showed that ectopically expressed Foxp3 could bind to the promoters of the CD25, CTLA-4 and GITR genes, remodel their chromatin and induce gene transcription (91). The disparities in the data generated by the ChIP-on-chip analyses indicate that further studies will be required to reconcile these differences and to resolve the role of additional signaling molecules that act through the Foxp3 signaling pathways to mediate T cell regulation.

Although the discovery of Foxp3 as a marker for T_R was accepted with great enthusiasm, the fact that it is only expressed in the nucleus made sorting and studying T_R based on its expression difficult. Even after reliable antibodies were generated, cells needed to be fixed and permiablized to stain for Foxp3 and this made it impossible to do *in vivo* studies of live cells. To solve this problem, Fontenot et.al.(81) and Wan et.al. (92) generated gene-targeted mice in which the complete GFP (Fontenot) or RFP (Wan) coding sequence was inserted in-frame into the coding region of the Foxp3 gene. In both mice, modified alleles ($Foxp3^{gfp}$ or $Foxp3^{rfp}$) encoded a chimeric Foxp3 fusion protein, having eGFP or mRFP

inserted upstream of the entire remaining Foxp3 gene and transcribed with the endogenous Foxp3 promoter. With these mice, you can clearly identify the lymphocytes expressing Foxp3 by measuring their GFP or RFP expression via flow cytometry. Both of these are a useful tool to identifying and study T_R and the $Foxp3^{gfp}$ were used in my studies, described later in this text.

Fontenot et.al used the $Foxp3^{gfp}$ mice to examine several important theories about T_R development. These mice have normal expression of Foxp3, as well as normal development and distribution of T_R, demonstrating that the insertion of GFP does not affect Foxp3 protein expression. Greater than 97% of the GFP^+ cells from the peripheral lymphoid organs fell into $CD4^+CD8^-$ live gate and these GFP^+ cells express high levels of CD25, CTLA-4, GITR and other markers previously characterized as typical to the T_R phenotype, demonstrating the strict lineage-specific expression of Foxp3. In the thymus, the majority of $Foxp3^{gfp}$ cells were CD4 SP cells and Foxp3 expression required interactions with MHC class I and class II establishing the requirement of TCR signals in T_R lineage selection within the thymus. Additionally, this group showed that CD4 lineage-specific ablation of Foxp3 expression resulted in multi-organ autoimmune disease that was strikingly similar to *sf* disease. Taken together, all these data support the hypothesis that Foxp3 is necessary and sufficient for the development of a specific lineage of $CD4^+$ T cells selected and sustained for the precise purpose of controlling autoreactive T cells.

Mechanisms of T_R Activity

Despite extensive research since the initial studies showing the importance of $CD4^+CD25^+$ cells in preventing autoimmunity, the mechanism by which these cells control immune responses is not completely understood. Initial *in vitro* studies postulated several possible strategies within which T_R meditated their suppression, such as the contribution of suppressive cytokines, cell-contact dependant mechanisms and specific homing properties(93). TGFβ1 was initially thought to induce T_R suppression, but, as I stated in the previous section, it was found to be more important for T_R homeostasis and dispensable for the suppressive activity of T_R (though this is still controversial). Concurrently, mouse studies of IBD induced by the transfer of $CD4^+CD45RB^{high}$ cells into SCID mice showed that the ability of co-transferred T_R to make IL-10 was critical in preventing the disease(30,94). Additional models of transplantation tolerance, GVHD, infection and autoimmune disease also suggested that IL-10 was critical to T_R function. Indeed, recent evidence has clearly shown that $Foxp3^+$ T_R make IL-10 in the intestinal tissues(95). In contrast, it was shown that IL-10-deficient T_R could mediate *in vitro* suppression normally and could prevent the autoimmune disease produced by the depletion of T_R(94). One explanation of these seemingly contrary results is the discovery of T_R1 cells or $CD4^+CD25^-$ cells without Foxp3 expression can secrete IL-10 through chronic antigen stimulation(70,96). These T_R1 cells have been shown in both mice and humans to be a separate lineage of non-$Foxp3^+$ regulatory T cells and are an important regulatory subset within the intestinal tissues. Though the presence of

T_R1 cells does not negate the possibility that T_R utilize IL-10 to mediate suppression in some situations, IL-10, like TGFβ1, may not be the primary mechanism of T_R-mediated suppression.

Additional evidence that cytokine-based suppression is not the primary mechanism behind the activity of T_R is that *in vitro* suppression depends on cognate cell-to-cell interactions between T_R, T_{eff} and APCs. It was shown that supernatants recovered from activated T_R were unable to induce suppression and no suppression was observed when a semi permeable membrane separated T_R and the T_{eff}/APC mixture (29). Research showing that T_{eff} from B7-1/B7-2-double-deficient mice lacked that ability to be suppressed by T_R(97)implied that T_R directly suppresses T_{eff} by co-stimulatory molecule interactions between B7-1/B7-2 on T_{eff} and CTLA-4 on T_R. However, recent advances in live tissue and intravital imaging technology suggest that direct T_R to T_{eff} interactions may not mediate suppression(98,99). These studies have allowed direct visualization of the interaction of T cells with APCs in intact lymph nodes (LN) during the priming of an immune response. By labeling T_R with one dye and T_{eff} with a different dye, the two subsets can be independently tracked in the same LN. In studies of the non-obese diabetic (NOD) mouse model of autoimmune diabetes, T_R actively suppressing autoreactive T_{eff} were not forming direct, stable interactions. The T_R to T_{eff} interactions observed in antigen draining LN were similar in duration and frequency to interactions observed in non-draining LN where there was no active suppression. Similar results were seen in cells responding to myelin basic protein in the experimental

autoimmune encephelomyelytis (EAE) model. These observations lead to the hypothesis that APCs were the target of signals by T_R. In fact, *in vitro* evidence showing that T_R can induce down-regulation in expression of MHC class II and co-stimulatory molecules on APC supports this idea(100-104). Together these findings suggest that T_R control the priming of autoreactive T_{eff} by preventing their persistent conjugation with APC. These data highlight the importance of understanding how T_R to APC interactions are initiated and sustained, as well as understanding the signals passing between them.

Tissue localization of T_R

Despite their importance for the prevention of autoimmunity, the homing properties and tissue distribution of T_R remain poorly characterized. The cells and tissues of the immune system are precisely organized to promote the cellular interactions required for the development, activation, function and regulation of diverse leukocyte populations(105-108). Tissue- and microenvironment selective lymphocyte homing is the basis for this organization, which in turn is mediated by their expression of specific combinations of adhesion and chemoattractant receptors. These homing receptors promote lymphocyte migration from the blood into tissues via a series of interactions between the lymphocyte and the vascular endothelium in specialized post-capillary venules (Fig. 4). Initially, low-affinity interactions (generally mediated by selectins or low-affinity integrins) tether the cell to the endothelium and cause it to roll along the endothelial surface. Signals through $G_{\alpha i}$-coupled chemoattractant receptors (especially chemokine receptors)

trigger upregulation of integrin affinity and avidity, resulting in the firm arrest of the cell on the endothelial wall. Finally, cells undergo diapedesis through the endothelium into the underlying tissue parenchyma, where they integrate chemokine gradients in the process of microenvironmental localization. Because post-capillary venules in different tissues and microenvironments express unique combinations of adhesion molecules and chemokines, they recruit distinct populations of lymphocytes that express the appropriate counter receptors. For example, naïve T and B cells display a remarkable tropism for the secondary lymphoid tissues (SLT) as a result of their high level expression of the 'rolling' receptor L-selectin (CD62L), the chemokine receptor CCR7, and the LFA-1 integrin (CD11a/CD18). However, upon activation these cells reprogram their tissue tropism, and upregulate homing receptors that target them to non-lymphoid sites of inflammation and infection. Because the homing receptors needed to access non-lymphoid tissues vary depending on the tissue and the type of inflammatory response, homing receptor expression by effector and memory T cells is remarkably heterogeneous. Moreover, expression of particular combinations of homing receptors can in some cases be used to predict the tissue-tropism of a given cell population(109).

As previously discussed, cell-to-cell contact between T_R and their targets (either APC, T_{eff} or both) is likely required for them to exert their suppressive function. Therefore, it follows that in order to effectively control autoimmunity, T_R must co-localize with their targets *in vivo*. Thus, the proper localization and tissue

distribution of T_R are likely to be essential for their ability to prevent autoimmunity. My data and several recently published studies in mice and humans show that like conventional T cells, T_R express diverse patterns of homing receptors(110-112). Among these are molecules that control T cell homing to secondary lymphoid tissues (CD62L and CCR7), to the intestines and other mucosal surfaces ($\alpha4\beta7$ integrin and CCR9), and to the skin and other sites of peripheral inflammation (E- and P-selectin ligands and CCR4). Interestingly, expression of adhesion receptors that target T_R to non-lymphoid tissues is associated with expression of the $\alpha E\beta7$ integrin. This suggests that through interactions with its ligand E-cadherin, which is widely expressed by epithelial cells, $\alpha E\beta7$ plays an important role in T_R localization and function in non-lymphoid tissues.

Of particular importance to my experiments, many of the homing receptors expressed by T_R have also been reported to mediate homing of pathogenic T cells into non-lymphoid tissues, such as the pancreas, skin and intestinal tissues. Indeed, in murine models of type-1 diabetes (T1D), T_R have been found in both the draining pancreatic lymph node and in the pancreatic islets(113). Additionally, the majority of T_R in human peripheral blood express the skin-associated homing receptor CLA, and subsequent analysis of $CD4^+$ T cells from human skin showed that a high percentage were $CD4^+CD25^{high}$ (114). Moreover, several other studies demonstrated that T_R are found in other non-lymphoid tissues such as the lung(104) and intestinal lamina propria(115). The relationship between T_R found in lymphoid

and non-lymphoid tissues has not been characterized. These populations may represent different stages in the differentiation of 'natural' T_R. Alternatively, the T_R in non-lymphoid tissues may primarily be the 'adaptive' T_R that have been proposed to have developed from chronically stimulated $CD4^+CD25^-$ conventional T cells (see Fig.2). In addition, the functional significance of T_R localization to lymphoid and non-lymphoid tissues for the prevention of autoimmunity has not been addressed. However, their differential localization suggests that T_R in the SLT and in non-lymphoid tissues target different populations of autoreactive T cells. T_R in the SLT may prevent the initiation of autoreactive immune responses by blocking the proliferation and/or functional differentiation of naïve T cells, perhaps via APC modulation, while T_R in non-lymphoid tissues likely interact with primed T cells, preventing them from inappropriately elaborating their destructive effector functions.

Questions to Address

Although $Foxp3^+$ T_R are clearly an essential subset in the protection against self-reactivity and much has been learned about their activity and homeostasis, many questions remain to be answered. As I previously described, studies in mice show T_R can suppress many T cell-mediated autoimmune diseases through a mechanism that is cell-contact dependant. Moreover, T_R suppressive function requires antigen recognition on APC, in conjunction with additional co-stimulatory signals. This interaction is not only essential for their function, but also may be necessary for T_R homeostasis and survival. There is little data to explain how T_R

are directed to the site of APC interaction and whether these activated T_R need to traffic to the site of tissue inflammation to mediate suppression. My preliminary studies and work by others show that, like conventional T cells, T_R express a diverse pattern of homing and adhesion receptors. It is not well established what role the expression of these different homing receptors has on the homeostasis, function and survival of T_R. *I hypothesize that T_R express a combination of homing receptors in order to target them to specific tissues and microenvironments in vivo. The expression of these molecules is driven by precise signaling events during the stimulation of T_R through their TCR by APCs. This expression may provide a mechanism to deliver specific immunoregulatory functions to distinct immune compartments in vivo.* These studies sought to clearly understand (a) how the expression of specific homing receptors impacts the homeostatic expansion, differentiation and survival of T_R (b) whether expression of particular homing receptors is required for T_R -mediated suppression in specific tissues (c) the contribution of specific signaling molecules to T_R differentiation and homeostasis after stimulation through their TCR.

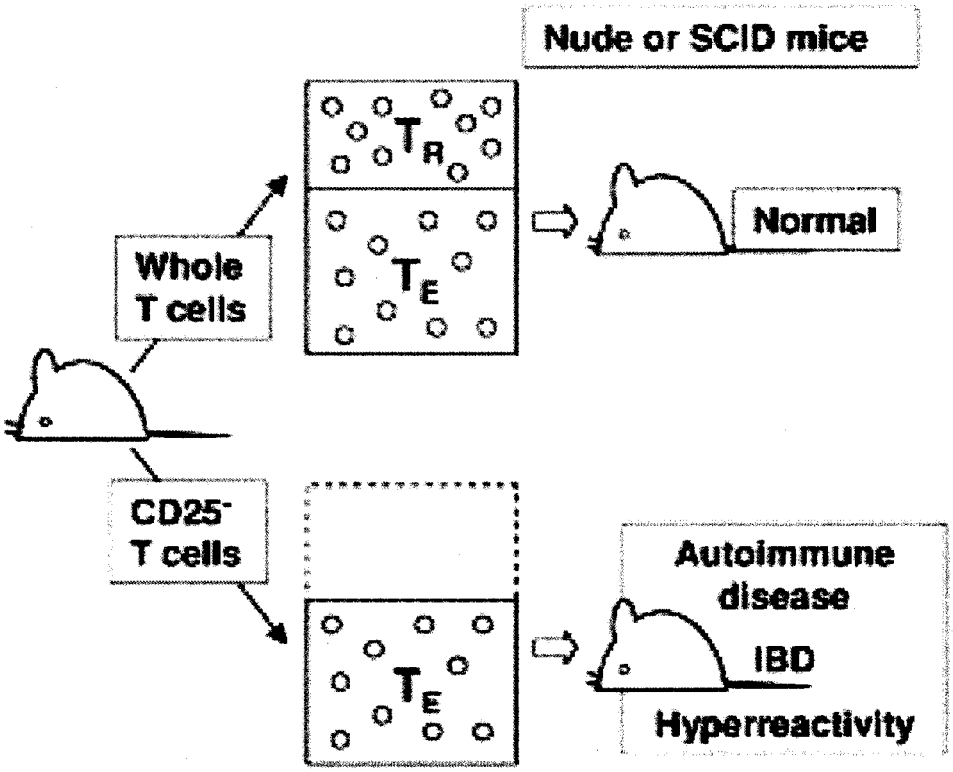


Figure 1.
Original T_R experiment performed by Sakaguchi et.al: These experiments demonstrated that the transfer of T cell suspensions depleted of $CD25^+CD4^+$ T_R cells into athymic nude mice or SCID mice induces autoimmune disease and IBD. *S. Sakaguchi, Annu. Rev. Immunol. 2004, 22:531-62*

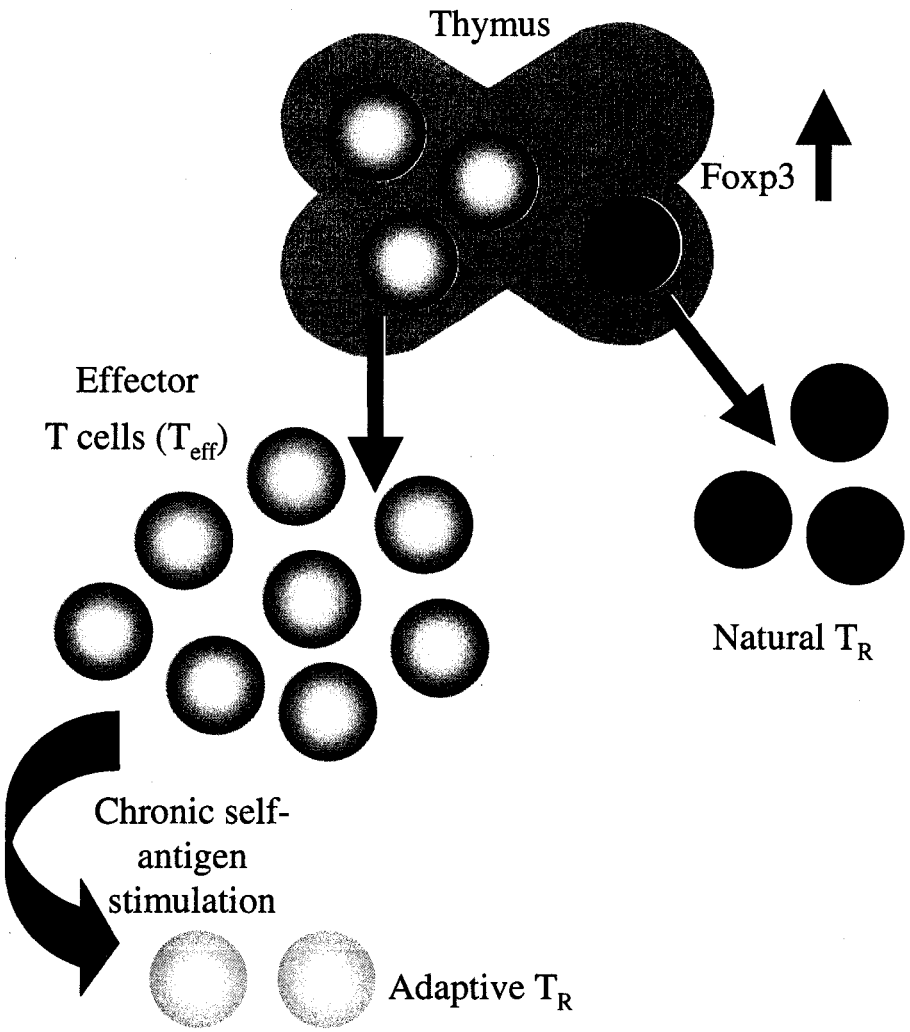


Figure 2.
Natural T_R vs. Adaptive T_R: during positive selection, natural T_R are selected via high affinity self-reactive TCR and upregulated Foxp3 at the same time as T_{eff} are selected via low-affinity self-reactive TCR. Both subsets move into the periphery to mediate the immune response. Adaptive T_R are proposed to arise during chronic immune responses from T_{eff} that are stimulated with excessive amounts of self-antigen in the absence of proper co-stimulation, likely from APCs that are not fully activated.

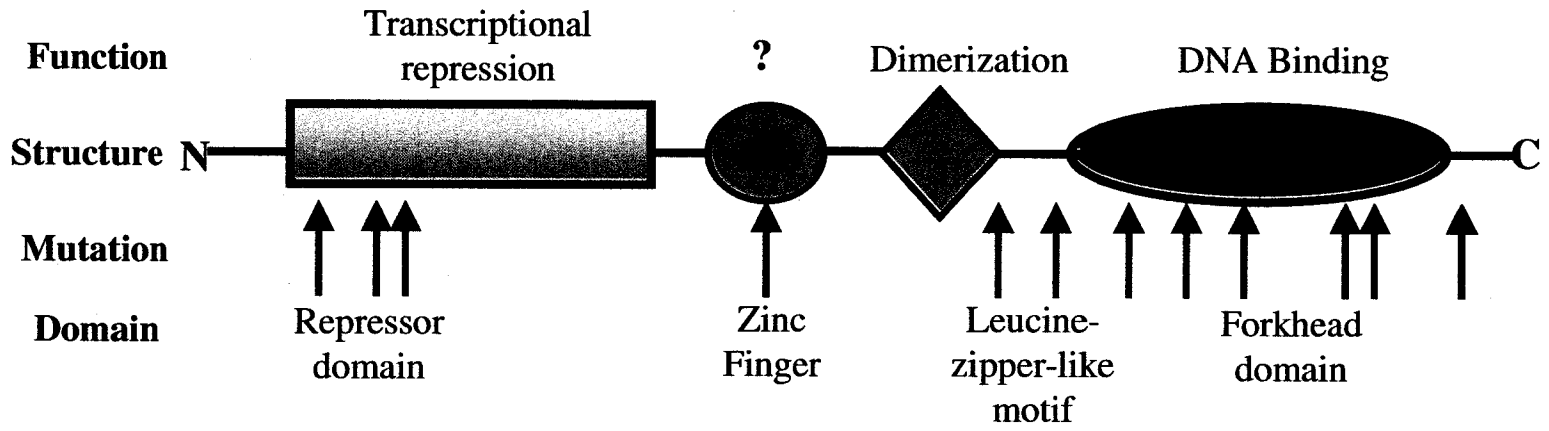
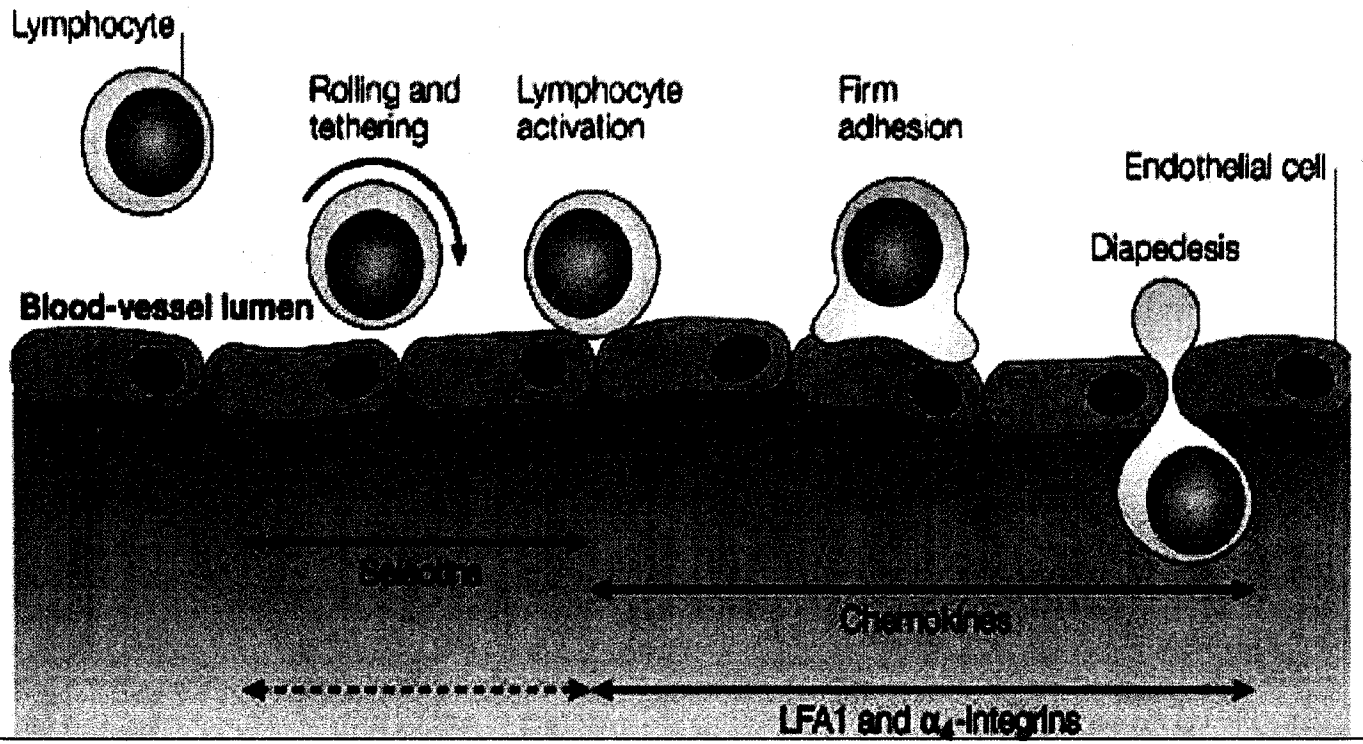


Figure 3.

Structure of Foxp3: the X-chromosome encoded Foxp3 gene encodes a conserved protein expressed exclusively in the nucleus of T_R. The protein consists of a N-terminal transcriptional repressor domain, a zinc-finger domain, a leucine-zipper-like motif (necessary for dimerization) and a C-terminal forkhead winged-helix domain (necessary for DNA binding). Arrows represent known mutations in the Foxp3 gene (in humans and mice) that result in reduction or loss of protein production and the presentation of an autoimmune phenotype.

Figure 4.

Schematic representation depicting the steps of lymphocyte migration from blood circulation into tissues: Lymphocytes must undergo four independently regulated receptor-ligand interactions to move from the blood into the surrounding tissue. First, rolling of lymphocytes along the endothelium is facilitated by leukocyte receptors that occur at high density on the tips of microvilli surface protrusions, such as PNAd, E-selectin, VCAM-1 and MAdCAM-1. Next, rolling lymphocytes respond to chemoattractants on endothelial cells because they express specific seven transmembrane receptors, chemokine receptors, which signal through G proteins. Different subsets of lymphocytes, depending on their tissue specificity, express different chemokine receptors. These activating signals induce rapid conformational change and activation of β2 integrins (LFA-1) which bind to ICAM-1 and mediate firm arrest of lymphocytes to the endothelium. Finally, lymphocytes undergo transendothelial migration into the underlying tissue parenchyma (diapedesis), where they are guided by chemokines and other chemoattractants to specific tissue microenvironments



Cell Type	Rolling & Tethering	Activation	Adhesion	Diapedesis & Migration
Naïve T cells	CD62L-PNAd	CCR7-SLC	LFA-1-ICAM-1	CCR7-ELC
Skin Memory T cells	CLA-E-selectin α4β7-VCAM-1	CCR4-CCL17 CCR10-CTACK	LFA-1-ICAM-1 αEβ7-E-cadherin	CCR10-CTACK
Small Intestinal Memory T cells	α4β7-MAdCAM-1	CCR9-TECK	LFA-1-ICAM-1 α4β7-MAdCAM-1	CCR9-TECK

Chapter 2: Altering the distribution of FoxP3$^+$ regulatory T cells results in tissue-specific inflammatory disease

Introduction

As I extensively discussed in the first chapter, CD4$^+$FoxP3$^+$ T_R are essential for the maintenance of self-tolerance, and T_R control pathology in many mouse models of organ-specific autoimmunity, including diabetes(59,116), experimental autoimmune encephelomyelytis(117,118), colitis(119), gastritis(120), and collagen-induced arthritis(110). Furthermore, CD25$^+$ T_R ameliorate graft-versus-host disease, and have been implicated in limiting immunopathology during responses to foreign antigens, including infectious agents such as *Leishmania major*(121). Although extensively studied, the mechanisms used by T_R to control T cell responses remain controversial, with different mechanisms invoked in various *in vitro* and *in vivo* settings(93). This suggests that T_R may utilize multiple tactics to limit autoimmunity, and may reflect functional heterogeneity among T_R subsets that localize to distinct tissue environments.

Although the activity of T_R within non-lymphoid sites is still poorly understood, expression of CD103 (also known as the αE integrin) has been suggested as a marker for 'effector-memory' T_R that preferentially migrate to and function within these sites(110,122). Indeed, several recent studies have implicated T_R activity in non-lymphoid tissues in limiting peripheral inflammation and autoimmunity. In the BDC2.5 adoptive transfer model of diabetes, co-transferred T_R did not inhibit the initial activation and expansion of the BDC2.5 effector T

cells, nor did they change the level of cellular infiltration of the pancreatic islets(123). Instead, their activity appeared to be restricted to controlling the progression and severity of islet destruction within the pancreas. Similarly, during *Leishmania major* infection, $CD103^+$ T_R migrated to the skin in a CCR5-dependent manner where they served to dampen the immune response to the pathogen(124,125). T_R migration to non-lymphoid tissues was also required for their ability to prevent acute graft-versus-host disease and cardiac graft rejection(126,127). Finally, migration of T_R to the skin was required for the amelioration of OVA-induced delayed-type hypersensitivity responses(112). Although these data indicate that T_R migration into non-lymphoid tissues is essential during inflammatory responses to foreign antigens or in acute models of autoimmunity, this requirement does not appear to be absolute. For example, in an adoptive transfer model of colitis, T_R can prevent disease even when their migration to the colon is severely impaired(128). Importantly, the role of T_R in maintaining tolerance in non-lymphoid tissues under steady state conditions has not been demonstrated.

A high percentage of T_R in human peripheral blood express the chemokine receptor CCR4 and display strong chemotactic responses to its ligands CCL17 and CCL22(114,129). CCR4-deficient mice fail to develop allograft tolerance following administration of anti-CD154 and donor spleen cells, and this is associated with decreased accumulation of Foxp3-expressing cells in the graft(126). In addition, high expression of the CCR4 ligand CCL22 has been

observed in ovarian tumors, as well as a high number of $CCR4^{+}Foxp3^{+}T_R$ present in the tumors themselves(130). These observations were associated with poor clinical prognosis suggesting that an excess of T_R within malignant non-lymphoid tissue allows tumors to escape immune attack. Nevertheless, the role of CCR4 in directing T_R migration and function *in vivo* is still poorly understood. Outside of the thymus, the CCR4 ligand CCL17 is constitutively expressed by endothelial cells in dermal post capillary venules and by bronchial epithelial cells(131,132). Notably, the expression of CCR4 ligands is upregulated in the inflamed skin of human dermatitis patients (CCL22) and bronchial epithelium of asthmatic patients (CCL17 and CCL22)(132,133). In addition, both CCL17 and a second CCR4 ligand, CCL22, are expressed by activated B cells, macrophages and by several DC subsets(134,134,135). Therefore, T_R may depend on the expression of CCR4 not only to localize in non-lymphoid tissues such as the skin and the lungs, but also to direct their migration to different populations of antigen-presenting cells (APC) within the secondary lymphoid tissues.

In the first segment of my thesis work, I examined how the tissue distribution of T_R is related to their ability to maintain immune tolerance and prevent inflammatory disease. I demonstrated that the majority of $Foxp3^{+}$ T_R in most murine non-lymphoid tissues are $CCR4^{+}$, and that T_R in the skin have a unique $CD103^{hi}CCR4^{+}$ surface phenotype. Analysis of antigen-specific T_R demonstrated that these cells upregulate CCR4, CD103 and other skin homing receptors when they are stimulated by their cognate antigen within sub-cutaneous

lymph nodes under pro-inflammatory conditions. Furthermore, I showed that CCR4 plays a role in the development or survival of skin-tropic $CD103^{hi}$ T_R, and in the accumulation of T_R in the skin and lung airways. To assess the impact of altering the tissue- and microenvironmental-distribution of T_R, I constructed mixed bone marrow-chimeras in which complete loss of CCR4 is restricted to the $Foxp3^+$ T_R compartment. In these animals, the lack of CCR4 expression on T_R resulted in peripheral lymphadenopathy, and in an increased frequency of $CD4^+$ effector T cells (T_{eff}) expressing skin-homing receptors. In addition, these mice spontaneously developed severe lymphocytic infiltration and inflammation in the skin and the lungs, while all other tissues examined remained normal. Thus, selectively perturbing the migration of T_R through removal of CCR4 impaired their ability to effectively control $CD4^+$ T cell activation and differentiation, and to prevent tissue-specific inflammatory disease.

Results

$CCR4^+$ T_R are enriched in non-lymphoid tissues

The activity of T_R isolated from lymphoid tissues has been extensively characterized, but the presence and activity of these cells within non-lymphoid tissues is not well understood, particularly under non-inflammatory conditions. To determine if T_R are resident within non-lymphoid tissues, I analyzed $CD4^+$ T cells isolated from *$Foxp3^{GFP}$* mice (described in Chapter 1(81)). In the *$FoxP3^{GFP}$* mice, there was a significant population of $CD4^+GFP^+$ cells in not only secondary lymphoid tissues such as the spleen, sub-cutaneous peripheral lymph nodes (PLN),

mesenteric lymph nodes (MLN), and Peyer's patches, but also in all non-lymphoid tissues examined, including the skin, lung, liver, peritoneal cavity, intestinal epithelium, and intestinal lamina propria (Fig. 5, and data not shown). Thus, T_R are distributed throughout the body in a wide array of non-lymphoid tissues, even in the absence of any overt inflammatory responses.

Two surface homing receptors previously associated with T_R migration into non-lymphoid tissues are the chemokine receptor CCR4 and the integrin CD103. Therefore, I examined expression of these molecules by T_R isolated from different organs. $CCR4^+$ and $CD103^+$ T_R were dramatically enriched in all non-lymphoid tissues examined, consistent with their proposed roles in directing T_R to these sites (Fig.5). These data are consistent with data recently published by Lee et.al. that showed high CCR4 expression on T_R from several different tissues during an antigen-specific immune response (111). Interestingly, nearly all GFP^+ T_R in the skin expressed a unique $CD103^{hi}CCR4^+$ phenotype that was uncommon among T_R from all other tissues examined (see boxed gate, Fig 5). CCR4 has been implicated in the migration of $CD4^+$ T cells to the skin and in the development or survival of skin-tropic $CD4^+$ T_{eff} that express CD103 and functional E-selectin ligands (E-lig)(136). Likewise, CD103 is associated with T cell accumulation in epithelial sites, where its ligand E-cadherin is expressed(137). Therefore, the $CD103^{hi}CCR4^+$ phenotype of cutaneous T_R suggests that the coordinated activities of these molecules may be particularly important for the proper localization and function of T_R within the skin.

Peripheral recognition of self-antigen alters T_R tissue-tropism

Naive $CD4^+$ T cells exit the thymus expressing homing receptors such as CD62L and CCR7 that mediate their continual recirculation through the various secondary lymphoid tissues. Upon stimulation with cognate antigen, T cells shift their tissue tropism and acquire expression of homing receptors that direct their migration into specific non-lymphoid sites(138,139). Similarly, T_R may upregulate expression of CD103, CCR4 and other homing receptors that direct their entry into non-lymphoid tissues following recognition of self-antigens in the secondary lymphoid tissues. To directly test this hypothesis, I examined ovalbumin (OVA)-specific T_R from double transgenic DO11.10xRIP-mOVA mice. In these animals, OVA (driven by the rat insulin promoter) is expressed in both the thymus and in the pancreatic islets. As a result, ~50% of the OVA-specific transgenic T cells (identified by staining with the TCR clonotypic antibody KJ126) differentiate into fully functional $CD25^+FoxP3^+$ T_R(31).

I transferred $1x10^5$ FACS-sorted $CD4^+CD25^+CD103^-CD62L^+$ T_R from DO11.10xRIP-OVA mice into wild type Balb/c recipients (Fig. 6a). Approximately 90% of the transferred cells were KJ1-26^+, expressed the lymph node homing receptor CCR7, but were negative for expression of CCR4 and the skin-homing receptor E-lig (Fig. 6b). To mimic recognition of cutaneous self-antigen in different conditions, I immunized the recipient animals with 200μg of soluble OVA by sub-cutaneous injection in PBS, either alone or in combination

with 1μg of cholera toxin (CT) as a pro-inflammatory stimulus. Five days after immunization, cells were harvested from the draining inguinal lymph nodes and their phenotype was analyzed. In both sets of mice, greater than 95% of the KJ1-26^+ cells remained $Foxp3^+$, indicating that immunization under these conditions did not alter their functional status as T_R (Fig. 7a). By contrast, the two different immunization strategies induced strikingly different homing receptor phenotypes on the transferred transgenic T_R (Fig. 7b). Upon stimulation with OVA + CT, the vast majority of KJ1-26^+ T_R became $CD103^{hi}$. In addition, many of these cells co-expressed CCR4 and E-lig, and nearly all cells downregulated CCR7. Although many KJ1-26^+ T_R also upregulated CCR4 in response to soluble OVA alone, few upregulated CD103 or E-lig, while many retained expression of CCR7 and CD62L. Thus, antigen-recognition in the PLN under pro-inflammatory conditions resulted in a dramatic shift in T_R tissue tropism, inducing expression of homing receptors required for migration to the skin while extinguishing expression of a chemokine receptor critical for T cell homing into secondary lymphoid organs. Indeed, 5 days after immunization with OVA+CT, I found $CD4^+$KJ1-26^+ T cells in the inflamed skin overlaying the injection site (data not shown). By contrast, a large fraction of T_R activated by soluble antigen appeared to retain their preferential tropism for the secondary lymphoid organs.

T_R accumulation in the skin and lungs in impaired in the absence of CCR4

To determine if CCR4 has a role in T_R development or localization, I constructed mixed bone marrow chimeras using congenically marked $CCR4^{-/-}$

(CD45.2$^+$) and wild type (WT) B6.SJL (CD45.1$^+$) donors. Then, I determined if specific subsets of T_R were at a competitive disadvantage due to their lack of CCR4, and whether CCR4$^{-/-}$$T_R$ accumulated in various tissues as efficiently as their WT counterparts.

In the spleens of the mixed bone marrow-chimeras, the ratio of Foxp3$^+$ T_R from the CCR4$^{-/-}$ and WT donors was similar to that observed for CD4$^+$ Foxp3$^-$ T cells, indicating there was no gross developmental disadvantage for CCR4$^{-/-}$$T_R$ (Fig. 8). However, within the sub-cutaneous PLN, T_R expressing high levels of CD103 were predominately derived from the WT donor (Fig. 9a). For each animal analyzed, we normalized the ratio of WT: CCR4$^{-/-}$ cells among the CD103^{hi} T_R in the PLN by the ratio found among total splenic T_R. On average, the ratio among the CD103^{hi}cells was ~3-fold higher than the ratio in the spleen, indicating a clear developmental or survival advantage for WT cells in this compartment (Fig. 9b). A similar 3-fold increase in the ratio of WT: CCR4$^{-/-}$ cells was observed among E-lig$^+$ T_R in the PLN (Fig. 10), consistent with the preferential expression of E-lig by CD103^{hi} cells following antigen stimulation of OVA-specific T_R (Fig. 7c). By contrast, the ratio of WT: CCR4$^{-/-}$ cells within the CD103^{low} and CD103 negative populations of T_R in the PLN, or in any T_R populations examined in the intestinal MLN, was nearly identical to that found among T_R in the spleen (Fig. 8 and Fig. 9b).

Because most T_R in non-lymphoid tissues expresses CCR4, we also examined the ratio of WT: CCR4$^{-/-}$TR in the skin, liver and lung airways of the

chimeric mice (Fig. 9). Similar to their CD103^{hi} counterparts in the PLN, we found that cutaneous T_R were largely of WT origin, with a normalized ratio ~3-fold higher than that found among T_R in the spleen. Surprisingly, among all tissues and T_R subsets examined, the highest ratio of WT:CCR4$^{-/-}$ cells was found among T_R isolated from the lung airways, with an average ratio ~6-fold higher than splenic T_R. By contrast, there was only a slight (<2-fold) preference for WT cells among T_R isolated from the livers of these mice that did not reach statistical significance when compared to the ratio of WT: CCR4$^{-/-}$ cells found in the CD103$^-$ T_R from the PLN. From these data, we conclude that in the absence of CCR4, the development and/or survival of cutaneous CD103^{hi} T_R is impaired, and there is a reduced accumulation of T_R in the skin and lung airways.

Lack of CCR4 expression on T_R results in cutaneous and pulmonary inflammation

The impaired accumulation of CCR4$^{-/-}$$T_R$ in the skin and lung airways demonstrates that this receptor helps direct T_R migration to these sites *in vivo*. However, CCR4$^{-/-}$ mice do not develop any spontaneous inflammatory disease indicative of a defect in T_R function(140). This may be because both T_{eff} and T_R migration are similarly impaired in these animals. Consistent with this notion, in the WT: CCR4$^{-/-}$ mixed bone marrow chimeras, we found that the accumulation of both Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells in the skin and lung airways were severely impaired in the absence of CCR4 (Fig. 9 and data not shown). Therefore, to assess the importance of CCR4 for T_R localization and function in the context of a largely WT Foxp3$^-$ T cell compartment, we constructed mixed-bone marrow chimeras by

transferring bone marrow from CD45.1$^+$ FoxP3-deficient scurfy *(sf)* mice(77) and CD45.2$^+$ CCR4$^{-/-}$ mice into irradiated RAG-1$^{-/-}$ recipients (Fig. 11a). In the resulting chimeras, only the CCR4$^{-/-}$cells can give rise to FoxP3$^+$ T_R, whereas all the other T cell compartments are a mixture of CCR4$^{+/+}$ and CCR4$^{-/-}$cells. As controls, irradiated RAG-1$^{-/-}$ mice were reconstituted with a bone marrow from *sf* and wild type (WT) CD45.2$^+$ mice, or with *sf* bone marrow alone. Hereafter, these mice will be referred to as CCR4/*sf*-, WT/*sf*, and *sf*-chimeras. In both CCR4/*sf*- and WT/*sf*-chimeras, normal numbers of lymphocytes develop and populate the periphery after bone marrow reconstitution. Furthermore, as expected all T_R developed from the CD45.2$^+$CD45.1$^-$CCR4$^{-/-}$ or WT donor (Fig. 11b).

All chimeras that received only *sf* bone marrow developed severe dermatitis and wasting, and were sacrificed by 40 days post transplantation. Histological analysis of these animals revealed extensive inflammatory disease in the skin, lungs and liver, the three major target tissues of autoimmunity in *sf* mice (Fig. 12, bottom panels). By contrast, the WT/*sf*-chimeras remained phenotypically normal for up to 300 days post transfer, and showed little or no inflammatory disease in all tissues examined (Fig. 12, middle panels). Therefore, WT T_R were able to suppress the inflammatory disease caused by *sf*-derived CD4$^+$T cells. However, all CCR4/*sf*-chimeras developed severe localized skin inflammation 50-150 days post-transplantation. When cutaneous inflammatory disease became severe, with visible crusting, alopecia and erythema in affected regions, each CCR4/*sf* chimera was sacrificed and analyzed along with a WT/*sf* counterpart (between 100 and 250 days

post-BM transplant). Within affected areas of the skin, there was extensive mixed leukocytic infiltration of the dermis accompanied by marked epidermal hyperplasia (Fig. 12, top panels). Inflammatory dermal infiltrates were composed largely of lymphocytes, neutrophils, eosinophils and mast cells (Fig. 13). In addition, there was consistent, albeit less severe, lymphocytic infiltration and inflammatory disease in the lungs, concentrated around the blood vessels and large airways (see arrows, Fig. 12). To quantify disease severity in these mice we developed a histological scoring system based on the severity and overall distribution of inflammatory infiltrates within each tissue section (Table 1). Blinded analysis of sections from the skin, lungs, and livers from a panel of CCR4/*sf*- and WT/*sf*-chimeras revealed that the ability of $CCR4^{-/-}T_R$ to prevent inflammatory disease in the skin and lungs is significantly impaired (Fig. 14). By contrast, WT and $CCR4^{-/-}$ T_R were both capable of preventing the extensive inflammatory hepatitis that developed in animals receiving only *sf* bone marrow.

Consistent with the cutaneous inflammation observed in the CCR4/*sf*-chimeras, these mice displayed severe lymphadenopathy selectively in the sub-cutaneous PLN (Fig. 15a). This suggested that the $CCR4^{-/-}T_R$ failed to efficiently control the activation and differentiation of the *sf*-derived $CD4^+$ T cells. Indeed, phenotypic analysis of the $CD4^+CD45.1^+$ *sf*-derived T cells in the PLN showed a significantly increased frequency of $CD44^{hi}CD45RB^{low}$ effector/memory cells when compared with cells from WT/*sf* chimeras (Fig. 15b). T cells activated in cutaneous lymph nodes upregulate skin homing receptors such as P-selectin ligand

(P-lig), E-lig and CCR4(138,141). Accordingly, there was a significant increase in the fraction of *sf*-derived $CD4^+$ T cells expressing these receptors in the cutaneous PLN of the CCR4/*sf*-chimeras (Fig. 16). This accumulation of activated, skin-tropic $CD4^+$ T cells in the PLN of CCR4/*sf*-chimeras suggests that these cells migrated to the skin and mediated the cutaneous inflammation observed in these animals. Indeed, within the skin of the CCR4/*sf*-chimeras, there was a substantial accumulation of $CD4^+CD45.1^+$ *sf*-derived T cells that was not found in the phenotypically normal WT/*sf*-chimeras (Fig.17). Importantly, this accumulation was not simply due to increased migration of *sf*-derived CD4+ T cells in response to pro-inflammatory cytokines because the observed enrichment was very comparable when I compared lymphocytes from affected skin verses unaffected skin (Fig. 18). Consistent with the pulmonary inflammation in the CCR4/*sf* chimeras, $CD45.1^+$ T cells were also enriched in the both the lung airways and parenchyma these mice (Fig17). In addition, among the $CD4^+$ T cells in the skin and lung airways, there was a 3-4-fold reduction in the fraction of $Foxp3^+$ T_R in the CCR4/*sf* chimeras when compared with their WT/*sf* controls (Fig. 19).

Adoptive transfer of purified $CD4^+CD25^+$ T_R into neonatal *sf* mice prevents the development of systemic autoimmune and inflammatory disease in these animals(80). Therefore, to further assess the importance of CCR4-dependent T_R localization *in vivo*, we compared the ability of WT and $CCR4^{-/-}T_R$ to rescue animals from disease in this model. Whereas WT T_R effectively prevented inflammatory disease in all tissues examined, *sf* mice given $CCR4^{-/-}T_R$ developed a

histological and cellular phenotype similar to the CCR4/*sf*-chimeras, visibly apparent after 50 days of age, with severe inflammatory disease evident in the skin and lungs, but not the liver (Fig 20). Thus, the cutaneous and pulmonary inflammation in the CCR4/*sf*-chimeras is not simply a by-product of the pro-inflammatory effects of the lethal irradiation used to condition the recipients, nor is it due to the lymphopenia present in the recipient animals. In addition, confirming previous results(126), we found that $CCR4^{-/-}T_R$ were as efficient as WT T_R at suppressing the proliferation of $CD4^+$ T cells *in vitro*, indicating that there is not a gross defect in the function of $CCR4^{-/-}T_R$ (Fig. 21). Instead, our data demonstrate altering the tissue and/or microenvironmental distribution of T_R by selective removal of CCR4 leads to excessive activation and differentiation of CD4+ cells in the PLN and their subsequent accumulation in the skin and lungs, resulting in the development of tissue-specific inflammatory disease.

I hypothesized that the reason I saw spontaneous skin and lung inflammation in our CCR4/*sf* chimeras and $CCR4^{-/-}$ *sf* neonatal transfers, whereas no such phenotype is seen in $CCR4^{-/-}$ mice is because both T_R and non-T_R CD4+ effector T cells are equally inefficient at gaining access to subsets of DC expressing CCR4 ligands. To test this hypothesis, I crossed the *sf* mice and the $CCR4^{-/-}$ mice and repeated the $CCR4^{-/-}$ T_R rescue into $CCR4^{-/-}$ male *sf* neonates. I found that $CCR4^{-/-}$ T_R rescued $CCR4^{-/-}$*sf* mice as efficiently as WT T_R and no disease pathology was seen in these mice up to 100 days post-transfer (n=4). This confirms that that pathology I was in the *sf* mice rescued with $CCR4^{-/-}$ T_R was not simply due

to the nature of the effector cells in these mice, but was likely due to inefficient localization of T_R compared to non-T_R. These data support our hypothesis and additional experiment are needed to delineate the mechanism by which CCR4 ligand interactions between DC, T_R and $CD4^+$ effector T cells suppress skin and lung-specific self-reactivity.

Discussion

The regulation of immune responses by T_R *in vivo* is a complex process, involving multiple T_R subsets that appear to use unique functional mechanisms to suppress the activation, differentiation and function of effector T cells within both lymphoid and non-lymphoid tissues. I examined the tissue distribution of T_R in the steady state, and determined the impact of altering this distribution by removal of the chemokine receptor CCR4. My results have several important implications for current models of T_R migration and function.

Early studies of T_R suggested they predominantly functioned within secondary lymphoid tissues by inhibiting the initial priming of autoreactive T cells(142). However, data from a number of models have now convincingly demonstrated that T_R can also regulate T_{eff} responses to both self- and foreign-antigens during acute inflammation at non-lymphoid sites(143). Although T_R have been isolated from normal human skin and intestines(95,114), their role in maintaining immune homeostasis in non-lymphoid sites in the absence of an inflammatory stimulus is not clear. My analysis of T_R distribution in the *Foxp3*^{GFP} mice further demonstrates that T_R are present within a wide variety of non-

lymphoid organs even in the absence of any overt inflammatory response. This suggests that T_R constitutively function to help maintain immune tolerance and prevent autoimmunity at these sites even in the absence of any acute inflammation.

The integrin CD103 has been proposed as a marker of 'effector-memory' T_R with tropism for non-lymphoid tissues(110). Our analysis of T_R in Foxp3^{gfp}animals demonstrated that CD103$^+$ T_R are present at a higher frequency in non-lymphoid tissues than in the lymph nodes, spleen and Peyer's patches. However, within most non-lymphoid compartments I examined, including the lungs, liver, intestinal lamina propria and intestinal epithelium, we found a mixture of both CD103$^+$ and CD103$^-$$T_R$. Thus, expression of CD103 alone cannot be used to define T_R with non-lymphoid tissue tropism. However, T_R isolated from the skin did uniformly express very high levels of CD103, the expression of which may be selectively induced following T cell migration into the skin. CD103 has previously been associated with cutaneous T cells(144), and the CD103 ligand E-cadherin is expressed by epidermal keratinocytes(145). Analogous to its proposed role in retaining T cells in the intestinal epithelium, CD103 may therefore facilitate T_R retention in the epidermis. This is consistent with the obligate function of CD103 in T_R-mediated immune regulation during cutaneous *Leishmania major* infection(124), and suggests that the CD103^{hi} subset of T_R is phenotypically specialized to localize to and function within the skin. Indeed, the phenotypic diversity of T_R resident in different tissues further supports the concept that the T_R population as a whole is made up of numerous subsets, each expressing different

combinations of homing receptors, which act to deliver specific immunoregulatory functions to distinct tissue sites *in vivo*.

My analyses of OVA-specific T_R demonstrated that T_R acquire the ability to migrate to non-lymphoid sites after the recognition of cognate antigen in the secondary lymphoid tissues, and that addition of a pro-inflammatory stimulus greatly augmented this shift in T_R tissue tropism. Most subsets of APC undergo a low level of constitutive trafficking from non-lymphoid tissues into the corresponding draining lymph nodes. Once there, they can present self-antigens they have acquired to the largely autoreactive T_R population(36). I propose that when T_R recognize self-antigen presented by immature APC during these non-inflammatory conditions (such as those found following administration of soluble antigen in our adoptive transfer model), they largely maintain their tropism for the secondary lymphoid tissues, where they function to suppress the initial priming and differentiation of self-reactive T cells. However, during a strong tissue inflammatory response (such as that induced by addition of cholera toxin in our model), T_R undergo extensive expansion, during which they acquire the ability to migrate to non-lymphoid tissues. This most likely occurs due to the increased migration of fully mature APC to the draining lymph node, which bear a wide range of T cell costimulatory molecules, and can drive T_R into a full program of expansion and differentiation. Once in non-lymphoid sites, T_R may act to limit effector T cell responses, resulting in effective pathogen control without corresponding collateral tissue damage and immunopathology. This type of

peripheral immune regulation may be especially important in tissues, such as the skin, lungs and intestines, that are frequently bombarded with foreign antigens and 'pathogen-associated molecular patterns' that effectively activate APC. Consistent with this notion, we found that an unusually large fraction (20-40%) of $CD4^+$ T cells in normal skin are $Foxp3^+$ T_R (Fig 5 and data not shown), suggesting that they have a critical function in preventing cutaneous inflammatory disease. Similarly, $Foxp3^+$ T_R have been isolated from normal human skin, and a high proportion of T_R in human peripheral blood express cutaneous homing receptors(114). The skin and lungs (along with the liver) are primary targets of autoimmunity in Foxp3-deficient *sf* mice(77,146), whereas humans deficient in FOXP3 and mice that lack T_R due to mutations in various components of the IL-2 pathway generally develop severe enteropathy(49,51,147). Thus, $Foxp3^+$ T_R appear to have a particularly important function in preventing autoimmune and inflammatory disease in these barrier tissues.

The chemokine receptor CCR4 has previously been associated with T_R activity in both humans and mice(126,129). In humans, CCR4 is expressed by most E-selectin ligand$^+$ skin-tropic $CD4^+$ T cells found in peripheral blood, and by nearly all $CD4^+$ T cells isolated from normal skin (131,148). Accordingly, CCR4 helps direct $CD4^+$ T cells to the skin during a contact hypersensitivity response to the hapten dinitro-fluorobenzene in mice(149,150). In addition, by constructing mixed bone marrow chimeras using WT and $CCR4^{-/-}$ mice as donors, Baekkevold et.al. demonstrated that CCR4 plays a significant role in the development and/or survival

of cutaneous $CD103^{+}$E-lig^{+} $CD4^{+}$ T cells(136). In similar mixed chimeras, I confirmed these results, and additionally demonstrated that the vast majority of $CD103^{hi}$ or E-lig^{+} T_R present in the skin-draining lymph nodes were derived from the WT donor (Fig. 9 and Fig. 10). Furthermore, both $Foxp3^{+}$ and $Foxp3^{-}$ $CD4^{+}$ T cells in the skin of these animals were predominantly of WT origin (Fig 9 and data not shown). Thus, my results confirm the critical role of CCR4 in the accumulation of $CD4^{+}$ T cells in the skin, and demonstrate that this requirement applies to both conventional $CD4^{+}$ $Foxp3^{-}$ T cells and $Foxp3^{+}$ T_R.

Surprisingly, loss of CCR4 also severely impaired the accumulation of both $CD4^{+}Foxp3^{-}$ T cells and T_R in the lung airways. The CCR4 ligand CCL17 is constitutively expressed in the lungs by bronchial epithelial cells and $CD11c^{+}$ APC(132,135). Additionally, expression of both CCL17 and CCL22 is strongly upregulated in the lungs during pulmonary inflammation(132,151). Although CCR4 is expressed by nearly all $CD4^{+}$ T cells recovered after bronchoaveolar lavage in humans, and at lower levels by most $CD4^{+}$ cells isolated from human lung parenchyma(152), the function of this receptor in $CD4^{+}$ T cell trafficking to the lungs remains poorly understood. My results support a model in which CCR4 plays a significant role in the constitutive migration of CD4+ T cells to the lung airways. However, in addition to any direct effects CCR4 may have on T cell migration, loss of this receptor may also impair the generation of lung-and skin-tropic subsets of $CD4^{+}$ T cells, contributing to their failure to accumulate at these sites.

Co-localization of T_R with their targets is thought to be important for their suppressive function *in vivo*. By constructing mixed bone marrow chimeras using CCR4$^{-/-}$and Foxp3-deficient *sf* mice as donors, I was able to determine how altering the distribution of T_R *in vivo* impacts their ability to prevent tissue-specific inflammatory disease. Indeed, the phenotype of the CCR4/*sf*-chimeras is indicative of multiple roles for CCR4 in T_R migration and function. Within the secondary lymphoid tissues, T_R can modulate the ability of APC to effectively prime naïve T cells(98,99). Upon maturation, most APC subsets produce both CCL17 and CCL22, and this may help attract recently activated CCR4$^+$ T cells for further activation(133,134,153). Notably, compared with splenic DC, both CCL17 and CCL22 are expressed at particularly high levels by epidermal Langerhans cells (LC) at a baseline level, and upon their maturation and migration to the skin-draining lymph nodes(154,155). CCR4 may therefore play an important role in guiding T_R to LC and other cutaneous APC subsets within the PLN. During T cell priming LC potently induce CD4$^+$ T cells expression of skin homing receptors such as P-lig, E-lig(141). Thus, if CCR4$^{-/-}$$T_R$ fail to effectively compete with WT T_{eff} for access to LC, I expect that this would result in uncontrolled activation and differentiation of skin-specific autoreactive T cells. This is consistent with the selective peripheral lymphadenopathy and increased frequency of *sf*-derived CD4$^+$CD44^{hi}CD45RB^{lo} skin-tropic T_{eff} observed in the CCR4/*sf*-chimeras, which may be further augmented by the actions of various pro-inflammatory cytokines and chemokines. Indeed, the preliminary data from the CCR4$^{-/-}$ *sf* mice rescued

with $CCR4^{-/-}$ T_R suggests that inefficient access to APC by $CCR4^{-/-}$ T_R in competition with CCR4-sufficient T_{eff} is mediating the disease process. Therefore, I propose that dysregulated T_R function within the PLN, coupled with the inefficient localization of $CCR4^{-/-}T_R$ to the skin, led to the severe cutaneous inflammation that developed in these animals. Similarly, the pulmonary inflammation that developed in these animals was most likely due to impaired T_R migration to the lung airways, and a corresponding failure to limit T_{eff} function at this site.

Despite the fact that CCR4 is expressed by most T_R in all non-lymphoid tissues we examined from the $Foxp3^{gfp}$ mice, T_R -mediated protection of tissues other than the skin and lungs appeared to function normally even in the absence of this receptor. However, among non-lymphoid tissues in unmanipulated mice, constitutive expression of CCL22 and/or CCL17 is largely restricted to the skin and lungs(135,156). As I have shown that CCR4 is rapidly upregulated by T_R upon antigen recognition even in the absence of inflammation, the high frequency of $CCR4^+$ T_R in other non-lymphoid tissues may simply reflect continual stimulation of T_R by self-antigen at these sites.

The critical function of T_R in maintaining immune tolerance *in vivo* has been firmly established. Indeed, acute depletion of T_R is adult mice leads to rapid development of systemic lymphadenopathy and splenomegaly accompanied by severe multi-organ inflammation, indicating that T_R -mediated immune suppression is critical throughout life (82). My data demonstrate that T_R are found in a wide variety of lymphoid and non-lymphoid organs, and that simply altering the tissue-

and microenvironmental-distribution of T_R results in spontaneous tissue-specific inflammatory disease. This indicates that the balance of T_R and T_{eff} activities in different tissues are precisely tuned to allow for effective immunosurveillance and pathogen control whilst preventing the development of chronic inflammatory and autoimmune diseases. In addition, the possibility that subsets of T_R are phentoypically specialized to function in specific tissues is important to consider when designing therapies involving T_R manipulation or enrichment.

Materials and Methods

Animals

C57BL/6J, Balb/cJ, $RAG^{-/-}$(B6.129S7-$Rag1^{tm1Mom}$/J), and DO11.10 (C.Cg-Tg(DO11.10)10Dlo/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME). $CD45.1^+$ B6.SJL mice (B6.SJL-$Ptprc^a$/BoyAiTac) were purchased from Taconic Farms (Germantown, NY). *Scurfy* mice (B6.Cg-$Foxp3^{sf}$/J) were obtained from Jackson Laboratories and crossed to B6.SJL mice to generate $CD45.1^+$ animals. $CCR4^{-/-}$ mice on the C57BL/6 genetic background were obtained from Dr. Steve Ziegler (Benaroya Research Institute, Seattle, WA). $Foxp3^{gfp}$ mice have been described previously(81). Balb/c mice expressing RIP-mOVA were provided by Dr. Abul Abbas (University of California, San Francisco). All animals were housed and bred under specific pathogen–free conditions in the Benaroya Research Institute animal facility. All experiments were approved by the Benaroya Research Institute Institutional Animal Care and Use Committee.

Lymphocyte isolation

After whole body perfusion with 50ml PBS, lymphocytes were isolated as follows. Single cell suspensions were prepared from thymus, spleen, peripheral (pooled inguinal, auxiliary, brachial and superficial cervical nodes) and mesenteric lymph nodes by tissue disruption with glass slides, and filtered thru a 40mM filter. Bronchoaveolar lavage fluid was collected by flushing the lungs with 10 ml cold sterile PBS. To isolate cells from the liver, lung and skin (after removal of sub-cutaneous fat by scrapping), tissues were finely minced with scissors and vigorously stirred in RPMI with 0.14U/ml blendzyme (Roche Pharmaceuticals, Switzerland) and 100ug/ml DNAse I (Roche Pharmaceuticals, Switzerland) for 20 minutes at 37°C. Supernatants were filtered through a 70μM cell strainer, and the remaining tissue fragments were digested twice more, pooling all released cells. After dissection and removal of Peyer's patches, intestinal lamina propria lymphocytes (LPL) were isolated as follows. The intestinal epithelium was stripped as previously described(157,158), and the remaining intestinal pieces were washed 3 times in 40ml of cold RPMI. Intestinal pieces were added to 50ml of RPMI plus 100 μl 0.5M MgCl2, 100μl 0.5M CaCl2, 500μl 100X HGPG (111.9mg/ml Hepes, 29.2 mg/ml L-glutamine, 1000U/ml penicillin, 1mg/ml streptomycin, 10mg/ml gentamycin all purchased from Invitrogen) and 150U/ml collagenase (Roche Pharmaceuticals, Switzerland). Samples were stirred at 37°C for 1 hour, and the released cells were then filtered through nytex. Cells isolated from the skin, lung, liver and lamina propria were pelleted and resuspended in 44%

percoll (GE Healthcare Biosciences-AB) in RPMI, layered over 67% percoll and spun at 2800rpm for 20 minutes. Lymphocytes were isolated from the interface and used for subsequent flow cytometry analyses.

Flow cytometry

For cell-surface staining, 10^6 cells per sample were incubated with various antibodies in staining buffer (HBSS and 3% FCS) for 20 minutes on ice. Anti-murine antibodies included: anti-CD25 (PC61.5), anti-CD4 (RM4-5), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45RB (C363.16A), anti-CD45.1 (A20), anti-CD45.2 (104), anti-DO11.10 TCR (KJ1-26) and anti-CD103 (2E7) from eBioscience, (San Diego, CA) To assess the expression of CCR4 and CCR7, cells were incubated with CCL22- or CCL19-IgG3 fusion proteins, followed by anti-human IgG-APC (Jackson ImmunoResearch). To assess expression of functional P- and E-selectin ligands, cells were sequentially incubated with either a P- or E-selectin–human IgM fusion protein (produced in COS-7 cells), followed by biotinylated goat anti–human IgM (Jackson ImmunoResearch) and streptavidin–PE (eBioscience). Foxp3 expression was assessed by staining with anti-Foxp3 (FJK-16s, eBioscience) according to the manufacturer's protocol. Data were acquired on a FACsCalibur (BD Biosciences, San Diego CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Adoptive Transfer and Immunization

$CD4^+CD25^+CD62L^+CD103^-$TR were isolated from the spleen and peripheral lymph nodes of 5-week-old double transgenic DO11.10xRIP-OVA mice by FACs.

Balb/c mice were given 10^5sorted TR by retro-orbital injection in 100µl of PBS. One day after transfer, the recipient mice were immunized by sub-cutaneous injection under the abdominal skin with 200µg OVA either alone or in conjunction with 1µg cholera toxin in 100µL PBS. Fiver days after immunization, mice were sacrificed, and lymphocytes were isolated from the draining inguinal lymph nodes for analysis by flow cytometry.

Bone Marrow Chimeras

Bone marrow cells were prepared by flushing the femurs and tibias with cold sterile PBS. The cells were filtered through a 40µM filter and incubated in hemolytic buffer for 2 min at room temperature. $CD4^+$ cells were depleted from *sf*-derived bone marrow by magnetic depletion using anti-CD4 microbeads (Miltenyi, Auburn, CA), and contained less than 1% contaminating $CD4^+$ T cells after depletion. The cells were counted, washed, resuspended in sterile PBS, and injected retro-orbitally into anesthetized, $RAG^{-/-}$ mice (612 wk old) that had received 2 doses (separated by 4 hr) of 450 Rad from a cesium irradiator. Recipients were given $2x10^6$ CD4-depleted bone marrow cells from *sf* donors, either alone or mixed with $1x10^6$ $CCR4^{-/-}$or WT cells. For the WT: $CCR4^{-/-}$ mixed chimeras, $Rag^{-/-}$ recipients (treated as stated above) received $3x10^6$ a 1:1 mixture of WT ($CD45.1^+$) and $CCR4^{-/-}$ ($CD45.2^+$) bone marrow cells.

Tissue histology

Tissues were immersion fixed in 10% neutral buffered formalin, paraffin embedded and cut into 5µm sections, which were stained with hematoxylin and eosin. All

tissue sections were examined by a blinded observer for inflammatory infiltrates and scored for severity (normal, minimal, mild, moderate, severe) and degree of distribution (focal, focally extensive, multifocal, coalescing, diffuse) in different sub-regions of each tissue. These sub-scores were then combined to generate a total histological score for each section (see Table 1 for additional details).

Neonatal transfers

$CD4^+CD25^+$ TR cells (>90% purity in all experiments) were isolated from the spleen and lymph nodes of 8-12 wk old B6.SJL ($CD45.1^+$) and $CCR4^{-/-}$ ($CD45.2^+$) as described above for the suppression assay. Neonatal *sf* mice (1-2 days old) were given 1-2x10^6 $CD4^+CD25^+$ TR in 20µl PBS by intraperitoneal injection. Mice were monitored for external signs of inflammatory disease and sacrificed either 28 (no transfer) or 70 (CCR4-/- or WT transferred) days post-transfer for histological and phenotypic analyses.

In vitro suppression assay

$CD4^+$ T cells were isolated from the spleen and lymph nodes of WT or $CCR4^{-/-}$ mice by negative selection with a Dynal CD4 T cell negative isolation kit (Invitrogen, Carlsbad, CA). These cells were further separated into $CD25^+$ and $CD25^-$fractions by staining with CD25-PE, and magnetic fractionation using anti-PE magnetic microbeads (Miltenyi Biotech). Final suspensions of $CD4^+CD25^+$ TR and $CD4^+CD25^-$Teff cells were >90% pure. $CD4^+CD25^-$Teff were incubated for 9 min at 37°C in 0.8 µM CFSE (Invitrogen, Carlsbad, CA) in PBS, washed with 100% FBS, resuspended in complete DMEM. In each culture well, 10^6 CFSE

labeled WT Teff were incubated with 10^6 irradiated (2500 Rad) WT CD4-depleted spleen cells as APC, with or without addition of WT or $CCR4^{-/-}CD4^+CD25^+$ TR. All cultures (except unstimulated control) were stimulated with 3μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 110 hours. Teff proliferation was measured by assessing relative CFSE dilution by flow cytometry.

Figure 5.

Foxp3^{+} CD4^{+} T cells are resident in both lymphoid and non-lymphoid tissues: (First and third rows) Flow cytometry analysis CD4 and GFP expression by lymphocytes isolated from the spleen, skin-draining peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), thymus, Peyer's Patches, skin, lung parenchyma, liver, and intestinal lamina propria of a 12-week-old *Foxp3^{GFP}* mouse. Percentage indicates the fraction of CD4^{+} cells expressing GFP. (Second and fourth rows) Expression of CD103 and CCR4 by gated CD4^{+}GFP^{+} cells from each tissue. Percentage indicates the fraction of T_R that are CD103^{high}CCR4^{+} as defined by the rectangular gate in the upper right quadrant. For the CCR4 negative control, spleen cells were stained with CCL22-Fc fusion protein that was pre-incubated for 5 minutes with a neutralizing anti-CCL22 monoclonal antibody. Results are representative of >6 mice analyzed in this fashion.

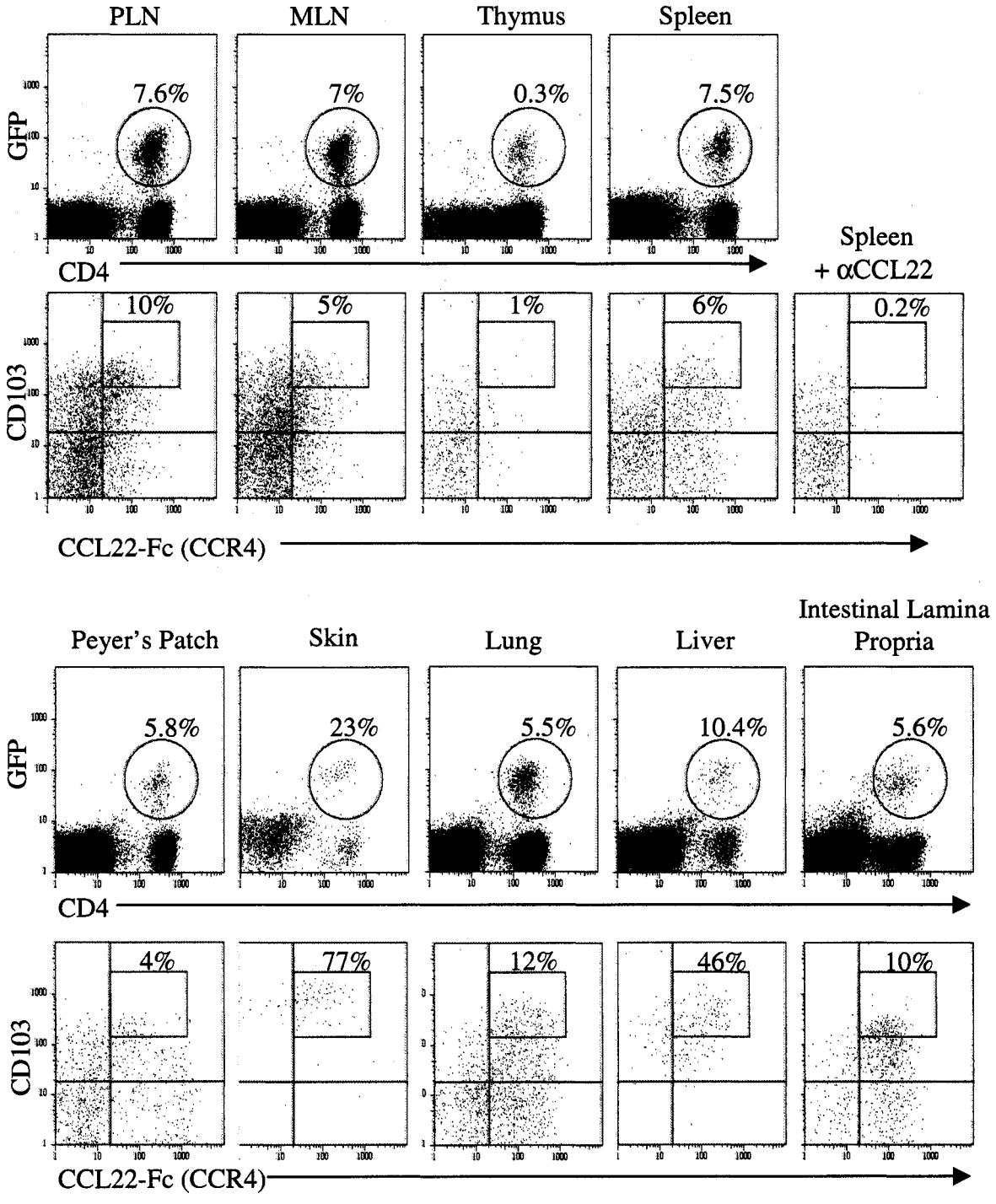
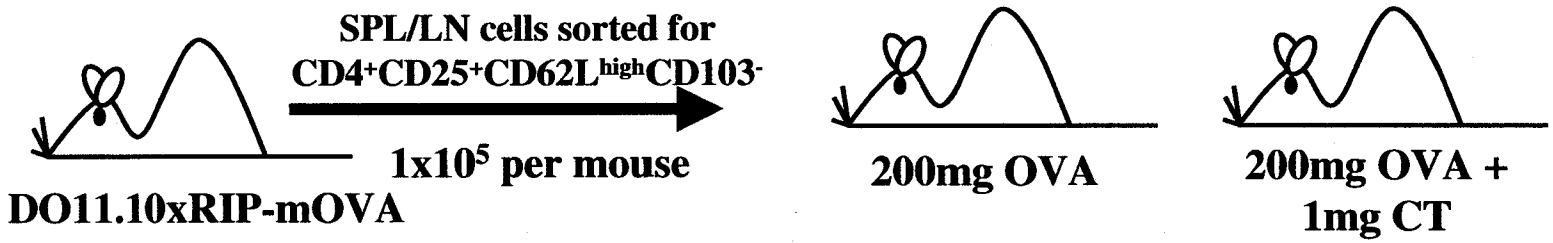

PLN
MLN
Thymus
Spleen
7.6%
7%
0.3%
7.5%
GFP
CD4
Spleen
+ αCCL22
10%
5%
1%
6%
0.2%
CD103
CCL22-Fc (CCR4)
Peyer's Patch
Skin
Lung
Liver
Intestinal Lamina
Propria
5.8%
23%
5.5%
10.4%
5.6%
GFP
CD4
4%
77%
12%
46%
10%
CD103
CCL22-Fc (CCR4)

Figure 6.

Experimental set-up of DO11.10 T_R transfer: (**A**) Schematic of experimental set-up; Naïve $CD4^+CD25^+CD62L^{high}CD103^-$ T_R were isolated from DO11.10xRIP-mOVA mice and $1x10^5$ cells were transferred into wild-type BALB/c mice (**B**) Flow cytometry analysis of CD62L and CD103 expression by gated $CD4^+CD25^+$ cells from the pooled spleen and PLN of a DO11.10xRIP-mOVA mouse before and after sorting of $CD62L^+CD103^-$ cells (left panels). (Right panels) Expression of the DO11.10 clonotypic TCR (KJ1-26), E-selectin ligand, CCR4 and CCR7 by the sorted $CD62L^+CD103^-$ T_R.

A



Harvest DLN 5 days after immunization

B

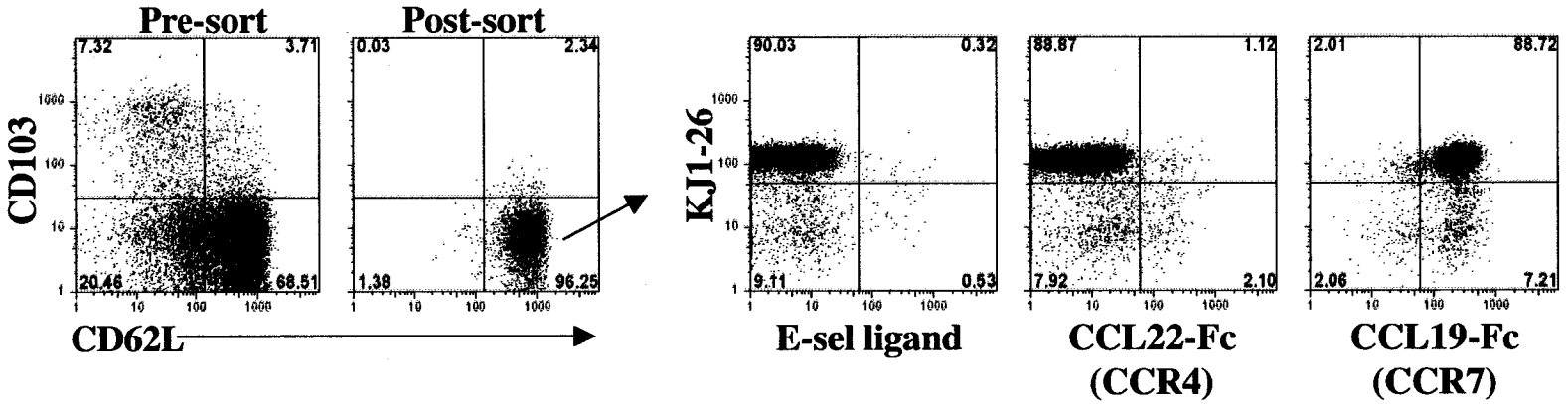
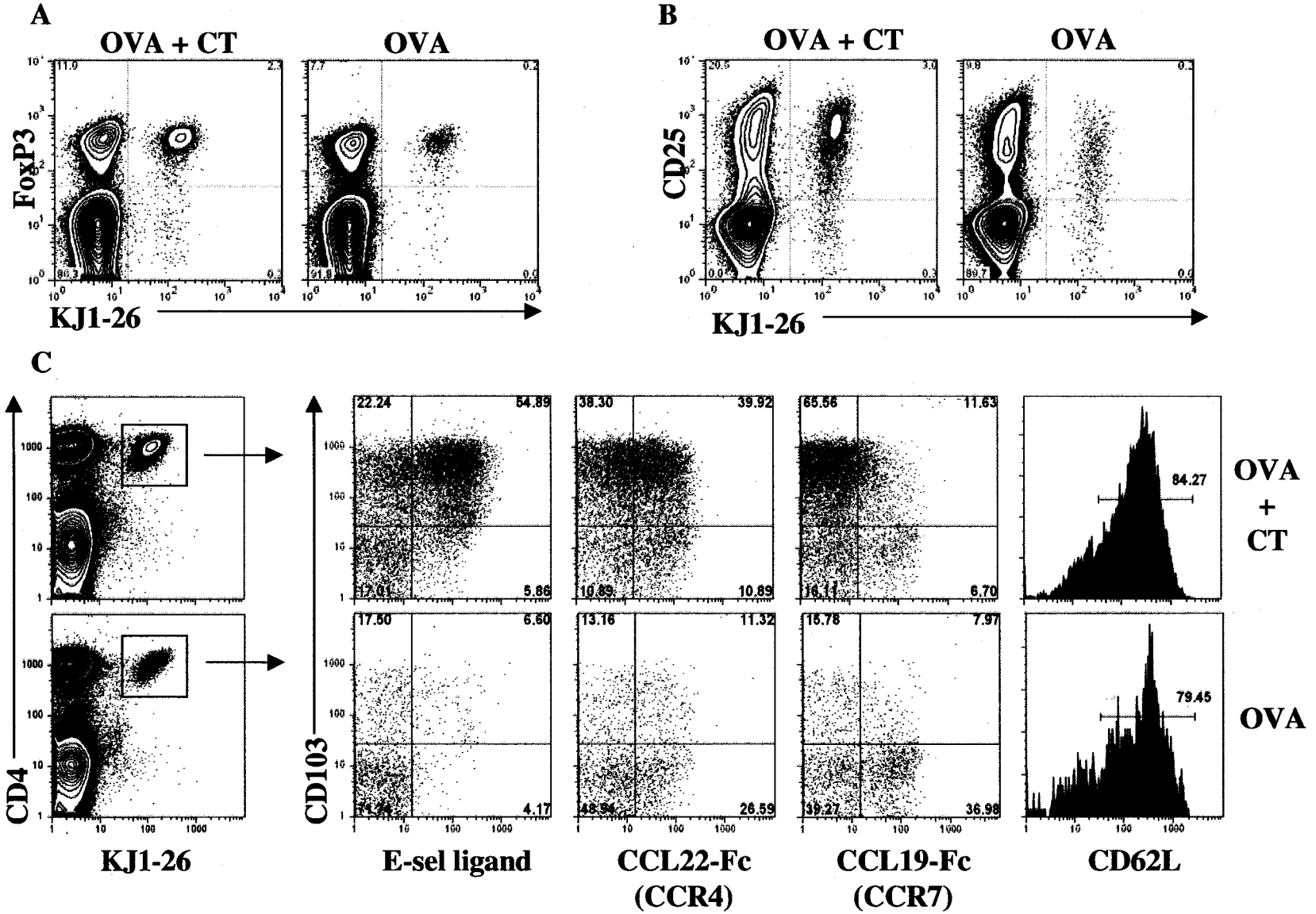


Figure 7.

T_R alter their tissue-tropism following antigen stimulation: Flow cytometry analysis of (**A**) Foxp3 expression and (**B**) CD25 expression on KJ1-26 transferred cells after stimulation with OVA+CT or OVA alone. (**C**) Flow cytometry analysis of lymphocytes from the draining inguinal lymph nodes 5 days after immunization with OVA+CT (top panels) or OVA alone (bottom panels). Left panels show gates used to define the OVA-specific T_R. Right panels show expression of CD103, E-selectin ligand, CCR4 and CCR7 by gated $CD4^+KJ1\text{-}26^+$ cells.


A
OVA + CT
OVA
FoxP3
KJ1-26
B
OVA + CT
OVA
CD25
KJ1-26
C
CD4
KJ1-26
CD103
E-sel ligand
CCL22-Fc
(CCR4)
CCL19-Fc
(CCR7)
CD62L
OVA
+
CT
OVA
84.27
79.45

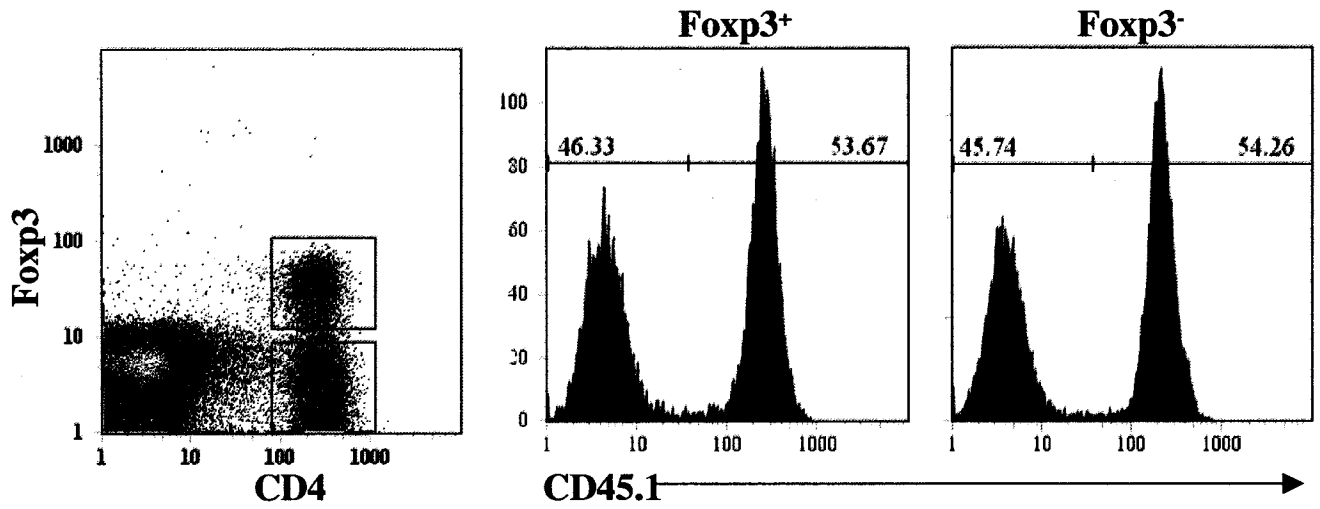
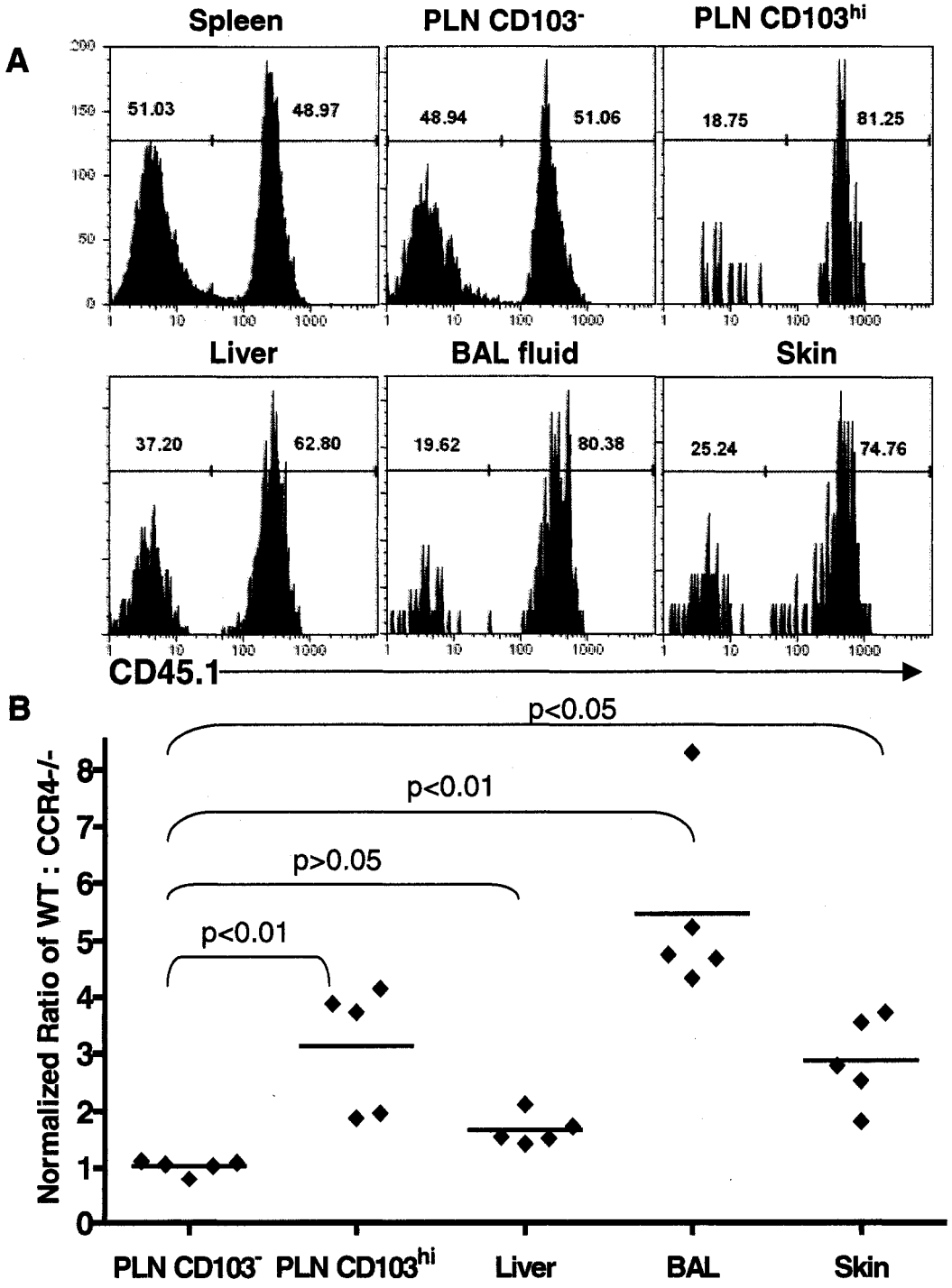


Figure 8.

Normal development of Foxp3+ T_R from both CCR4-deficient donor cells: Lymphocytes were isolated from the spleen of irradiated RAG-$1^{-/-}$ mice that were reconstituted with a 1:1 mix of bone marrow from WT ($CD45.1^+$) and CCR4-deficient ($CD45.1^-$) mice 8 wks prior to analysis. Lymphocytes were stained for CD4, CD45.1 and Foxp3 and the contribution of each donor to the $Foxp3^+$ and $Foxp3^-$ populations was measured.

Figure 9.

Impaired accumulation of CCR4$^{-/-}$ T_R in the skin and lung airways: (**A**) Representative flow cytometry analysis of CD45.1 expression by gated CD4$^+$Foxp3$^+$ cells from the indicated tissues of a WT + CCR4$^{-/-}$ mixed-bone marrow chimera. WT cells are CD45.1$^+$. (**B**) The normalized ratio of WT to CCR4$^{-/-}$ T_R in the indicated tissues/compartments was derived by dividing the ratio of WT:CCR4$^{-/-}$ T_R in each by the ratio of WT:CCR4$^{-/-}$ T_R in the spleen. Each data point represents the normalized ratio from one individual chimera. Horizontal lines indicate the average normalized ratio (n=5) in each tissue/compartment. Statistical analysis was performed using a one-way repeated measures ANOVA (overall p-value<0.0001). p-values for the indicated pairwise comparisons were then computed using Dunnett's Multiple Comparison Test.


A
Spleen
PLN CD103-
PLN CD103hi
51.03
48.97
48.94
51.06
18.75
81.25
Liver
BAL fluid
Skin
37.20
62.80
19.62
80.38
25.24
74.76
CD45.1
B
p<0.05
p<0.01
p>0.05
p<0.01
Normalized Ratio of WT : CCR4-/-
PLN CD103-
PLN CD103hi
Liver
BAL
Skin

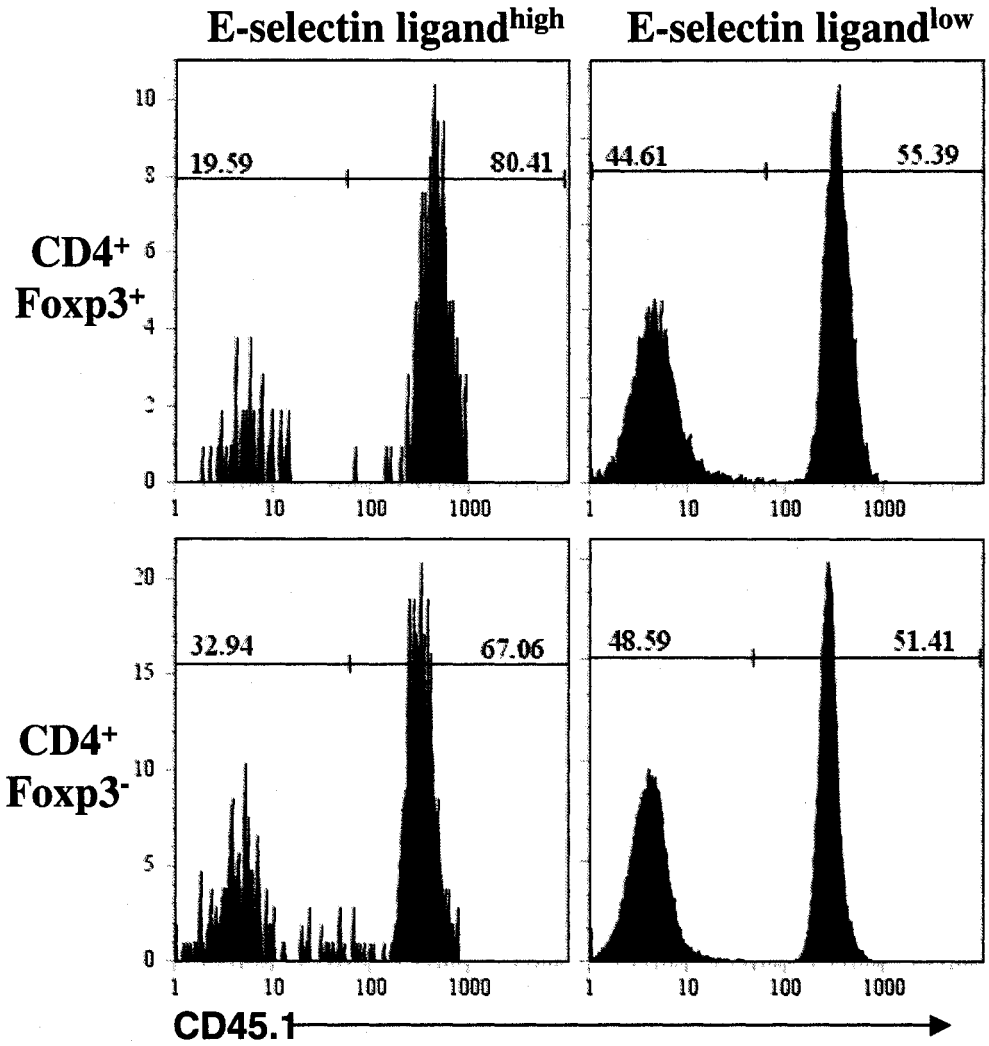


Figure 10.
Impaired development of E selectin ligand^{high} cells developing from CCR4$^{-/-}$ bone marrow:
Representative flow cytometry analysis of CD45.1 expression by gated CD4^{+}FoxP3^{+} and CD4^{+}FoxP3^{-} cells from the PLN of a WT + CCR4$^{-/-}$ mixed-bone marrow chimera. WT cells are CD45.1^{+}.

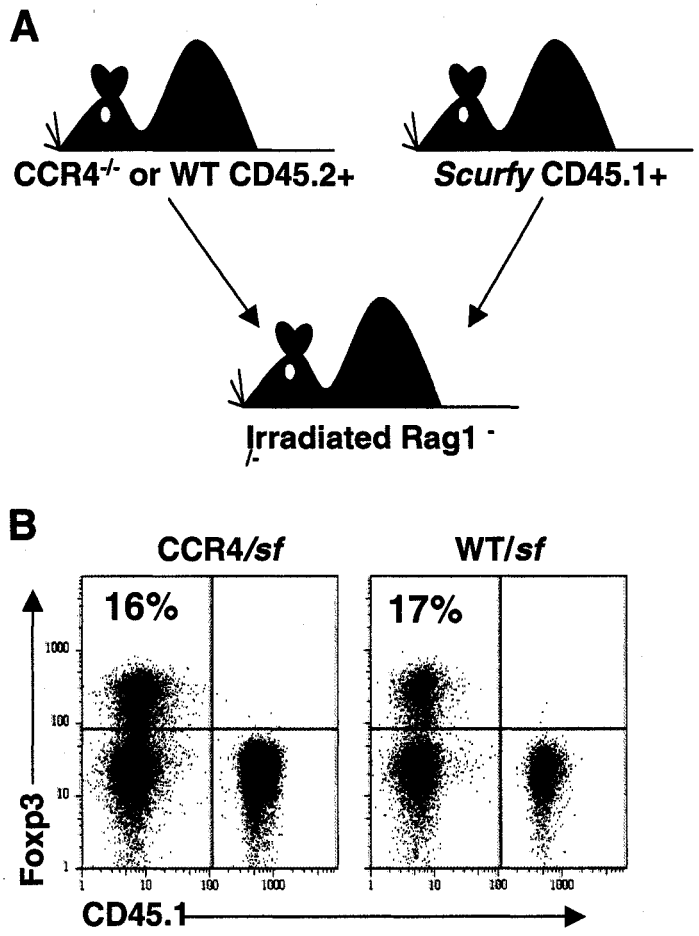


Figure 11.
Schematic of mixed bone marrow chimera experimental set-up and Foxp3⁺ T cell development: (**A**) Mixed bone marrow chimeras were made by transferring a 60:40 mix of CD4-depleted *sf* bone marrow (CD45.1⁺) with either CCR4$^{-/-}$ or WT (both CD45.2⁺) bone marrow into irradiated Rag-1$^{-/-}$ recipients (**B**) Representative flow cytometry analysis of CD45.1 and FoxP3 expression by gated CD4⁺ T cells from the PLN of CCR4/*sf* and WT/*sf* chimeras sacrificed 153 days post-BM transplant.

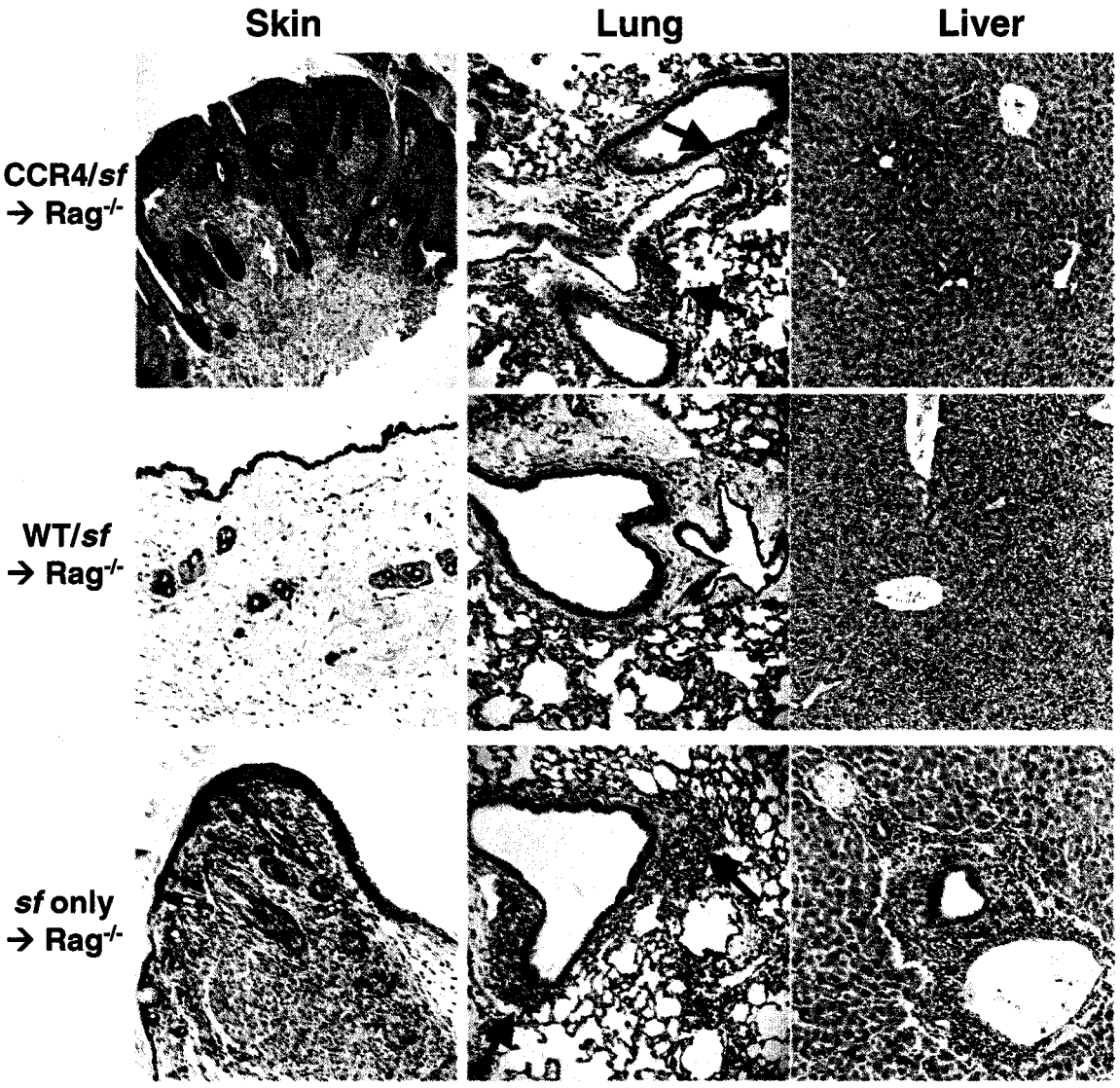


Figure 12.

Inflammatory disease in the skin and lungs of CCR4/sf- chimeras.

Photomicrographs (20x) of hematoxylin and eosin stained sections of the skin, lung and liver from CCR4/*sf*-, WT/*sf*- (sacrificed 114 days post BM transplant) or *sf*- only chimeras- (sacrificed 38 days post BM transplant). Green arrows indicate the location of inflammatory infiltrates in the lungs of CCR4/*sf*- and *sf*-chimeras.

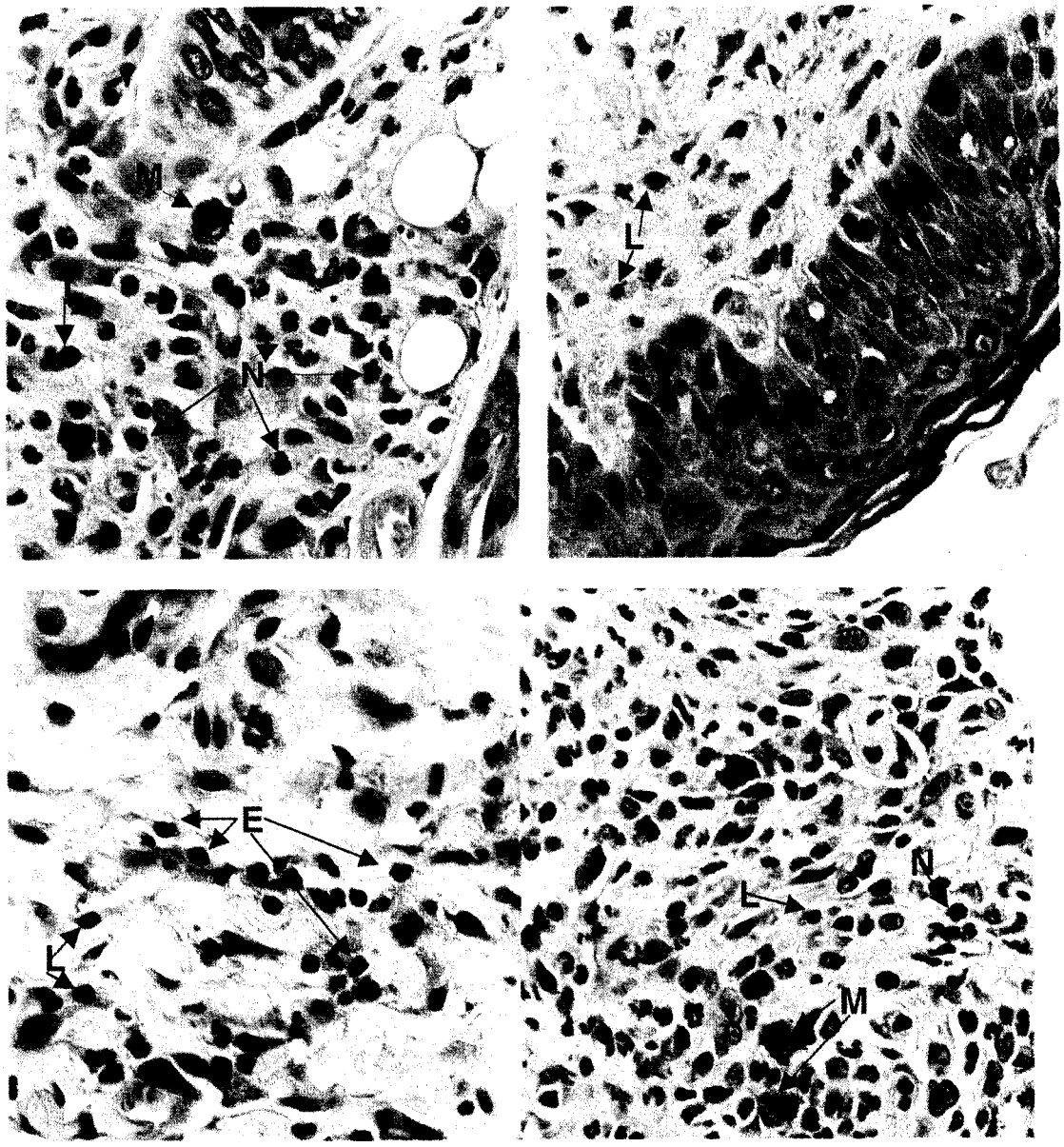


Figure 13.
Dermal infiltrates in CCR4/sf chimeras are composed of neutrophils, eosinophils and mast cells: Photomicrographs (100x) of hematoxylin and eosin stained sections of affected skin from CCR4/sf-chimeras. Green letters and arrows mark representative cell types; L=lymphocytes, N = neutrophils, E = eosinophils and M = mast cells.

Table 1.

Histological Scoring System: Histological examination and scoring was performed on hematoxylin and eosin-stained tissue sections. Each tissue section (lung, liver or skin) was given an inflammation severity score (ISS) and distribution severity score (DSS) for each of the indicated regions as appropriate. For the skin, inflammation in the epidermis and epidermal changes such as hyperplasia, hyperkeratosis, ulceration, erosion or crusting were considered separately. Inflammation sub-scores for each region were calculated by multiplying the ISS and DSS. Finally, a total histological score was then computed by summing the inflammation sub-scores (see Figure 17). Ranges of possible scores for each tissue were as follows: Lung=0 to 100, Liver=0 to 80, Skin=0 to 60.

Tissue	Regions analyzed	Inflammation Type Epidermal Changes (Skin)	Inflammation Severity Score (ISS)	Distribution Severity Score (DSS)
Lung	Perivascular Peribronchiolar/peribronchial Subpleural Alveolar Intrabronchial/bronchiolar	Lymphohistiocytic Lymphoplasmacytic Histiocytic/ Histiocytosis Acidophilic macrophages Lymphoid aggregates	0- None, normal 1- Minimal 2- Mild 3- Moderate 4-Marked	1-Focal 2-Focally extensive 3-Multifocal 4-Coalescing 5-Diffuse
Liver	Centrilobular Periportal Midzonal Random	Lymphohistiocytic Lymphoplasmacytic Histiocytic Lymphoid aggregates Microgranuloma	0- None, normal 1- Minimal 2- Mild 3- Moderate 4-Marked	1-Focal 2-Focally extensive 3-Multifocal 4-Coalescing 5-Diffuse
Skin	Dermal Epidermal inflammation Epidermal changes	Lymphohistiocytic Lymphoplasmacytic Histiocytic Granulocytic Lymphoid aggregates Epidermal changes: Hyperplasia Hyperkeratosis Ulceration Erosion Crust	0- None, normal 1- Minimal 2- Mild 3- Moderate 4-Marked	1-Focal 2-Focally extensive 3-Multifocal 4-Coalescing 5-Diffuse

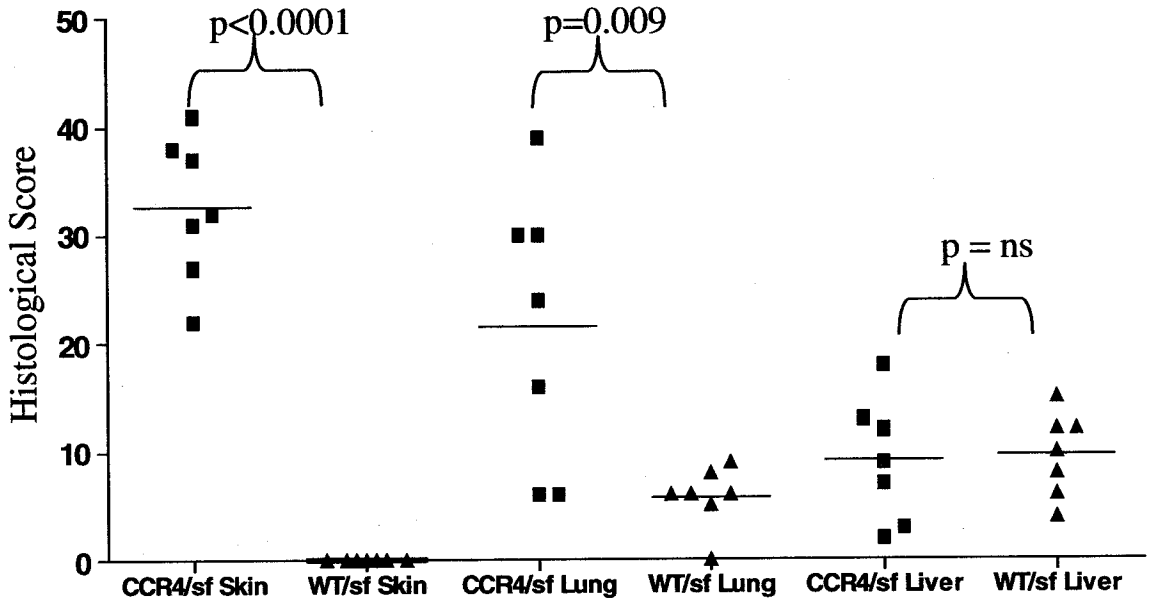
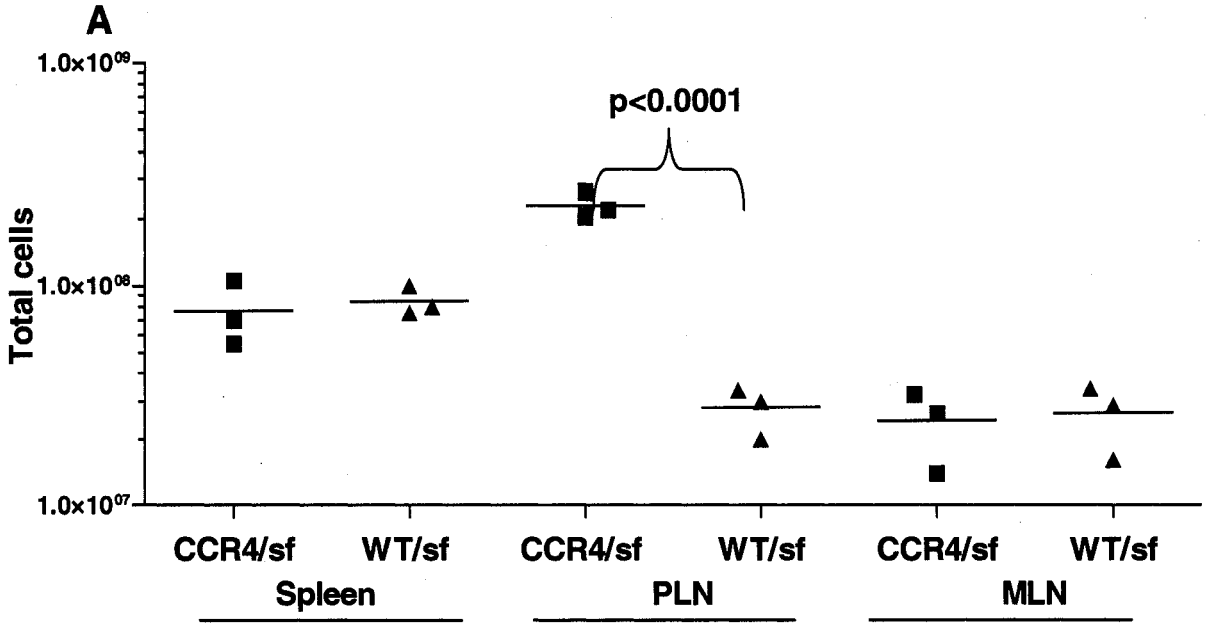


Figure 14.

Blinded analysis of tissue sections from 7 pairs of CCR4/sf- and WT/sf-chimeras: All mice in blinded analysis were sacrificed between 114-250 days post BM transplant. Each section was scored based on the severity and extent of inflammation. Statistical analysis was performed using two-tailed, paired student-t test. ns, not significantly different ($p>0.05$).

Figure 15.
Peripheral lymphadenopathy and enhanced T_{eff} differentiation in CCR4/sf-chimeras: (**A**) Lymphocytes were isolated and counted from the spleen, subcutaneous peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) of a matched group of 3 CCR4/*sf*-chimeras (squares) and 3 WT/*sf*-chimeras (triangles) sacrificed 140 days post -BM transplant. Data are representative of 6 experiments. Statistical analysis was performed using a two-tailed, unpaired student-*t* test. (**B**) Representative flow cytometry analysis of CD44 and CD45RB expression by gated $CD4^+CD45.1^+$ *sf*-derived T cells from the PLN of WT/*sf*- and CCR4/*sf*-chimeras sacrificed 140 days post -BM transplant. Graph on right shows the frequency of $CD44^{hi}$ cells among gated $CD4^+CD45.1^+$ PLN cells from 9 matched pairs of WT/*sf*- and CCR4/*sf*-chimeras (all sacrificed between 114-250 days post BM transplant). Statistical analysis was performed using a two-tailed, paired student-*t* test.


A
1.0x10^09
1.0x10^08
1.0x10^07
Total cells
p<0.0001
CCR4/sf
WT/sf
CCR4/sf
WT/sf
CCR4/sf
WT/sf
Spleen
PLN
MLN

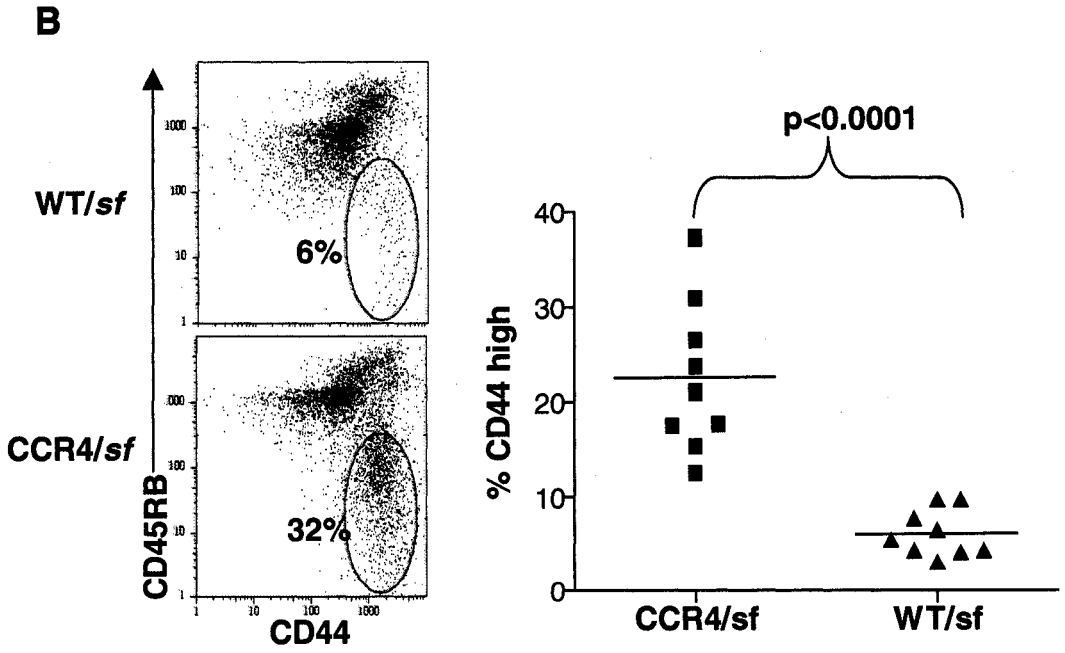

B
WT/sf
CCR4/sf
6%
32%
CD45RB
CD44
p<0.0001
% CD44 high
40
30
20
10
0
CCR4/sf
WT/sf

Figure 16.

CCR4/sf chimeras have an elevated frequency of skin-tropic T_{eff}*:* (Top) Representative flow cytometry analysis of CCR4, P-selectin ligand, and E-selectin ligand expression by gated $CD4^+CD45.1^+$ *sf*-derived T cells from the PLN of WT/*sf*- and CCR4/*sf*-chimeras (open histograms) sacrificed 140 days post-BM transplant. Shaded histograms indicate background staining in the presence of αCCL22 (left) or 10mM EDTA (middle and right). (Bottom) Graphs indicating the frequency of gated $CD4^+CD45.1^+$ cells expressing the indicated receptor in the PLN of 5 matched pairs of WT/*sf*- and CCR4/*sf*-chimeras (all sacrificed between 114-250 days post BM transplant). Statistical analysis was performed using a two-tailed, paired student-*t* test.

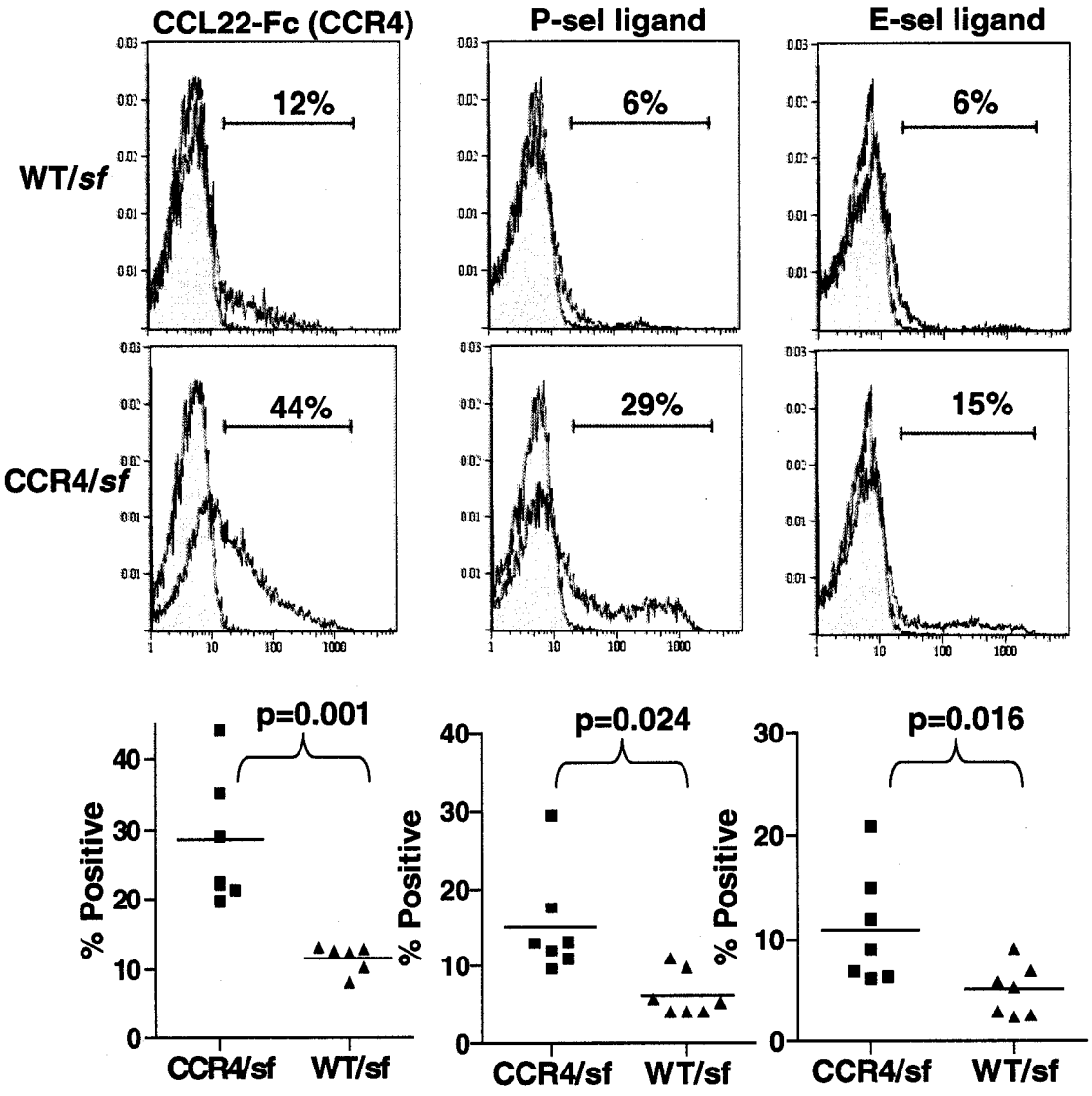

CCL22-Fc (CCR4)
P-sel ligand
E-sel ligand
WT/sf
CCR4/sf
12%
6%
6%
44%
29%
15%
p=0.001
p=0.024
p=0.016
% Positive
CCR4/sf
WT/sf

Figure 17.

sf-derived CD4⁺ T cells accumulate in the skin and lungs of CCR4/sf-chimeras: Representative flow cytometry analysis of CD45.1 expression by gated CD4⁺ T cells isolated from the indicated tissues of two pairs of WT/*sf*- and CCR4/*sf*-chimeras. *sf*-derived cells are CD45.1⁺. Data are representative of >8 mice analyzed in each group.

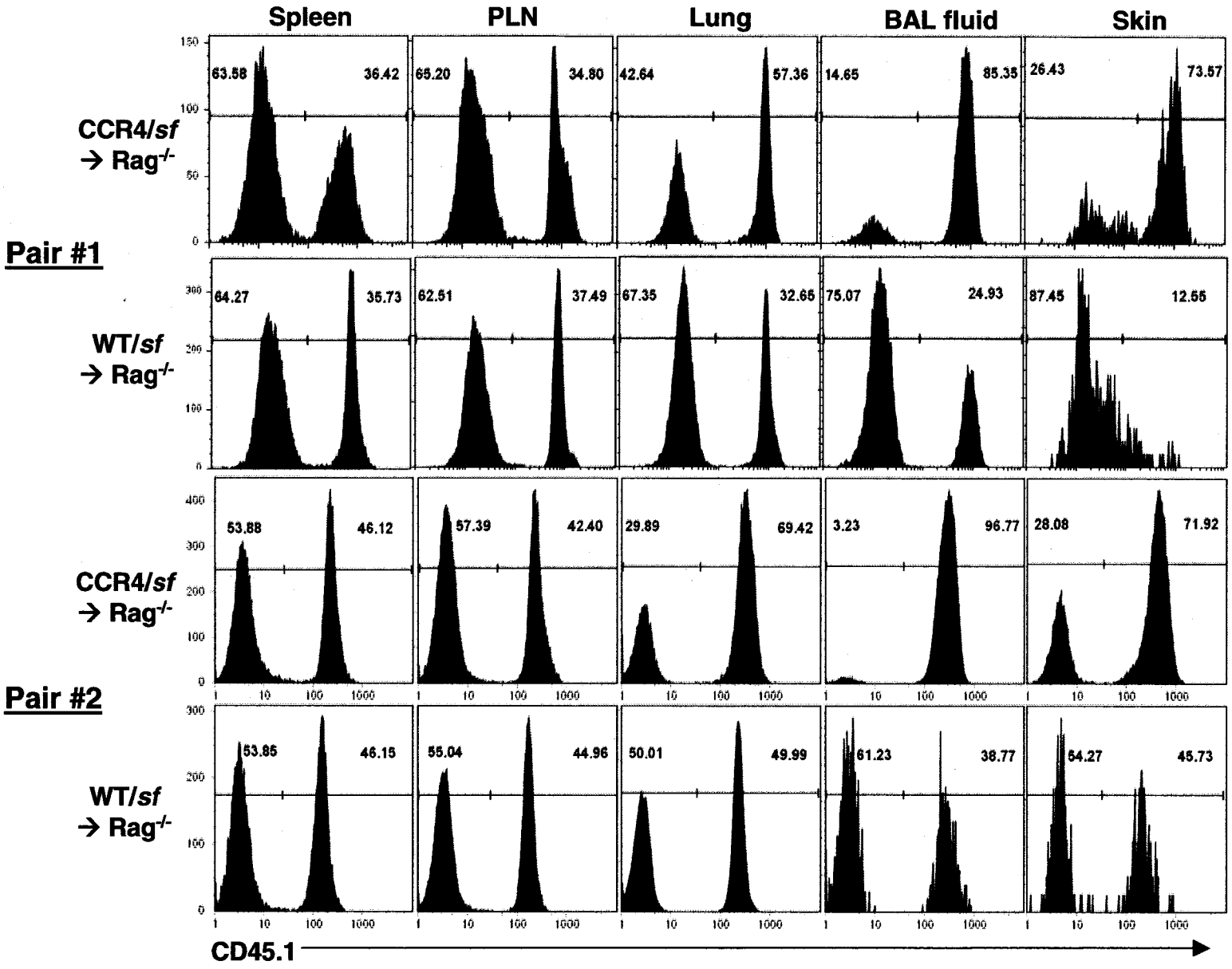

Spleen
PLN
Lung
BAL fluid
Skin
Pair #1
CCR4/sf → Rag-/-
63.58
36.42
65.20
34.80
42.64
57.36
14.65
85.35
26.43
73.57
WT/sf → Rag-/-
64.27
35.73
62.51
37.49
67.35
32.65
75.07
24.93
87.45
12.55
Pair #2
CCR4/sf → Rag-/-
53.88
46.12
57.39
42.40
29.89
69.42
3.23
96.77
28.08
71.92
WT/sf → Rag-/-
53.85
46.15
55.04
44.96
50.01
49.99
61.23
38.77
54.27
45.73
CD45.1

Figure 18.
Accumulation of sf-derived CD4$^+$ T cells was comparable in affected and unaffected skin: Lymphocytes were isolated from the spleen, unaffected skin and affected skin of two CCR4/*sf* mixed bone marrow chimeras (both between 110 and 250 days post-BM transfer). Cells were stained with monoclonal antibodies for CD4 and CD45.1. All plots were gated on the total live CD4$^+$ lymphocytes. CD45.1$^-$ cells represent cells derived from the non-*sf* bone marrow and CD45.1$^+$ cells are *sf* derived.

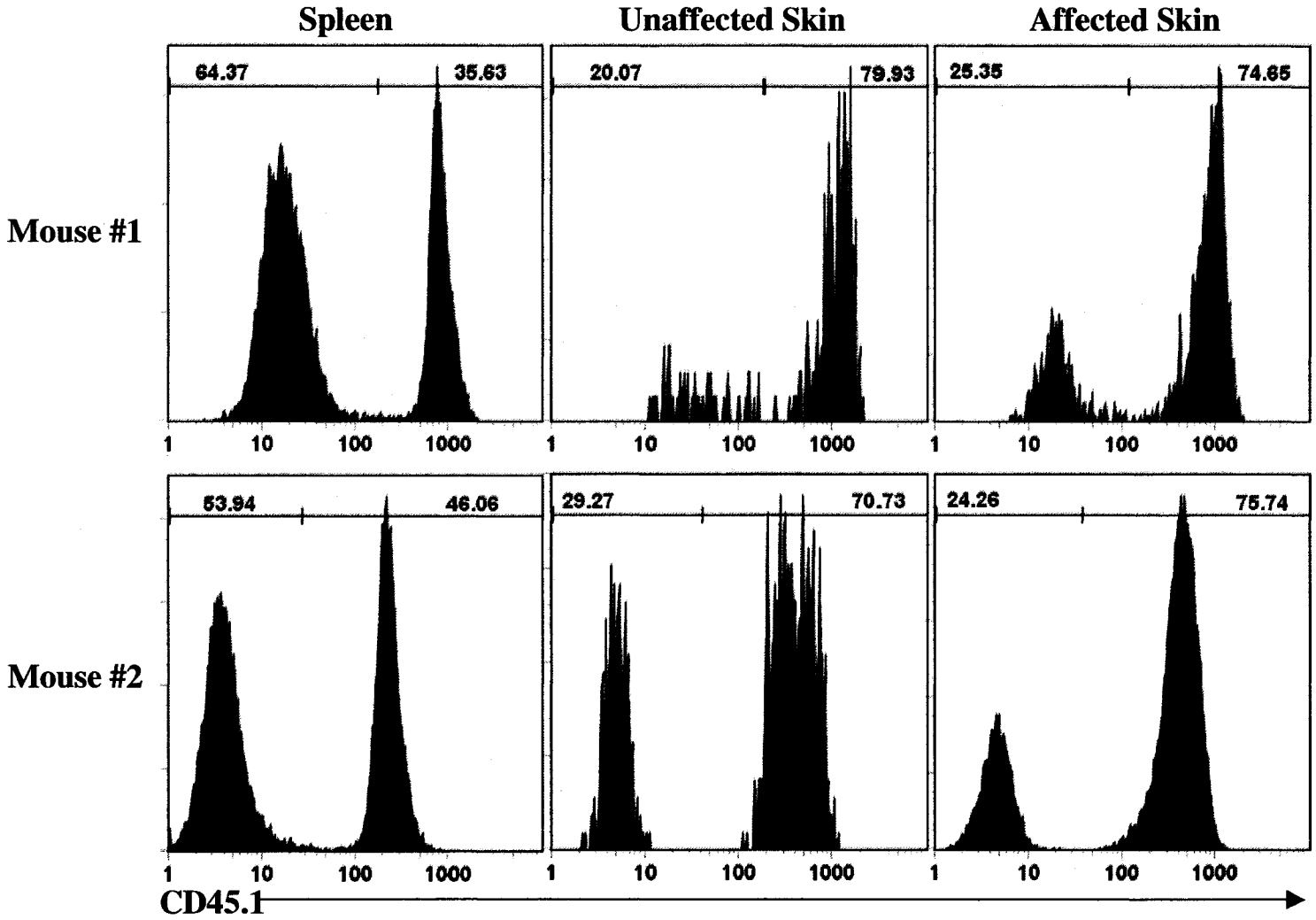

Spleen
Unaffected Skin
Affected Skin
Mouse #1
Mouse #2
64.37
35.63
20.07
79.93
25.35
74.65
53.94
46.06
29.27
70.73
24.26
75.74
1
10
100
1000
CD45.1

Figure 19.

Reduction in Foxp3$^+$ T_R in the skin and lung airways of CCR4/sf chimeras: Lymphocytes were isolated from the bronchiolar lavage fluid (BAL) or skin of two pairs of CCR4/*sf* and WT/*sf* mixed bone marrow chimeras (both between 100 and 250 days post-BM transfer). Cells were stained with monoclonal antibodies for CD4, CD45.1 and Foxp3. All plots were gated on the total live CD4$^+$ lymphocytes . CD45.1$^-$ cells represent cells derived from the non-*sf* bone marrow and CD45.1$^+$ cells are *sf*-derived.

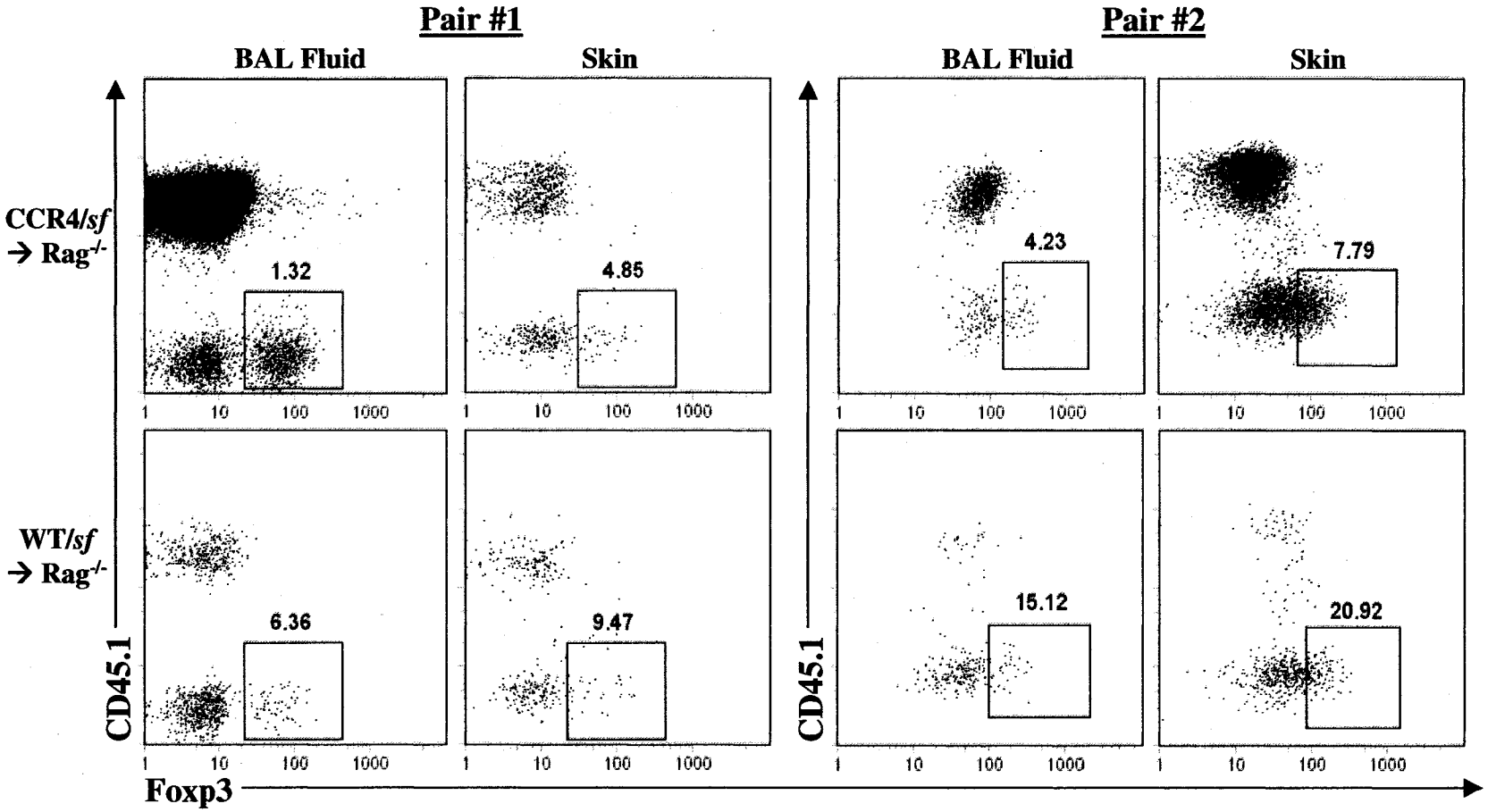

Pair #1
Pair #2
BAL Fluid
Skin
BAL Fluid
Skin
CCR4/sf → Rag-/-
WT/sf → Rag-/-
1.32
4.85
4.23
7.79
6.36
9.47
15.12
20.92
1
10
100
1000
CD45.1
Foxp3

Figure 20.

CCR4$^{-/-}$ T_R fail to prevent cutaneous and pulmonary inflammation following transfer into neonatal sf mice: Photomicrographs (20x) of hematoxylin and eosin stained sections of skin, lung and liver from either an unmanipulated *sf* mouse (bottom panels) or from *sf* mice given purified WT or CCR4$^{-/-}$ CD4^{+}CD25^{+} T_R (top and middle panels) shortly after birth. Analysis of unmanipulated *sf* mice done when mice were 25 days old and transferred *sf* mice were 70 days old. Data are representative of >8 mice analyzed in each group.

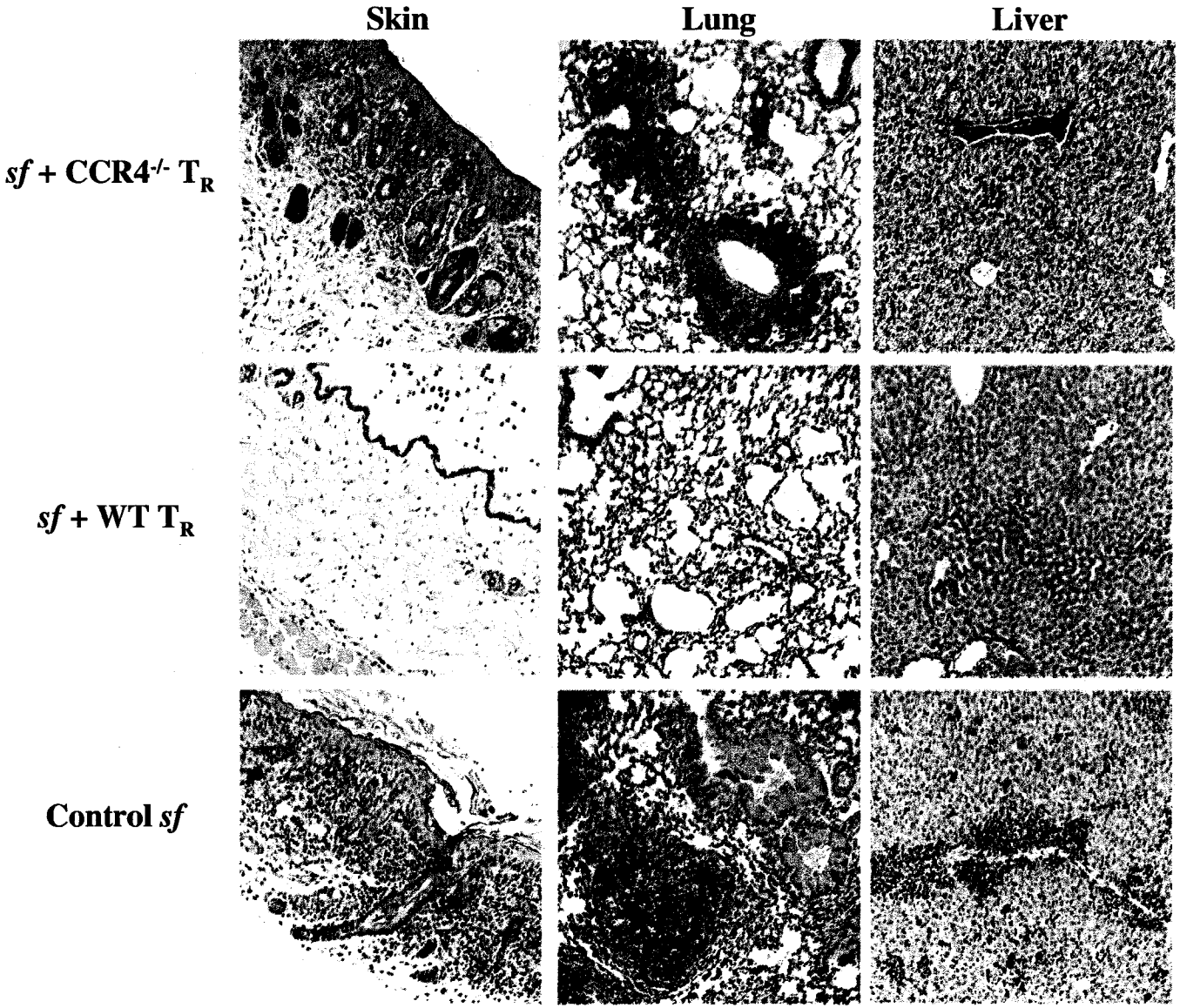
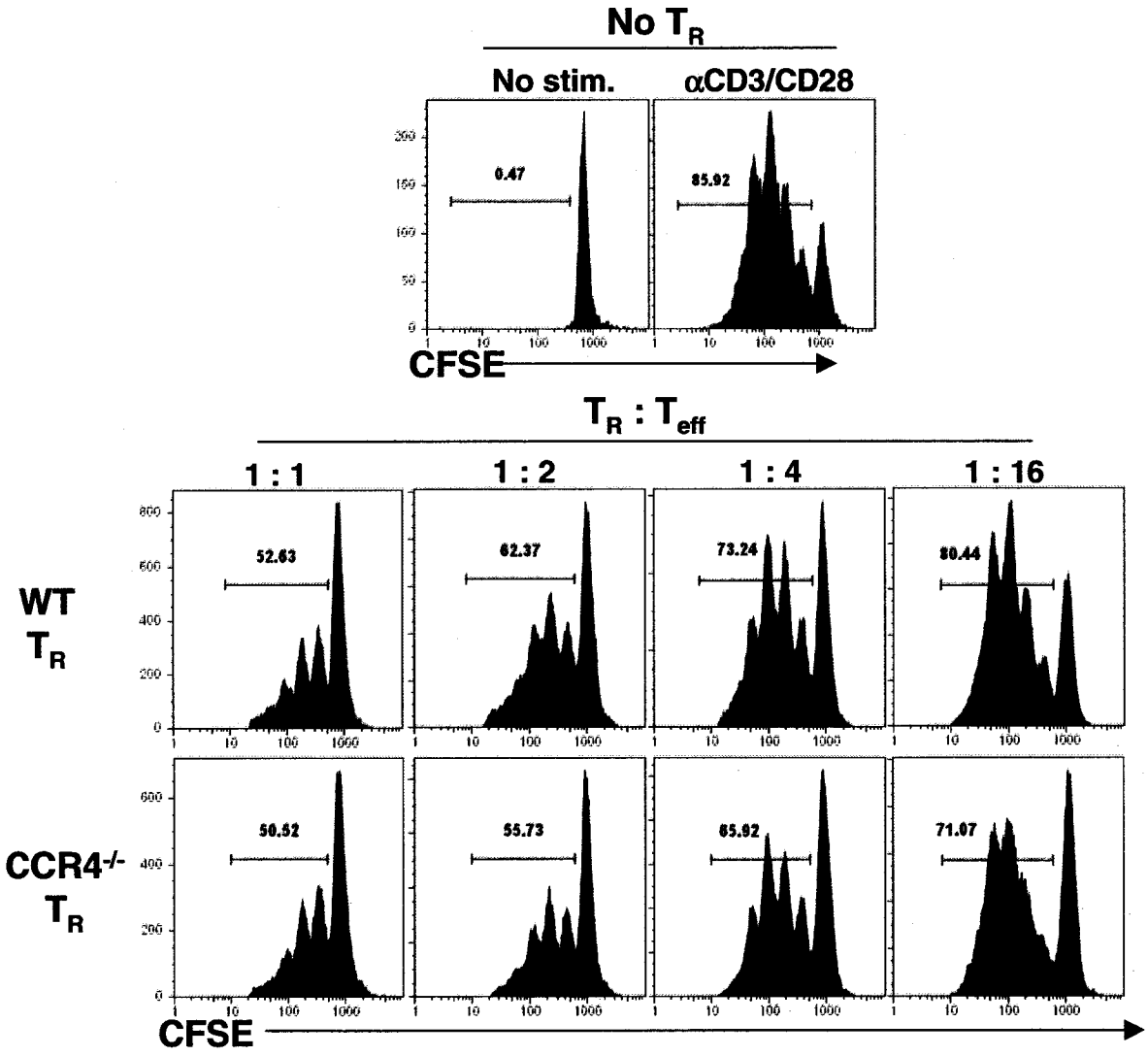

Skin
Lung
Liver
sf + CCR4-/- TR
sf + WT TR
Control sf

Figure 21.

CCR4$^{-/-}$ T_R function normally in vitro: Flow cytometry analysis showing the proliferation of CFSE-labeled WT T_{eff} following stimulation with αCD3 and αCD28 monoclonal antibodies for 110 hours. Relative CFSE dilution was measured on cells co-cultured with the indicated ratio of either WT (upper panels) or CCR4$^{-/-}$ (lower panels) T_R. Left panels indicate the extent of proliferation with and without αCD3/αCD28 treatment in the absence of T_R.


No T_R
No stim.
αCD3/CD28
0.47
85.92
CFSE
T_R : T_{eff}
1 : 1
1 : 2
1 : 4
1 : 16
WT T_R
52.63
62.37
73.24
80.44
CCR4$^{-/-}$ T_R
50.52
55.73
65.92
71.07
CFSE

Chapter 3: The Role of CCR4 in T_R Homeostasis

Introduction

CCR4 clearly plays a role in the proper accumulation of T_R to the skin and lung, and likely is important for targeting subsets of T_R to APCs expressing CCL17 and CCL22. Interactions with APCs and other non-T_R cells are vital in controlling tissue-specific immune responses. In the absence of an ongoing immune response, these interactions may be essential to T_R homeostasis. As I mentioned in Chapter 1, there are several signals that T_R must receive for proper peripheral homeostasis. Consequently, the proper the placement of T_R in the vicinity of lymphocytes, APCs of a particular activation state and cellular environments producing homeostatic signals is critical to their survival.

Extensive research has been performed to understand the signals necessary for the expansion of T_R, both *in vitro* and *in vivo*. Clearly IL-2 is important for maintaining T_R peripheral homeostasis *in vivo* (43,44), and can also be used to expand T_R *in vitro* in conjunction with strong TCR stimulation (27). *In vivo* IL-2 comes from surrounding non-T_R $CD4^+$ T cells. Thus, for proper homeostatic expansion (33) T_R would presumably need to be near these cells while undergoing homeostatic expansion. Non-T_R $CD4^+$ cells produce IL-2 in response to TCR stimulation and co-stimulatory signals such as CD28 and CD80/86 provided by APCs. Therefore, all three populations of cells, APC, T_R and T_{eff}, need to be in close proximity for IL-2-induced T_R homeostatic expansion, as well as T_R-mediated suppression.

In addition to IL-2, TGFβ may also be critical to T_R homeostasis, as I discussed in Chapter 1. The development of immature DC is promoted by TGFβ and these cells also produce it, whereas mature DCs do not make measurable amounts of TGFβ{623}. Given that the stimulation of naïve T cells with immature DCs results in T cells with an anergic phenotype and suppressive properties, TGFβ production by immature DC may promote the homeostasis of T_R. In addition to TGFβ, immature DC in the LN also make CCR4 ligand CCL17 (134) and we, and others, have shown that a large percentage of T_R in express CCR4 (110,111,129). Therefore, chemotaxis of T_R to immature DC via CCR4-CCL17 interactions may be necessary to promote T_R homeostasis via TGFβ and other signals delivered by immature DCs.

To test whether expression of CCR4 by T_R mediates efficient homeostasis, the ability of CCR4$^{-/-}$ T_R to undergo homeostatic expansion needs to be tested in direct competition CCR4-sufficient T_R. To do this, I utilized neonatal *sf* rescue model that was described in chapter 2 (Figure 20) To establish a competitive situation for homeostatic signals, I transferred CCR4$^{-/-}$ T_R at a 1 to 1 ratio with WT T_R. I demonstrated that early in homeostatic expansion, the CCR4$^{-/-}$ T_R are able to expand and fill the "T_R niche" at a similar rate as WT. However, over time they were not able to sustain themselves efficiently and were eventually out-competed by WT T_R. The mechanism of this seems to be mediated by proliferation rates and/or survival during the maintenance phase of homeostasis as WT T_R incorporate BRDU at a higher level than CCR4$^{-/-}$ T_R. These data suggest that during ongoing T_R

homeostasis, CCR4 expression by T_R is necessary for their proliferation and/or survival in the periphery.

Results

CCR4-deficient T_R expand at the same rate as WT T_R

To directly compare the homeostatic proliferative potential of CCR4$^{-/-}$ T_R with WT T_R, I isolated CD4$^+$CD25$^+$ T_R from CCR4$^{-/-}$ (CD45.2) and WT (CD45.1) mice, labeled with CFSE to measure their proliferation and transferred them into 3-day-old neonatal *sf* mice (CD45.1/CD45.2 heterozygotes) (Fig. 22a). After four days, the mice were sacrificed and the percentage of donor T cells was measured, as well as their proliferation in the spleen and the LN. I found that at this early time-point, the number of WT T_R remaining was slightly higher than CCR4$^{-/-}$ T_R in both the spleen and LN (Fig. 22b). Interestingly, when I measure the proliferation of the two populations, the CCR4$^{-/-}$ cells seemed to be proliferating at a higher level then the WT in both the spleen and the LN (Fig. 23). Among the CCR4$^{-/-}$ donor population, there were a higher number of cells that had undergone any divisions at all (Fig.23a), as well as more cells that had undergone multiple divisions (Fig.23b), when compared to the WT donor cells. Though the difference was small, the result was reproducible. Taken together, this data suggests that CCR4$^{-/-}$ T_R can respond to homeostatic signals and undergo the initial expansion to fill the "T_R niche" when in competition with WT. However, the fact that fewer CCR4$^{-/-}$ donor cells were present when compared to WT after 4 days suggest they may not be efficiently receiving survival signals from their environment.

CCR4-deficicient T_R are out-competed by WT T_R during homeostasis

Even though CCR4$^{-/-}$ T_R were present at slightly lower numbers when compared to WT after 4 days of homeostatic expansion in competition, the difference was quite small. Clearly CCR4$^{-/-}$ T_R can expand and, when transferred alone, they do initially rescue *sf* mice from their disease. Therefore, they may have more of a defect in homeostatic maintenance than in their initial expansion. To test this possibility, I transferred CCR4$^{-/-}$ and WT T_R at a 1 to 1 ratio into male *sf* mice (in the same manner Fig. 22&23) and followed the levels of each donor population over time in their PBL. By weaning age, the numbers of donor T_R from both the WT and CCR4$^{-/-}$ donor were almost equal, with a slight advantage for the WT T_R, similar to what I had seen in the mice I analyzed at the early time-point. Interestingly, the percentage of T_R that were CCR4$^{-/-}$ derived declined over time and eventually the majority of the donor T_R were WT derived (Figure 24). This typically occurred between 40 and 60 days post-transfer, though there was some variability in the timing of decline. These data suggest that CCR4$^{-/-}$ T_R are less efficient at homeostatic maintenance when in competition with WT T_R.

CCR4-deficient T_R do not cycle at the same rate as WT T_R during homeostatic maintenance

The CCR4$^{-/-}$ T_R were able to expand at close to the same rate as WT T_R when the two populations expanding to fill the "T_R niche", but CCR4$^{-/-}$ are eventually outcompeted, suggesting they may have a reduced ability to divide homeostatically when compared to WT T_R. To test this possibility, CCR4$^{-/-}$ and

WT T_R were transferred into neonatal *sf* mice in the same manner as described previously (Fig. 22-24) and their level of proliferation was measured while undergoing homeostasis via Bromodeoxyuridine (BrdU). BrdU is a synthetic thymidine analog that gets incorporated into a cell's DNA when the cell is dividing (159). BrdU can be added to a mouse's drinking water and cells that are cycling will readily incorporate it into their DNA. Antibodies against BrdU that are conjugated to fluorescent markers can be used to label these cells, thereby providing visual evidence of cell division. Since most of the mice I had tested showed the CCR4$^{-/-}$ T_R being out-competed by WT T_R between 40 and 70 days post-transfer, I added BrdU to the drinking water for several days during this time window to a group of *sf* mice that had been neonatally rescued with a mix of CCR4$^{-/-}$ and WT T_R. Since I was unsure when CCR4-/- T_R began their decline, I tested two time-points within this window. The first pair of mice that I tested were given BrdU in their water at 60 days and then the level of incorporation was measured after four days and the second group of four mice was tested a bit earlier at 40 days and bled after 5 days. Strikingly, I found that in all mice tested at both time-points, a higher percentage of WT-derived donor T_R stained positive for BrdU, in comparison with the CCR4$^{-/-}$ derived T_R (Fig.25). There are several possible interpretations of this data. WT cells could be undergoing cell division at a higher rate than CCR4$^{-/-}$ T_R, which in turn would result in the WT T_R eventually out-competing the CCR4$^{-/-}$ T_R during ongoing homeostasis. Conversely, CCR4$^{-/-}$ T_R may undergo cell division at a similar rate as WT T_R, but may die at a higher rate

after division, resulting in fewer $BrdU^+$ $CCR4^{-/-}$ derived T_R. Finally, its possible that $CCR4^{-/-}$ T_R may cycle slower and die at a faster rate than WT T_R. Additional experiments to measure cell death via Annexin V staining and BrdU incorporation are needed to delineate this answer, but experimental restraints on the number of fluorochromes necessary to perform this experiment have not allowed me to answer this question as of the writing of this thesis. Future work will likely address this question.

Discussion

In this portion of my thesis work, I explored whether CCR4 expression by T_R was important for their homeostasis, in addition to their tissue-specific localization. In my earliest neonatal *sf* transfer experiments, when I used a minimal number of $CCR4^{-/-}$ T_R, fewer donor T_R were present in the rescued adult *sf* mice, when compared to mice transferred with the same number of WT donor cells. Though I did not see this difference when I increased the number of donor T_R transferred, I hypothesized that this earlier result may be due to the inefficient expansion and homeostasis of $CCR4^{-/-}$ T_R. By following $CCR4^{-/-}$ T_R expanding in direct competition with WT T_R, I found that, though $CCR4^{-/-}$ T_R may expand at a slightly faster rate than WT T_R, $CCR4^{-/-}$ T_R were outcompeted by WT T_R during on-going homeostasis. Additionally, incorporation of BrdU by WT T_R during homoeostatic maintenance was higher than $CCR4^{-/-}$ T_R at all time-points tested, suggesting they were outcompeted during this later phase due to the lack of specific signals mediating their turn-over rate, survival or both.

Though the data from this group of experiments is preliminary, it suggests that CCR4 expression by T_R may be important for their localization to cells that deliver homeostatic signals. Clearly the signals they receive from APC can contribute in different ways, depending on the context. Previous data showing the differential expression of CCR4 ligands by immature or mature DC (132,135,151) suggests that T_R CCR4 expression may mediate the interaction with specific DC subsets more efficiently then others. Additionally, the data demonstrating that T_R undergo their initial expansion primarily in peripheral lymph nodes (PLN) (44), in conjunction with the high expression of the CCR4 ligand CCL17 by immature $CD11c^+$ DC(134) in the PLN implies that CCR4-CCL17-mediated microlocalization of T_R with immature DC in the LN may be one initiator of T_R homeostasis. It is possible that the CCR4 signal itself mediates the transcription of particular T_R homeostatic programs, but this possibility remains unclear and requires further research.

To fully understand the role of CCR4 in T_R homeostasis, it's important to determine which DC populations are interacting with T_R during this process. Recent work in a cardiac transplant model suggested that $CCR4^+Foxp3^+$ cells were induced by plasmacytoid DC (pDC), and removal of this population or restriction of homing of pDC to the PLN resulted in a loss of $CCR4^+T_R$ and a loss of graft tolerance(160). Though this was an induced model, these data may indicate that the pDC population is necessary for the expansion of $CCR4^+$ T_R. Further study of the activation state of these pDC during the expansion of $CCR4^+T_R$, as well as the

signals that they mediate during tolerance induction are needed to clarify whether pDC are also important in ongoing T_R homeostasis, as well as for to graft tolerance.

The preliminary data in this chapter opens several avenues of research about the importance of CCR4 expression by T_R during homeostasis. It is essential to show that the inefficient homeostasis of $CCR4^{-/-}$ T_R is due to a reduced interaction with APC and not an inefficient signal from some other cell in the lymphoid environment. It is possible that CCR4 ligands produced by endothelial cells interact with CCR4 on the T_R surface and induce changes in T_R adhesion molecules, allowing them to more stably adhere to specific areas of the lymphoid environment. Or conversely, these CCR4-ligand interactions could induce the up-regulation of specific co-stimulatory receptors that are important for their homeostasis. The answers to these questions, and others, will be critical to fully understand why CCR4 expression by T_R seems to be important for their homeostasis.

Materials and Methods

Animals

$CD45.1^+$ B6.SJL mice (B6.SJL-*Ptprc*^{a}/BoyAiTac) were purchased from Taconic Farms (Germantown, NY). *Scurfy* mice (B6.Cg-Foxp3^{sf}/J) were obtained from Jackson Laboratories and crossed to B6.SJL mice to generate $CD45.1^+$ animals. $CCR4^{-/-}$ mice on the C57BL/6 genetic background were obtained from Dr. Steve Ziegler (Benaroya Research Institute, Seattle, WA). All animals were housed and bred under specific pathogen-free conditions in the Benaroya Research Institute animal facility. All experiments were approved by the Benaroya Research Institute

Institutional Animal Care and Use Committee.

Lymphocyte isolation

Single cell suspensions for T_R isolation were prepared from pooled spleen, peripheral (pooled inguinal, axillary, brachial and superficial cervical nodes) and mesenteric lymph nodes by tissue disruption with glass slides, and filtered thru a 40mM filter. PBL were obtained via saphonious vein blood collections. In all cell suspensions, red blood cells were lysed using ACK lysis buffer.

Flow cytometry

For cell-surface staining, 10^6 cells per sample were incubated with various antibodies in staining buffer (HBSS and 3% FCS) for 20 minutes on ice. Anti-murine antibodies included: anti-CD4 (RM4-5), anti-CD45.1 (A20) and anti-CD45.2 (104), from eBioscience, (San Diego, CA). FoxP3 expression was assessed by staining with anti-Foxp3 (FJK-16s, eBioscience) according to the manufacturer's protocol. For CFSE labeling, T_R were incubated for 9 min at 37°C in 0.8 μM CFSE (Invitrogen, Carlsbad, CA) in PBS, washed with 100% FBS, resuspended in sterile PBS for neonatal transfer. Staining for BrdU was assessed by staining with anti-BrdU (PRD-1, eBioscience) according to the manufacturer's protocol. Data were acquired on a FACsCalibur (BD Biosciences, San Diego CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Neonatal transfers

$CD4^+CD25^+$ T_R cells (>90% purity in all experiments) were isolated from the spleen and lymph nodes of 8-12 wk old B6.SJL ($CD45.1^+$) and $CCR4^{-/-}$ ($CD45.2^+$)

as described in materials and methods for Chapter 2 (*in vitro* suppression assay section). For competition experiments, neonatal *sf* mice (1-2 days old) were given $1.5x10^6$ $CD4^+CD25^+$ T_R from both $CCR4^{-/-}$ and WT mice (1 to 1 ratio) in 20µl PBS by intraperitoneal injection. Mice were monitored for external signs of inflammatory disease, bled at the given time-points and sacrificed after 100 days post-transfer.

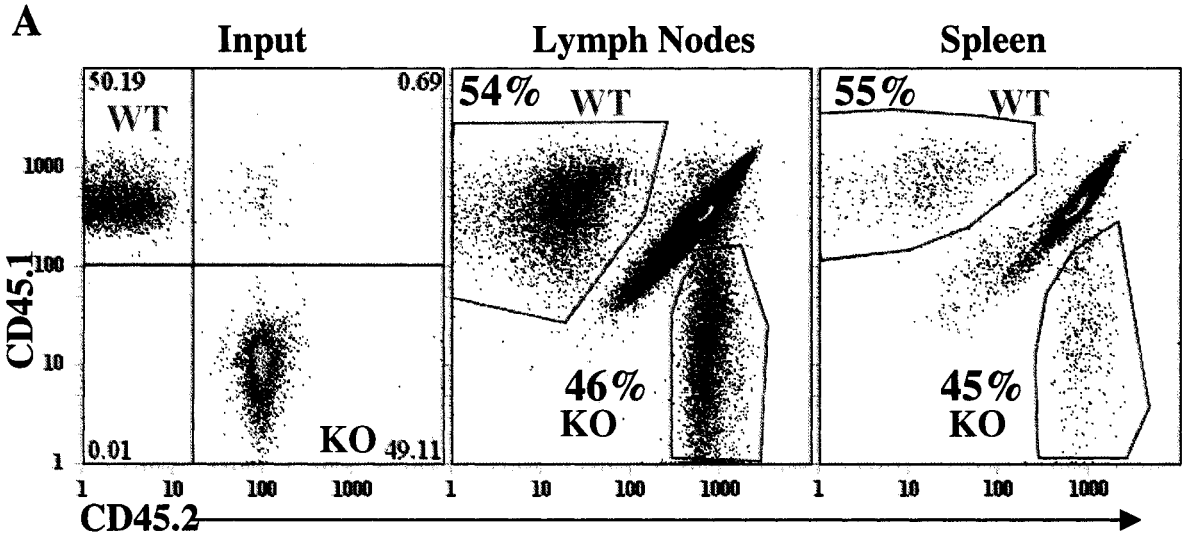
BrdU incorporation

Mice were given 0.8 mg/ml Bromodeoxyuridine (BrdU) in their drinking water for 4-5 days and levels of BrdU incorporation was measured via intranuclear staining of DNA according to the manufacturers protocol (BD Biosciences).

Figure 22.

$CCR4^{-/-}$ T_R are slightly outcompeted by WT T_R during initial homeostatic expansion: $CCR4^{-/-}$ (CD45.2) and WT(CD45.1) $CD4^+CD25^+$ cells (<90% pure) were isolated, CFSE labeled and $1.5x10^6$ of each was transferred i.p. into two 3-day-old male *sf* (CD45.1/CD45.2 heterozygotes). After four days, the transferred mice were sacrificed and the donor cells from the spleen and peripheral LN was analyzed. Cells were stained for CD4, CD45.1 and CD45.2 to distinguish the two different donor populations from the recipient cells. (**A**) The input ratio for all mice (left plot) and the donor cells from the PLN and SPL of one representative mouse. (**B**) Shows the percentage of total donor cells derived from either $CCR4^{-/-}$ or WT donor in the input cells and the spleen and PLN from two mice.

A


Input
Lymph Nodes
Spleen
50.19
WT
0.69
0.01
KO 49.11
54% WT
46% KO
55% WT
45% KO
CD45.1
CD45.2
1000
100
10
1

B

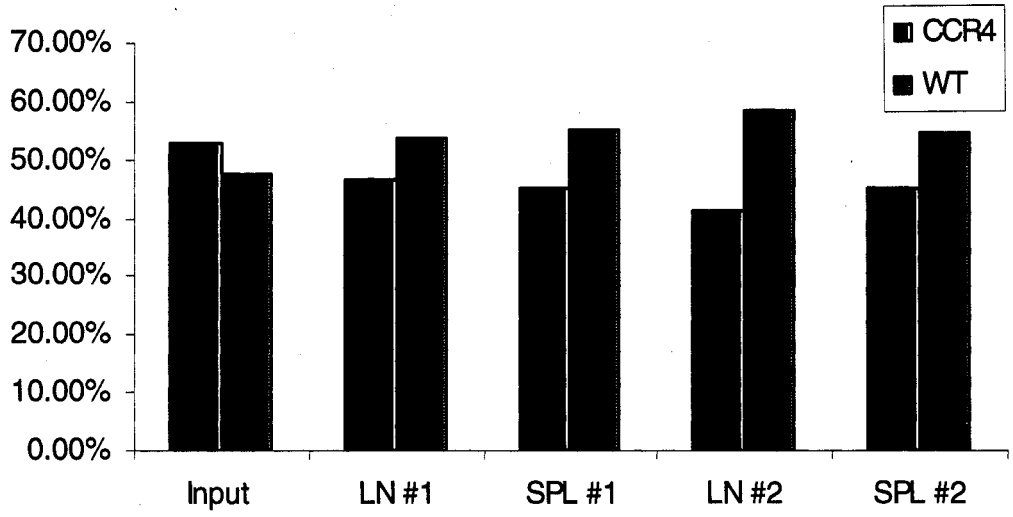

CCR4
WT
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Input
LN #1
SPL #1
LN #2
SPL #2

Figure 23.

$CCR4^{-/-}$ T_R proliferate at a faster rate than WT T_R during initial homeostatic expansion: $CCR4^{-/-}$ were isolated, CFSE labeled and $1.5x10^6$ of each was transferred i.p. into two 3-day-old male *sf* (CD45.1/CD45.2 heterozygotes). After four days, the transferred mice were sacrificed and the donor cells from the spleen and PLN was analyzed. Cells were stained for CD4, CD45.1 and CD45.2 to distinguish the two different donor populations from the recipient cells. from the recipient cells. (**A**) Graph shows the percentage of each donor population that has undergone any cell division (any cells below the primary peak of CFSE). (**B**) FACs plot of the spleen and PLN from one mouse showing the percentage of the total donor population within each division peak.

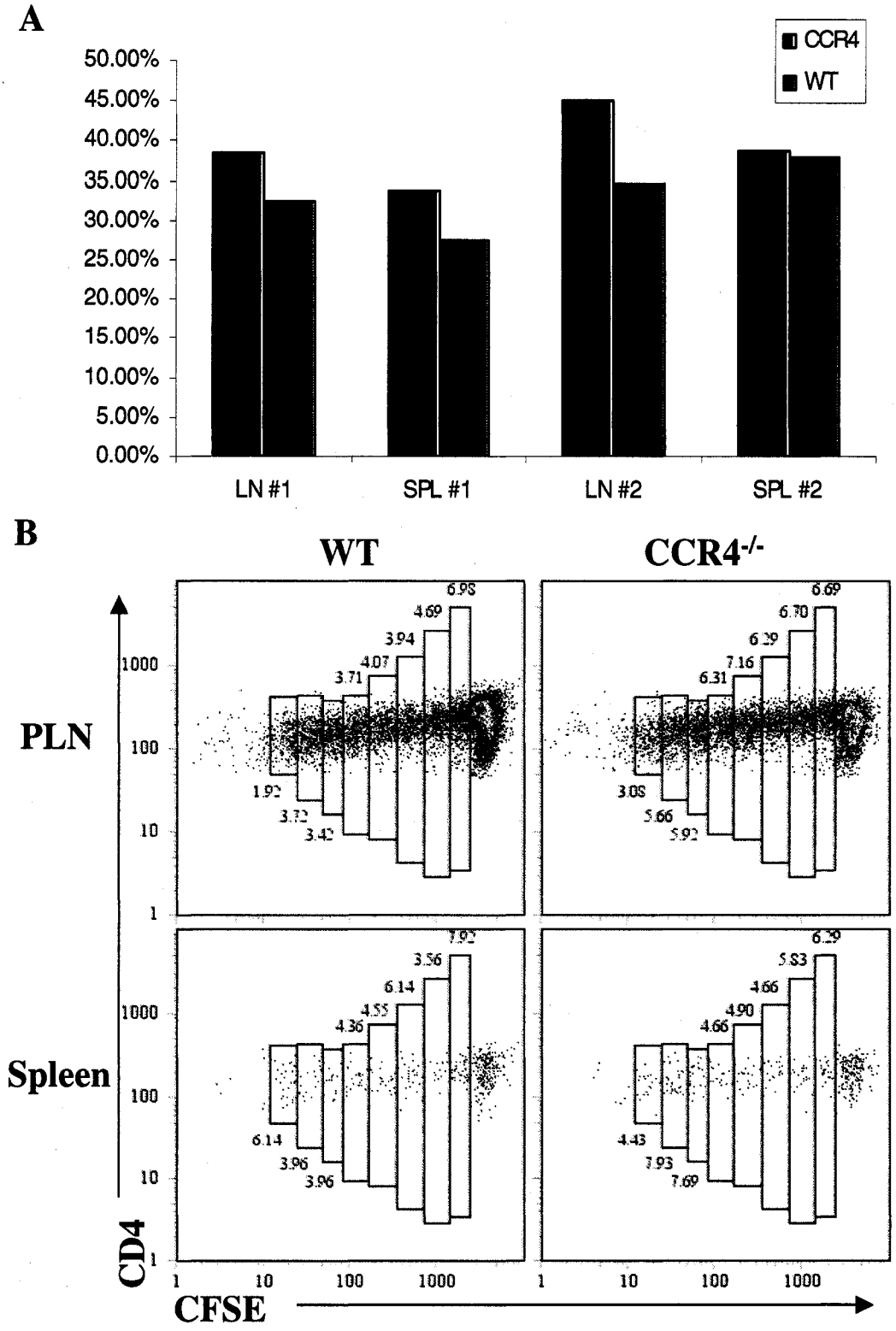
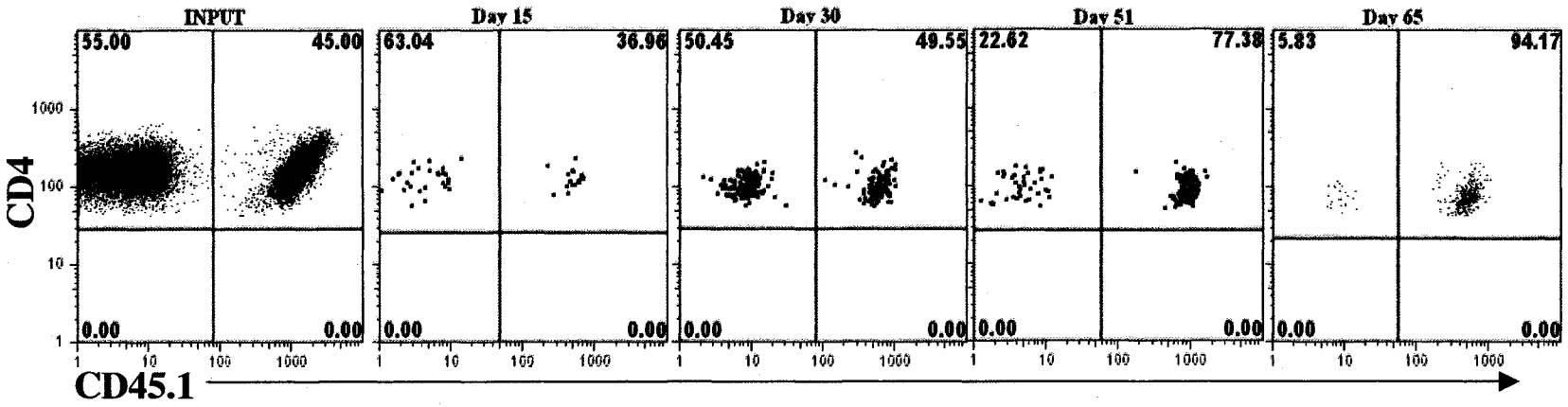

A
CCR4
WT
50.00%
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
LN #1
SPL #1
LN #2
SPL #2
B
WT
CCR4-/-
PLN
Spleen
CD4
CFSE
1
10
100
1000
6.98
4.69
3.94
4.07
3.71
1.92
3.72
3.42
6.69
6.70
6.29
7.16
6.31
3.08
5.66
5.92
7.92
3.56
6.14
4.55
4.36
6.14
3.96
3.96
6.29
5.83
4.66
4.90
4.66
4.43
7.93
7.69

Figure 24.

$CCR4^{-/-}$ T_R are outcompeted over time by WT T_R during ongoing homeostatic expansion: $CCR4^{-/-}$ (CD45.2) and WT (CD45.1) $CD4^+CD25^+$ cells (<90% pure) were isolated and $1x10^6$ of each was transferred i.p. into two 3-day-old male *sf* (CD45.1/CD45.2 heterozygotes). Starting between 18 and 25 days post-transfer, mice were bled and PBL were stained for CD45.1, CD45.2, CD4 and Foxp3 to measure the percentage of donor cells remaining from each donor population. (**A**) Data from one representative mouse; first panel shows the percentage of each donor population in the input cells and the subsequent panels show the percentage of each donor population present in the PBL at each time-point. (**B**) Graph of the amount of $CCR4^{-/-}$ derived donor cells in each individual mouse followed over time. First data point for each mouse show percentage of input derived from $CCR4^{-/-}$ donor and each subsequent data point shows the percentage of total donor cells derived from $CCR4^{-/-}$ donor at the time of bleed. Red line represents linear trendline of all mice tested.

A


INPUT
Day 15
Day 30
Day 51
Day 65
55.00
45.00
63.04
36.96
50.45
49.55
22.62
77.38
5.83
94.17
0.00
CD4
CD45.1
1000
100
10
1
1
10
100
1000

B

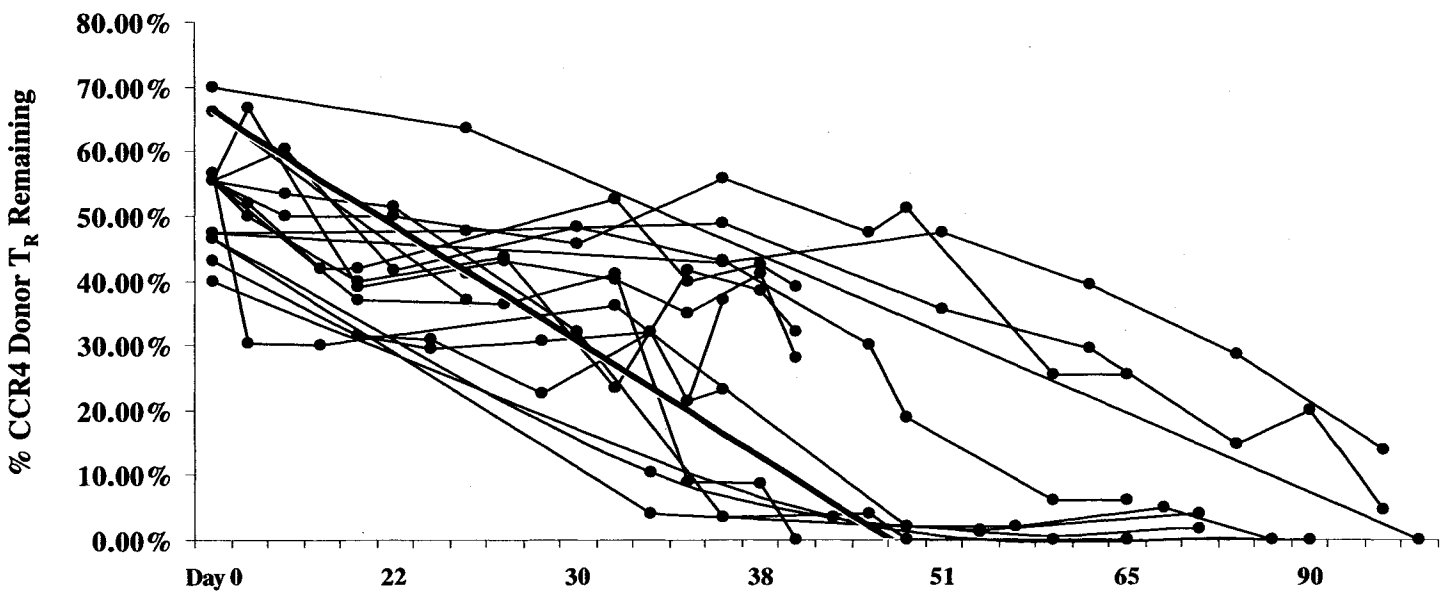
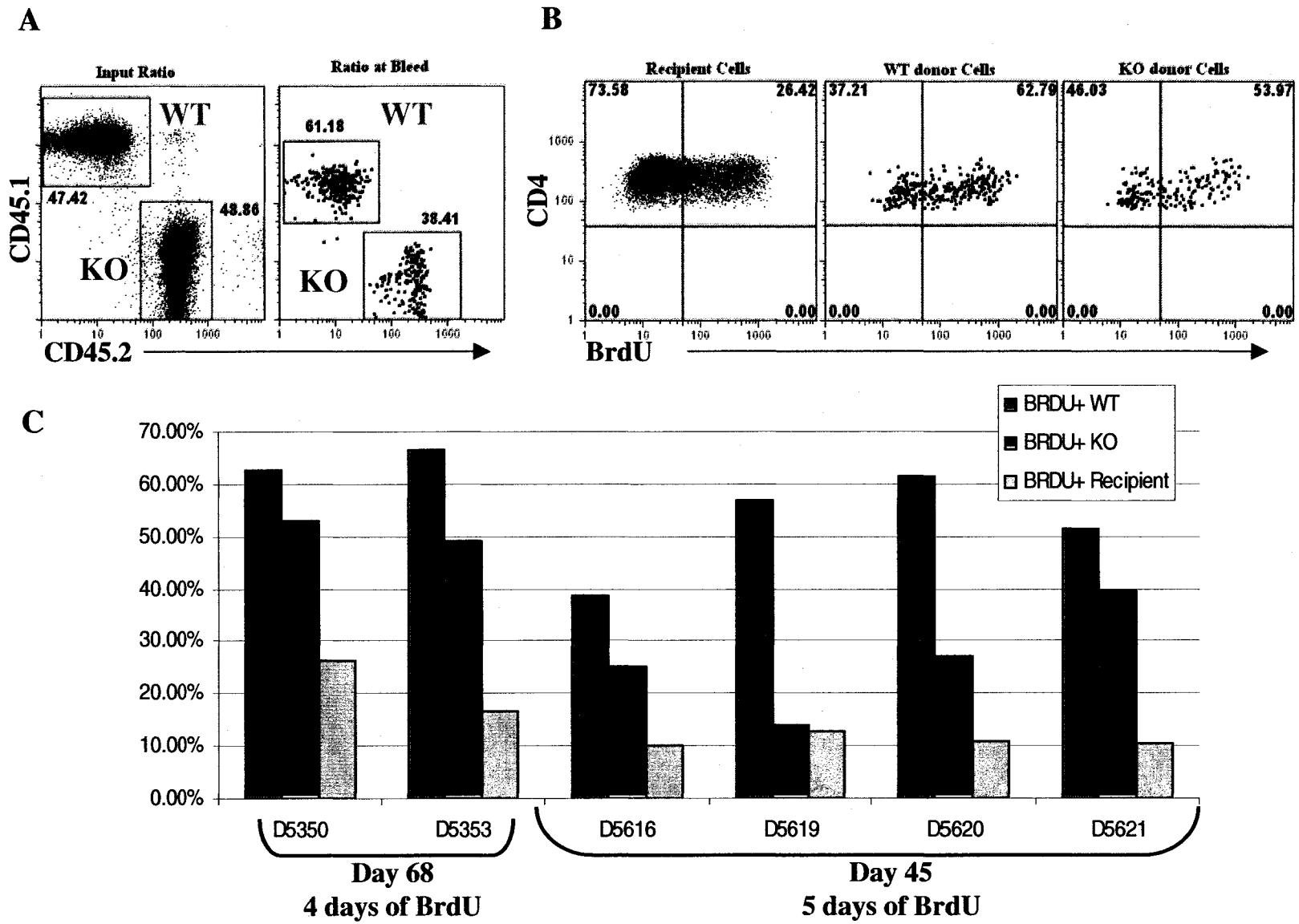

% CCR4 Donor T_R Remaining
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Day 0
22
30
38
51
65
90

Figure 25.

$CCR4^{-/-}$ T_R incorporate less BrdU during homeostatic proliferation when in competition with WT T_R: $CCR4^{-/-}$ (CD45.2) and WT (CD45.1) $CD4^+CD25^+$ cells (<90% pure) were isolated and $1x10^6$ of each was transferred i.p. into 3-day-old male *sf* (CD45.1/CD45.2 heterozygotes). Starting at day 40 or day 64 (two experimental groups shown) mice were fed BrdU (0.8m/ml) in their drinking water. After 4 or 5 days the mice were bled and the PBL was stained for CD45.1, CD45.1, CD4 and BrdU. (A&B) Data from one representative mouse (mouse D5350 on bottom graph) (**A**) The first panel shows the percentage of each donor population in the input cells and the second panel shows the percentage of each donor population at the time of the bleed. (**B**) $CD4^+$ cells were gated on $CD45.1^+/CD45.2^+$ recipient cells (farthest left), $CD45.1^+$ WT cells (middle) and $CD45.2^+$ $CCR4^{-/-}$ donor cells (farthest right) and the level of BrdU incorporation was measured in each population. (**C**) Graph of the percentage of each population staining positive for BrdU at the time of bleed for each individual mouse. Gated for each population were set as stated in part (**B**).


A
Input Ratio
Ratio at Bleed
WT
KO
47.42
48.86
61.18
38.41
CD45.1
CD45.2
B
Recipient Cells
WT donor Cells
KO donor Cells
73.58
26.42
37.21
62.79
46.03
53.97
0.00
CD4
BrdU
C
BRDU+ WT
BRDU+ KO
BRDU+ Recipient
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
D5350
D5353
D5616
D5619
D5620
D5621
Day 68
4 days of BrdU
Day 45
5 days of BrdU

Chapter 4: Wiskott Aldrich Syndrome Protein is Required for Regulatory T Cell Homeostasis

Introduction

While much is known about the generation and functional properties of T_R, the signals mediating their *in vivo* activation, differentiation and tissue localization remain to be clarified. Signaling events propagated by TCR binding are probably crucial in mediating the regulatory activities of activated T_R. In particular, signaling molecules that strengthen the signals received during TCR engagement may be crucial in the differentiation of thymocytes and naïve T cells into T_R. The final chapter of my thesis describes a collaborative study with the laboratory of Dr. David Rawlings in which we explored the importance of one such signaling molecule, WASp, in the peripheral homeostasis of T_R.

Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency disorder characterized by opportunistic, viral and bacterial infections due to abnormal lymphocyte function. Affected individuals also have thrombocytopenia with small platelets, eczema, and increased risk of autoimmune disorders and malignancies(161). Worldwide, more than 200 unique mutations have been described within the gene encoding the Wiskott-Aldrich Syndrome protein (WASp) (Fig. 26)(162). Mutations leading to loss of WASp expression correlate with a more severe disease phenotype(163). WASp mRNA is expressed in all hematopoietic lineages and WASp participates in multiple signal transduction pathways in a range of cell types. WASp deficiency, however, is most prominently associated with defects in T lymphocyte function. $WASp^{-/-}$ T cells fail to

polymerize and reorganize actin in response to αCD3 stimulation and formation of the T cell immunological synapse is defective. Thus, WASp deficiency appears to directly interfere with assembly of the TCR "signalosome" resulting in incomplete cellular activation and consequently decreased cell proliferation and cell survival.

WAS patients exhibit a very high prevalence of autoimmune disease. In one study, greater than 70% of patients (40/55 evaluated) had one or more autoimmune episodes including autoimmune cytopenias, arthritis, vasculitis, inflammatory bowel disease, or renal disease(161,164). Autoimmune manifestations in WAS typically present very early in life and are largely unresponsive to medical therapy. In addition, even patients with otherwise mild disease (thrombocytopenia only) due to mutations permitting low level expression of intact protein, or of a partially functional protein, can develop life-threatening autoimmune sequelae(163). The high prevalence for autoimmunity in WAS might result from escape of self-reactive T cells from negative selection due to defective TCR-mediated signals and reduced apoptosis at this checkpoint. Alternatively, chronic inflammatory stimuli, defects in IL-2 production, or alterations in antigen presenting cell or macrophage function have each also been suggested to explain these disease associations(165,166). In this study, we tested whether these paradoxical observations, immunodeficiency in association with life-threatening autoimmunity, might be explained on the basis of defects in T_R function and altered dominant tolerance.

We show that $WASp^{-/-}$ mice, like WAS patients, develop early onset, high titer autoantibodies. We also show that restoration of WASp expression in humans promotes the expansion of T_R. Consistent with both of these observations, $WASp^{-/-}$ T_R fail to

compete effectively in vivo and are unable to maintain immunologic tolerance in T_R deficient mice. Finally, we show that T_R expressing activation markers and adhesion molecules and chemoattractant receptors required for tissue entry are uniformly reduced in $WASp^{-/-}$ T_R suggesting a defect in peripheral T_R activation. Taken together, our findings indicate that T_R homeostasis is critically reliant upon signals integrated by WASp; and suggest a crucial role for WASp in antigen-driven T_R expansion and control of basal T and B cell activation in normal hosts.

Results

Expansion of differentiated $WASp^+$ T_R in a WAS patient with a revertant mutation

We recently identified a WASp null patient that exhibited an improved clinical picture in association with new evidence for WAS expression within his T, B, and NK cells. This teen-age patient had suffered from life-long, recurrent episodes of autoimmune hemolytic anemia beginning at 15 months of age. WASp re-expression correlated with stabilization of both his RBC count and reduction in his steroid therapy over the preceding 6-9mo. Previous diagnostic studies had identified a single nucleotide deletion in *WAS* leading to a frameshift and premature stop codon; and absence of WASp expression. These genetic studies were repeated using peripheral blood lymphocytes and a newly derived T cell line and identified a new single nucleotide insertion at the same genomic site. This change was predicted to restore the normal amino-acid sequence and WASp expression (T.Torgerson, H.Ochs and D.J. Rawlings, unpublished data).

This revertant mutation provided a unique opportunity to evaluate the consequences of restored WASp function in newly generated lymphoid populations. Consistent with the reversion impacting a limited progenitor pool, only ~2% of naïve

CD4 T cells (e.g. $CD4^+CD45RA^+CD27^+CD62L^+$cells) expressed WASp (Fig. 27, C-D). In contrast, we observed a striking increase in relative percentage of $WASp^+$ T_R (25-35% $CD4^+FOXP3^+$ cells expressed WASp; Fig.27, B). Similar results were obtained in three independent analyses over time. To define the relative expression of WASp within maturing T_R, we also used markers to identify $CD45RA^+CD27^+$, $CD45RA^-CD27^+$, vs. $CD45RA^-CD27^-T_R$ (Fig. 27, E). Based upon these staining criteria, very few $CD27^+CD45RA^+$ naïve cells were present within the $CD4^+FOXP3^+$ T_R population. We also evaluated the expression of CD62L within the $CD4^+FOXP3^+$ T_R population. $WASP^+$ cells comprised 24% and 27% of the $CD45RA^-CD62L^+$ and $CD45RA^-CD62L^-$subsets, respectively (Fig.27, F-G). In contrast, $CD45RA^+CD62L^+$ T_R comprised less than 1% of total T_R population; and the very small number of such cells precluded analysis of the relative percentage of $WASp^+$ cells within this naïve population (due to the lymphopenia present in this patient). These results suggested that $WASp^+$ human T_R manifested a strong *in vivo* selective advantage and raised the question as to whether WASp plays a critical role in T_R homeostasis.

$WASp^{-/-}$ mice develop high titer anti-DNA autoantibodies and autoimmune disease

WASp deficient ($WASp^{-/-}$) mice (167,168) provide a useful model for human WAS. These animals exhibit clear defects in T cell function including abnormal actin cytoskeletal organization, reduced CD3 and CD3/CD28 proliferative responses, and markedly reduced IL-2 production. Despite the striking clinical data in human WAS, however, there is only limited evidence that $WASp^{-/-}$ mice develop autoimmunity. The 129SvEv (129)/$WASp^{-/-}$ strain (167)develops spontaneous, and radiation-induced, colitis that resembles autoimmune inflammatory bowel disease. We have also observed

spontaneous sub-clinical colitis and frequent rectal prolapse in $WASp^{-/-}$ mice backcrossed into the C57Bl/6 background (data not shown). Thus this inflammatory disease association, while less severe, does not appear to be strain specific as previously suggested(169).

Notably, no previous studies have evaluated whether $WASp^{-/-}$ mice exhibit defects in B cell tolerance or develop humoral autoimmune features analogous to those commonly observed in patients with WAS. To address this question, we initially screened a cohort of aged $WASp^{-/-}$ and control animals for evidence of anti-nuclear antibodies and observed a marked increase in anti-doublestranded (ds)-DNA antibodies (Fig. 28). To determine the timing and frequency of autoantibody production, we followed cohorts of $WASp^{-/-}$ and control mice of both sexes and of both genetic backgrounds. Compared with age-matched wild type (WT) controls, the $WASp^{-/}$ mice demonstrated a consistent increase in anti-ds-DNA within 3 mo. of age (Fig.28). Significantly, titers of anti-DNA antibodies in 6 mo. $WASp^{-/}$mice were equivalent to those in 6-9 mo. female NZB/W F1 mice, a well-characterized murine model of systemic lupus erythematosus(170). Our data demonstrate that $WASp^{-/}$mice develop high-titer anti-nuclear antibodies with high frequency early in life, indicating that WASp deficiency promotes alterations in B cell tolerance.

Chimeric BM transplantation promotes WT T_R expansion and rescues $WASp^{-/-}$ from irradiation-induced colitis

Irradiation promotes the rapid onset of inflammatory colitis in $WASp^{-/-}$ mice (129 strain) and this complication is prevented by transplantation with WT but not $WASp^{-/-}$ bone marrow. For example, 11/12 $WASp^{-/-}$ animals developed severe colitis requiring

sacrifice following radiation doses of 550-950 cGy and transplantation with $WASp^{-/-}$ BM; whereas no animals transplanted with WT BM developed this complication (data not shown). Based upon these observations, we determined whether induction of colitis in this model was due to a cell intrinsic defect in tolerance induction of the $WASp^{-/-}$ cells. To this end, bone marrow ($1x10^7$ containing a 1:3 chimeric mixture of WT vs. $WASp^{-/-}$ cells) was transplanted into lethally irradiated 129 $WASp^{-/-}$ mice to establish stable mixed chimeras. In lieu of congenic markers, we utilized WASp intracellular staining as a highly sensitive assay to identify hematopoietic cells derived from $WASp^+$ vs. $^-$ donor marrow. Recipient animals exhibited a progressive accumulation of $WASp^+$ B and T cells (Fig.29, A; reaching ~40% at 10 wk; and 65-75% by 50 wk post transplantation). Similar findings were previously reported(169) and indicate that WASp expression provides a selective advantage to both T and B cells under these conditions. Interestingly, while detailed phenotypic analysis of T cell subsets indicated only limited selection for $WASp^+$ cells within the thymus and naïve T cell pool, we observed a preferential expansion of $WASp^+$ T_R. The relative numbers $WASp^+$ T_R reached nearly 100% at the time of sacrifice (12 mo post transplantation; Fig.29, B). In contrast, we observed no advantage for $WASp^+$ $GR1^+$ and Mac^+ myeloid cells which were maintained at levels equivalent to the initial chimeric mixture (25-30%; Fig.29, A; and data not shown). Notably, none of the transplanted mice developed overt signs of colitis and histological analysis at the time of sacrifice was indistinguishable from that of age-matched WT controls (data not shown). Thus, wild type cells could ameliorate the colitis mediated by $WASp^{-/-}$ cells and this correlated with a preferential expansion of $WASp^+$ T_R within $WASp^{-/-}$ hosts.

Transfer of WASp$^{-/-}$ T_R fails to control autoimmunity in neonatal Scurfy recipient mice

Because WASP^{+} T-effectors and B cells were also partially selected *in vivo* in our chimeric bone marrow transplant experiments, these studies were insufficient to determine whether defects in T_R function were principally responsible for the autoimmune features observed in WASp$^{-/-}$ mice. Therefore, we utilized the neonatal *sf* rescue model (described in Chapter1&2) to directly test for cell intrinsic *in vivo* defects in WASp$^{-/-}$ T_R function independent from any contribution of functional defects in WASp$^{-/-}$ T-effector cells. Non-irradiated male neonatal *sf* mice (CD45.1) received purified WASp$^{-/-}$ or WT CD4+CD25+ T_R (CD45.2) via adoptive transfer. As previously described, unmanipulated *sf* animals exhibited severe lymphadenopathy, splenomegaly, and phenotypic evidence for T cell activation and autoimmunity in all tissues examined. CD4 T cells isolated from the spleen and lung parenchyma expressed elevated levels of the activation marker, CD44, and reduced expression of the naïve T cell marker CD45RB (Fig. 30A, panel 3). Histological analysis of liver and lung, two particularly susceptible tissues in *sf* mice, revealed extensive lymphocytic infiltration and inflammation surrounding blood vessels in the liver and large and small airways in the lung (Fig. 31, panel 3). In contrast, animals receiving adoptively transferred WT T_R exhibited minimal T cell activation (Fig. 30A-B) and little or no histological evidence of lymphocytic inflammation in all tissues analyzed (lung & liver shown; Fig. 31; panel 2). These findings were similar to those in unmanipulated WT control mice (Figures 30, panel 4, Fig. 31, panel 4, respectively). Strikingly, recipients of WASp$^{-/-}$ T_R were unable to control aberrant activation of effector T cells (Fig. 30, panel 1); and developed marked splenomegaly, and pulmonary and hepatic inflammatory changes that were similar to

those in untreated *sf* mice (Fig.31, panel 1). Inflammatory cell infiltrates were also observed in other tissues including skin and kidney (data not shown). Importantly, all $CD45.1^+$ cells were uniformly $Foxp3^+$ indicating that the transferred cell populations were comprised entirely of either $WASp^{-/-}$ or $WASP^+$ T_R, respectively (data not shown). Taken together, these data demonstrate that $WASp^{-/-}$ T_R fail to effectively mediate dominant tolerance *in vivo*.

$WASp^{-/-}$ mice generate normal numbers of $Foxp3^+$ T_R cells within the thymus

Based upon the *in vivo* functional deficit exhibited by $WASp^{-/-}$ T_R, we next sought to determine whether WASp deficiency specifically impacted either T_R generation, *in vitro* T_R function, or *in vivo* homeostasis. To address the role for WASp in T_R production, we identified T_R in WT vs. $WASp^{-/-}$ animals using two independent staining protocols (Fig.32, A). T_R were readily identified in $WASp^{-/-}$ mice as based on either the $CD3^+/CD4^{int}/CD25^{hi}/CD69^-$ or $CD4/FoxP3^+$ cell phenotype. There was no difference in either the relative percentage or absolute numbers of $CD4^+Foxp3^+$ cells in the thymi of WT vs. $WASp^{-/-}$ mice in either the C57Bl/6J (Bl6) or 129 strains (Fig. 32b and data not shown).

To more precisely assess the role for WASp thymic T_R production, we evaluated WASp expression in thymic T cell subsets isolated from $WASp^{+/-}$ heterozygote female mice. This approach allowed us to identify any developmental stage(s) at which $WASp^+$ cells might manifest a selective advantage based upon nonrandom inactivation of the X-linked *WAS* gene. One previous study has suggested that BM progenitor cells derived from $WASp^{+/-}$ heterozygote mice exhibit early non-random X-inactivation(171). However, intracellular WASp staining of both bone marrow B cell progenitors and

myeloid cells from heterozygote animals was consistent with a random X-inactivation pattern with 50% of cells in both lineages expressing WASp (data not shown). Our data suggest that previous findings may reflect a selective advantage of $WASp^+$ cells for growth in colony forming assays. The relatively limited role for WASp in murine vs. human HSC function might reflect redundant activity of N-WASp or related proteins.

Using this staining method we observed little or no difference in the relative percentage of $WASp^+$ cells within any thymic developmental stage [from double negative (DN) to single positive (SP)]. The median number of $WASp^+$ cells increased slightly between the DN and SP stages reaching a level of slightly greater than 50% in CD4 and CD8 SP thymocytes (Fig.32, C). While a previous report suggested that WASp deficiency impairs the DN3 to DN4 transition(168), we also observed no significant change in the relative percentage of WASp expressing cells at this stage (data not shown). Most notably, the median level for WASp expression in thymic $Foxp3^+$ T_R was essentially identical to that of the total CD4 SP thymocytes in young (6-8.5 wk) and aged (6 mo) heterozygote mice (Fig. 32,B; and data not shown). Thus, WASp is not essential for generation of T_R in the thymus.

$WASp^{-/-}$ T_R exhibit in vitro suppressive activity

We used *in vitro* T cell suppression assays to directly test whether WASp was required for T_R suppressive activity. Spleen and lymph node (LN) $CD4^+CD25$- effector T cells were labeled with CSFE, or left unlabeled, and then cultured with increasing numbers of WT or $WASp^{-/-}$ $CD4^+CD25^+$ T_R. All cultures also contained irradiated CD4-depleted WT antigen presenting cells (APC), and were stimulated with agonistic αCD3 and αCD28 monoclonal antibodies. When tested with WT effector T cells, $WASp^{-/-}$ T_R

exhibited moderately reduced suppressor activity at all target ratios based upon CSFE dilution as a measure for effector proliferation (Fig.33, A). To better mimic the *in vivo* situation in $WASp^{-/-}$ mice, we also evaluated the relative suppressor activity WT vs. $WASp^{-/-}$ T_R with respect to $WASp^{-/-}$ effectors. Under these conditions (Fig. 33, B), WT and $WASp^{-/-}$ T_R functioned equivalently at all target ratios. Additionally, we compared the suppressive activity of WT verses $WASp^{-/-}$ T_R across a range of αCD3 cross-linking to determine whether WASp-deficiency impaired T_R function in the context of sub-optimal CD3 engagement. Again, WT and $WASp^{-/-}$ T_R functioned similarly at each CD3 concentrations evaluated (data not shown). Together, these findings indicated that, in contrast to the marked *in vivo* functional deficit observed following adoptive transfer, T_R activation and function *in vitro* is largely intact in the absence WASp. Further, because $WASp^{-/-}$ T_R efficiently suppressed $WASp^{-/-}$ effectors, alterations in T_R function appeared insufficient to explain the autoimmune phenotype WASp-deficient mice or humans.

$WASp^{-/-}$ T_R fail to compete effectively in vivo

While WASp was not required for the generation or *in vitro* activity of T_R, we reasoned that defects in T_R homeostasis might account for the failure of $WASp^{-/-}$ T_R to control self-reactivity *in vivo*. To address this issue, we first analyzed the relative numbers of $WASp^{+}$ vs. WASp-null T_R within peripheral lymphatic tissues of $WASp^{+/-}$ heterozygous female mice. While only ~55% of naïve T cells were $WASp^{+}$, >90% of peripheral T_R were $WASp^{+}$ (Fig.34, A). This skewing was obvious by 6 wk of age, maintained in older animals, and evident in all lymphoid tissues, as well as in cells isolated from non-lymphoid sites including the peritoneal cavity [peritoneal cavity exudate cells (PEC)] and lung airways [bronchoaveolar lavage fluid cells, (BAL)]

(Fig.34, B and data not shown).

We also determined whether the apparent selective advantage of WT T_R observed in heterozygote $WASp^{+/-}$ mice, was reflected in alterations in the relative numbers of T_R in $WASp^{-/-}$ animals (Fig.35). While the frequency of thymic T_R was not different in 6, 16, and 24wk old WT vs. $WASp^{-/-}$ animals, the percentage of $CD4^+FoxP3^+$ T_R was consistently reduced in all peripheral lymphoid compartments in $WASp^{-/-}$ animals. This reduction was present in mice at all ages evaluated in both Bl6 and 129 $WASP^{-/-}$ strains (Fig. 35; and data not shown). Because $WASp^{-/-}$ also exhibit a modest reduction in total $CD4^+$ T cell numbers, this change resulted in an approximate 30% reduction in the number of total $CD4^+FoxP3^+$ splenic T_R compared with WT animals. Notably, we observed no significant differences in relative percentage(s) of $CD3^+$, $CD4^+$ or $CD8^+$ cells within the peripheral lymphatic compartment compared with controls. This finding was consistent with a preferential loss of T_R within the peripheral T pool in $WASp^{-/-}$animals.

Purified $WASp^{-/-}$ T_R expand poorly in neonatal recipient mice

As I discussed extensively in the previous chapters, homeostatic maintenance of T_R in non-lymphopenic hosts is dependent upon both TCR signals and exogenous IL-2 provided via $CD4^+CD25^{low}Foxp3^-$T cells(33,34). Exogenous IL-2 levels are predicted to be decreased in $WASp^{-/-}$ mice due to the deficit in TCR-mediated IL-2 production(167,168), and this might limit the expansion/function of $WASp^{-/-}$ T_R. To test this possibility, WT (CD45.1) and $WASp^{-/-}$ (CD45.2) T_R were isolated and co-transferred ($2\text{-}6x10^6$ total T_R/recipient) into non-irradiated, C57Bl/6 (CD45.1/CD45.2) neonatal *sf* recipients. By co-transferring WT and $WASp^{-/-}$ T_R into neonatal *sf* mice, we could directly compare their ability for homeostatic expansion and survival in the context of

normal IL-2 production and in the absence of endogenous T_R. Adoptively transferred T_R were identified using antibodies to the CD45.1 vs. CD45.2 allotype markers beginning at 14d post transfer (Fig.36, A). $WASp^{-/-}$ T_R were rapidly out-competed by the co-transferred WT T_R, indicated by their progressive decline from spleen and lymph nodes within 20-30 days post-transfer (Fig. 36, A-B). Thus, IL-2 provided in "trans" by normal T effector cells does not appear to be sufficient to rescue homeostatic proliferation of $WASp^{-/-}$ T_R.

Impaired peripheral differentiation of $WASp^{-/-}$ T_R

The majority of T_R undergo cell division following transfer into non-lymphopenic hosts(33,34), and proliferating T_R in naïve animals acquire a distinct activated cell surface phenotype including down-modulation of CD62L and increased expression of CD44, CD69, and other activation markers(34). To determine if WASp may play an important role in T_R activation, we asked if $WASp^{+}$ vs. $WASp^{-/-}$ T_R derived from $WASp^{+/-}$ carriers differed with regard to expression of these markers. $WASp^{+}$ T_R consistently expressed higher levels of both CD44 and CD69; and reduced levels of CD62L (Fig.37). Essentially identical results were obtained using either $CD4^{int}CD25^{hi}$ or $CD4^{+}Foxp3^{+}$ gates to define T_R subsets. These findings suggested that T_R activation preferentially promoted the expansion of $WASp^{+}$ cells.

As I previously discussed, T_R are likely targeted to non-lymphoid sites only after self-antigen recognition within lymphoid tissues. Thus, T_R have been subdivided based on homing receptor expression into populations with differential tropism for lymphoid vs. non-lymphoid tissues(172). T_R expressing the αE chain (CD103) of the integrin, αEβ7, also preferentially express homing receptors that target cells to non-lymphoid tissues

including P-/E-selectin ligand, β1 integrin, CCR4, and CCR6(110,122). While $CD103^-$ and $CD103^+$ T_R exhibit similar *in vitro* suppressor activities, they exhibit distinct capacity to suppress *in vivo* immune responses. $CD103^-T_R$ effectively suppress naïve $CD4^+$ T cell activation in lymphoid tissues, whereas $CD103^+$ T_R home to inflammatory sites and preferentially suppress disease activity in inflammatory models(110,112) or modulate the T-effector response to local infection(124). To determine the relative requirement for WASp in generation of tissue tropic T_R subsets, we characterized the cell surface expression profile of $WASp^+$ vs. $WASp^-$ T_R with regard to a series of candidate adhesion and homing receptors. Essentially identical data were obtained in analysis of T_R isolated from spleen, or from peripheral or mesenteric lymph nodes (Fig.38; and data not shown). The vast majority of T_R in lymphoid tissues expresses CCR7, and accordingly we observed no appreciable difference in the relative expression of this receptor on $WASp^+$ vs. $WASp^{-/-}$ T_R. In contrast, both the relative percentage (Fig. 38) and relative MFI of CD103 expression (data not shown) were significantly reduced in $WASp^-$ T_R. Accordingly, the relative expression of P-/E-selectin ligand and CCR4 were also significantly reduced in $WASp^{-/-}$ T_R (Fig. 38). Although the number of cells available for analysis was relatively limited, we also observed an increase in CD103 expression in $WASp^+$ T_R isolated from the BAL and PEC (data not shown). Together, this phenotypic analysis supports the idea that WASp-dependent signals are required for optimal activation and functional differentiation of T_R in the periphery.

Discussion

Our data strongly support a model in which signals mediated by WASp are essential for T_R homeostasis, peripheral activation, and *in vivo* function. While WASp

exerts little or no role in thymic T_R production, WASp is required for peripheral T_R expansion and survival. WASp deficiency leads to decreased peripheral T_R numbers and markedly impacts the activated T_R pool as shown by the decrease in T_R that express activation markers and homing receptors associated with activation. Consistent with these observations, $WASp^{-/-}$ T_R exhibit decreased competitive fitness in three independent *in vivo* murine models including: heterozygous female carriers; chimeric bone marrow transplants; and adoptive transfer into T_R deficient hosts. Further, expansion of $WASp^{+}$ T_R correlated with rescue of $WASp^{-/-}$ recipients from radiation-induced inflammatory colitis. Conversely, $WASp^{-/-}$ T_R failed to mediate dominant tolerance after transfer into neonatal *sf* mice, nor could they control colitis in the $CD45RB^{hi}$ adoptive transfer model (173). Finally, consistent with each of these observations in mice and with the marked propensity for WAS patients to develop autoimmune sequelae, we observed a strong selective advantage for $WASp^{+}$ human T_R in vivo in a patient with a revertant mutation leading to re-expression of WAS in developing T_R. In this case, the presence of this population correlated with decreased autoimmune disease activity and an improved clinical condition.

Thymic T_R development is dependent upon FoxpP3 expression, cytokine signaling via the common gamma chain, and TCR mediated positive selection(80,174). Interestingly, WASp appears to play a very limited role in this process. The number of thymic T_R and their surface phenotype were identical in WT and $WASp^{-/-}$ mice and Foxp3 was also expressed at normal levels in $WASp^{-}T_R$. Also, analysis of X-inactivation in $WASP^{+/-}$ mice revealed no evidence for altered fitness in thymic T_R. We also failed to identify any differences in the TCR repertoire of $WASp^{-/-}$ vs. WT thymic T_R in

heterozygote animals using a panel of TCR Vβ antibodies (data not shown). While this analysis does not assess differences in TCR affinity, these combined observations suggest that TCR-mediated, T_R selection is largely WASp-independent. Further, while T cell mediated IL-2 production is deficient in $WASp^{-/-}$ mice, this deficit played no role in the thymic T_R production, a finding consistent with recent work(43).

T_R comprise a stable proportion of the steady state $CD4^+$ T cell population. Maintenance of the T_R pool is dependent upon signals provided via the TCR, IL-2, and co-stimulatory molecules including CD28/B7-1/B7-2(33,59,80). Mounting evidence, including the data presented here, argue that T_R homeostasis differs in crucial ways from homeostatic cycling of naïve $CD4^+CD25^-$T cells. First, compared with $CD25^-$T cells, T_R appear to be significantly longer lived(34). Second, the basal proliferative response in T_R is 2-5 fold greater than $CD4^+CD25^-$T cells and >80% of splenic T_R undergo multiple cell divisions within 30 days of transfer into non-lymphopenic hosts(33,34). Third, *in vivo* proliferating T_R in unimmunized, healthy animals acquire a distinct "activated" cell surface phenotype that includes: down-modulation of CD62L, expression of activation markers (CD44, CD69, GTIR, CD134/OX40, CD122/IL2Rβ and others) (34)and of tissue homing receptors including CD103(110). Thus, the cycling $CD44^{high}$ T_R and $CD103^+$ T_R populations largely overlap. Together, these observations suggest T_R homeostasis is mediated by encounter with cognate self-antigen presented within the draining lymph nodes or spleen; and this promotes expression of activation markers and receptors essential for homing and tissue entry. This activated T_R population mediates dominant tolerance via at least three alternative means: inhibiting the priming of co-localized naïve T cell in the secondary lymphoid tissues; entry into germinal centers and

modulation of B cell activation; and suppression of activated T effector cells within non-lymphatic tissue sites. In contrast to this scenario, homeostatic cycling of naïve $CD4^+CD25^-$T cells proceeds in the absence of co-stimulatory molecules, and does not lead to a stable alteration in activation markers or tissue tropism.

Our combined findings support the conclusion that WASp is essential for homeostatic T_R activation, and suggest, but do not prove, the idea that WASp is required for optimal self-antigen driven proliferation *in vivo*. Interestingly, $WASp^{-/-}$ T_R exhibited normal suppressive activity against $WASp^{-/-}$ T effector cells and only modestly reduced suppressive activity against WT T effectors, data that was confirmed by two recent studies by Marangoni et.al. and Maillard et.al.(173,175). Because thymic, naïve, and activated T_R behave similarly *in vitro* (34,110), it is perhaps not surprising that this assay failed to identify defects secondary to alterations in the relative number of activated T_R in $WASp^{-/-}$ animals. In contrast to our *in vitro* findings, WASp function is clearly required for peripheral T_R survival and/or expansion based on our data and others(173). WASp functions to facilitate efficient T cell: APC synapse formation and sustained TCR signaling. Thus, while not directly tested in this work, our data suggest that WASp is required for efficient responses to cognate antigen presented in a physiologic context *in vivo*. Indeed, recently published work showed that $WASp^{-/-}$ T_R could not control antigen-specific proliferation *in vivo* by WT OVA-specific CD4+ cells, and this was due to a lack of proliferation and proper localization of the $WASp^{-/-}$ T_R (175). Further, while alterations in APC function might also limit T_R activation in $WASp^{-/-}$ mice, our adoptive transfer data in *sf* recipients demonstrate that cell intrinsic defects are sufficient to abrogate $WASp^{-/-}$ T_R function in vivo.

Expression of CD103, E/P-selectin ligand, α4β7, CCR4, and CCR6 were all reduced in $WASp^{-/-}$ T_R. In addition, migration of $WASp^{-/-}$ T cells is impaired due to their defects in cytoskeletal rearrangement(176,177). Together, these defects are predicted to limit the capacity of $WASp^{-/-}$ T_R to enter and function within inflamed tissue. Consistent with this, nearly all tissue resident T_R in heterozygote mice were $WASp^{+}$ (data not shown). Defective T_R activation and migration may play an important role in the spontaneous and radiation induced inflammatory colitis present in $WASp^{-/-}$ mice. Also, recent work indicates that activated T_R can enter B cell follicles in a chemokine-dependent fashion; and modulate B cell activation(178-180). Thus, the striking increase in anti-DNA antibody levels in $WASp^{-/-}$ animals may derive from both failure to regulate T effector cell activation, as well as inefficient T_R homing to germinal centers and modulation of B cell activation. As BCR signaling is intact in $WASp^{-/-}$ B cells(167,168), loss of dominant tolerance directed towards activated B cells may have an enhanced phenotype in this context. Further studies are required to test this possibility and to determine whether restoration of WT T_R alone is sufficient to abrogate autoimmunity in $WASp^{-/-}$ animals.

Although T_R cannot produce the IL-2 needed for their sustained homeostasis, their activation is tightly linked to IL-2 produced by activated $CD4^{+}CD25^{-}$T effectors, tying T_R responses to the inflammatory signals they modulate. While dispensable for T_R production and *in vitro* T_R activity, IL-2 provided "in trans" orchestrates a non-redundant growth and survival program in peripheral T_R (43). Although $WASp^{-/-}$ effector T cells exhibit defects in IL-2 production, restoring IL-2 producing capacity with normal effector T cells appears to be insufficient to rescue the peripheral expansion of $WASp^{-/-}$ T_R. This

interpretation is consistent with the inability of $WASp^{-/-}$ T_R to compete effectively with WT cells in either heterozygous mice or T_R deficient *sf* hosts. Defects in IL-2 production may, however, accentuate the competitive disadvantage of $WASp^{-/-}$ T_R in $WASp^{-/-}$ mice and in human patients. As naïve and activated $Foxp3^-$ T cells rely on survival signals distinct from IL-2, this difference may further contribute to the overall reduction in peripheral T_R vs. T effector pool size observed in $WASp^{-/-}$ mice.

While $WASp^{-/-}$ mice exhibit a marked defect in T_R activation and function, the autoimmune features of $WASp^{-/-}$ mice and WAS patients are less severe than those present in either Foxp3 mutant mice or humans. Previous studies of WASp function may provide insight into these differences. First, concurrent defects in T effector cell activation may offset deficient T_R function by limiting basal self-reactivity, expansion, or survival of the effector pool. Second, the initial events driving T effector cell activation may also be blunted due to intrinsic alterations in antigen presenting cell function. Third, altered T-helper or follicular dendritic cell function might limit the capacity of activated B cells to generate high affinity autoantibodies.

Human *WAS* carriers exhibit non-random X-inactivation in hematopoietic lineages including T_R due to a competitive advantage for $WASp^+$ hematopoietic stem cells(162). This effect normally precludes any analysis of the relative competitive advantage for WASp function within human T_R. However, identification of a WAS patient with a revertant mutation presumably affecting a lymphoid stem cell allowed us to directly demonstrate relative selective advantage for WASp expressing T_R in humans. At present, our findings are insufficient to directly link this improvement in competitive fitness with the clinical improvement in this individual patient. However, in light of our

data in WASp$^{-/-}$ animals, these observations strongly suggest that alterations in T_R function may explain the high frequency of autoimmunity in WAS patients. Notably, two previous groups have identified WAS pedigrees with revertant mutations in lymphoid progenitors(181-183). Evaluation of WASp expression within T_R in these other pedigrees; and correlation of these data with autoimmune manifestations, and analyses of the TCR repertoire of expanded T_R in the patient described here and by others, should provide important additional insight into the events mediating homeostatic T_R activation.

Materials and Methods

Animals

129 WASp$^{-/-}$ and *sf*/Bl6 mice were obtained from Jackson Laboratories. All studies were performed using both the 129 WASp$^{-/-}$ (167) and a Bl6 WASp$^{-/-}$ strain generated by backcrossing with Bl6 mice for 6-10 generations; and WT controls. Mice were maintained in SPF facilities of Seattle Children's Hospital or the Benaroya Research Institute and handled according to NIH and institutional guidelines.

Cells and reagents

Single cell suspensions were prepared form lymphoid tissues (thymus, spleen, peripheral and mesenteric lymph nodes.) Peyers patches were excised from the intestine, disrupted into single cell suspension and filtered through 100um nytex for lymphocytes. Peritoneal or bronchiolar fluids were collected after lavage with cold sterile PBS. Erythrocytes were depleted by lysis with NH4CL2 solution. Murine cells were cultured in RPMI with 10% fetal calf serum (FCS) plus supplement (glutamine, 2-mercaptoethanol, penicillin and streptomycin and 10mM Hepes). Peripheral blood was obtained from WAS patients, carriers, and controls following informed consent obtained according to the Institutional

Review Board guidelines of Children's Hospital of Seattle. Human peripheral blood mononuclear cells were isolated using with Ficoll-hypaque (Amersham-Pharmacia) gradient centrifugation as previously described. Platelets were separated by low speed centrifugation.

Flow cytometry

For cell-surface staining, 10^6 cells per sample were incubated with various antibodies in staining buffer (PBS and 3% FCS) for 15 minutes on ice. Anti-murine antibodies included: CD25 (PC61.5), CD8 (53-6.7), CD62L (MEL-14), CD3 (1452C11), Gr-1 (RB6-8C5) and CD103 (2E7) from eBiosciences; CD4 (RM4-5), CD44 (IM7), CD69 (H1.2F3), CD11b (M1/70) from BD-Pharmigen. Anti-human antibodies included: CD27 (O323), CD62L (Dreg56) from eBiosciences; CD4 (RPA-T4), CD27 (M-T271), CD45RA (HI100 and L48) from BD Pharmigen; and CD4 (13B8.2) from Beckman Coulter. Chemokine-IgG3 fusion proteins were used for flow cytometry of CCR4, CCR6, and CCR7 expression as previously described(184). To assess binding of $CD4^+$ T cells to P- and E-selectin, cells were sequentially incubated in either a P- or E-selectin– human IgM fusion protein (provided by J. Lowe, University of Michigan; produced in COS-7 cells as previously described(185), followed by biotinylated goat anti–human IgM (Jackson ImmunoResearch) and streptavidin–PE (eBioscience). Murine and human FoxP3 antibody staining reagents were used according to the eBioscience or Biolegend protocols, respectively. Purified polyclonal rabbit anti-WASp antibody was generated as previously described(186) and intracellular staining was performed according BD Pharmigen Fixation/Permeabilization Solution Kit using FITC, Cy5, or PE-conjugated anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch or Southern

Biotechnology Associates). Data were acquired on a FACSCalibur or a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software.

In vitro suppression assays

For CSFE assays, $CD4^+CD25^-$ (effector) cells and $CD4^+CD25^+$ (T_R) cells were isolated from spleen and LN of WT or $WASp^{-/-}$ mice via CD4 negative isolation kit (Dynal) followed by separation of $CD25^+$ and $CD25^-$ fraction by CD25-PE plus anti-PE microbeads and fractionation via magnetic column (Miltenyi Biotech). CD4-negative spleen cells from WT mice were irradiated 5000 rads and used as APC's in all cultures. Effector cells were incubated for 9 min at 37^0 in 0.8 uM CFSE in PBS, washed with 100% FBS, resuspended in complete DMEM, and cultured as described in Figure 5.

ELISA assays

For detection of anti-dsDNA antibodies, 96 well Immuno-Plates (Nunc) were coated with 0.01% poly-L-lysine solution in PBS (Sigma) and then with 100 ug/ml salmon sperm dsDNA from calf thymus (Sigma). After blocking with 0.5% BSA/PBS, serially diluted serum samples in 0.05% Tween 20/0.5% BSA/PBS were added to the plates in triplicate. Plates were washed with 0.05% Tween 20/PBS using a plate washer (SkanWasher 400; Molecular Devices), and goat-anti-mouse IgG HRP (Southern Biotechnology) diluted 1/2000 into 0.05% Tween 20/0.5% BSA/1% goat serum/PBS was added to each well. Peroxidase reactions were developed using BD Bioscience OptEIA TMB substrate and stopped with 2N H2SO4. OD405 was read using Victor 3 multilabel microplate counter (Perkin Elmer). The OD readings from three wells were averaged for each serum sample.

Murine BM transplants and adoptive transfer purified T_R

For mixed BM chimera analysis, WT and $WASp^{-/-}$ BM was isolated, RBCs were lysed,

and 10^7 total BM cells (containing 1:3 ratio WT and KO cells) were transplanted via intravenously injection into lethally irradiated (1050rads) $WASp^{-/-}$ recipient animals. Peripheral blood was periodically analyzed for WASp expression in different hematopoietic lineages. For neonatal transfers, $CD4^+CD25^+T_R$ cells (>85% purity in all experiments) were isolated from the spleen and LN of 8-12 wk old WT and $WASp^{-/-}$ mice (Bl6 strain) as described for the CFSE assay. A minimum of 10^6 T_R were transferred in 20ul PBS via IP injection into >3 day old male neonatal *sf* mice alone; or as a 50:50 ratio of WT and $WASp^{-/-}$ T_R. Mice were monitored for *sf* phenotype and bled to measure donor chimerism (via staining for CD45.1, CD45.2, CD4 and Foxp3) starting at 14d and sacrificed between 30 and 50d post-transfer for full histological and phenotypic analysis.

Tissue histology

Tissues were immersion fixed in 10% neutral buffered formalin, processed into paraffin, and stained with hematoxylin and eosin (H&E), periodic acid Schiff's (PAS) or periodic acid silver methenamine by standard protocols. Immunofluorescence was done on acetone fixed frozen sections, as previously described.

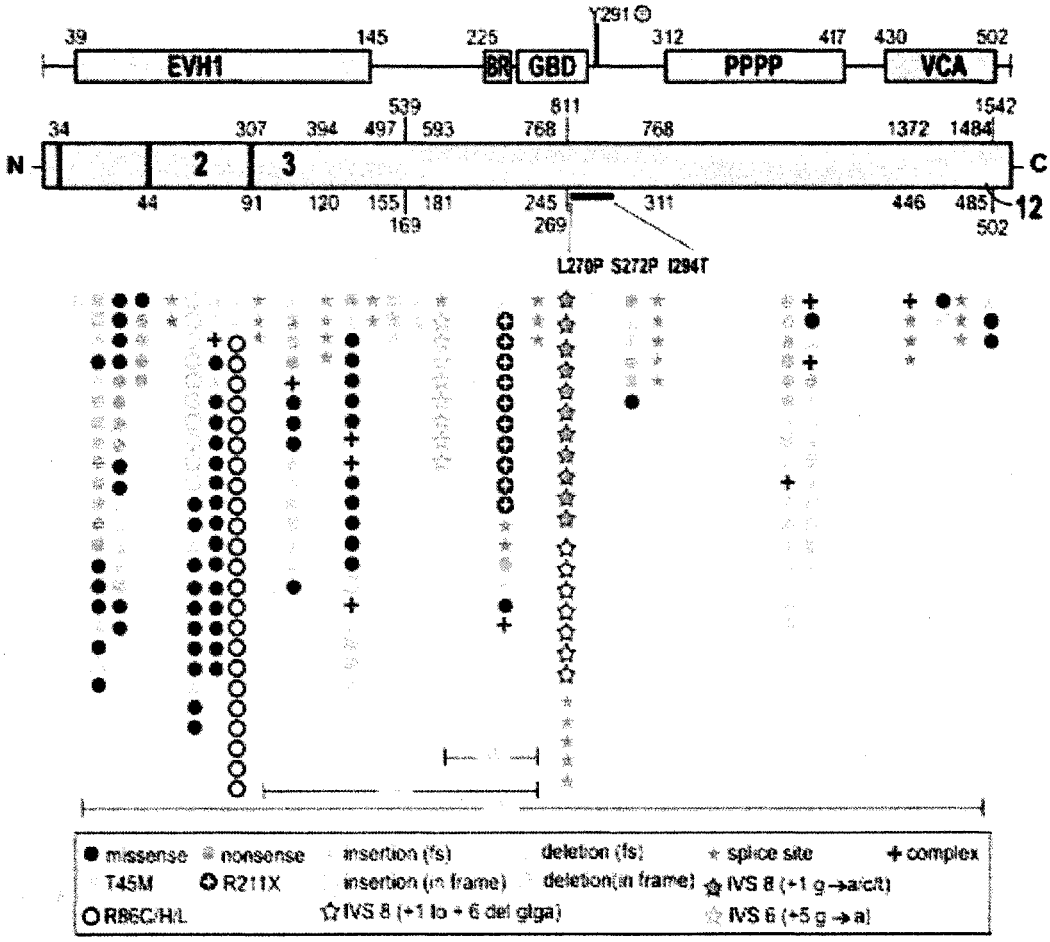


Figure 26.

Schematic representation of the WASP gene: The WASP gene encodes a protein with 12 exons and 5 major functional domains. The mutations identified in 270 unrelated WAS families are visualized according to their location in the exons and the exon-intron junctions. Each symbol represents a single family with WASP mutation. Missense mutations are located mostly in exons 1 through 4. Deletions, insertion and nonsense mutations are distributed throughout the WASP gene. Splice-site mutations are found predominantly in introns 6,8,9 and 10. The symbols for specific WASP mutations shown in the box represent 6 hots spots where mutations are commonly found. *EVH1*, Ena/VASP homology domain; *BR*, basic region; *GBD*, GTPase binding domain; *PPPP*, Proline-rich region, *VCA*, verpolin cofilin homology domains/acidic region.

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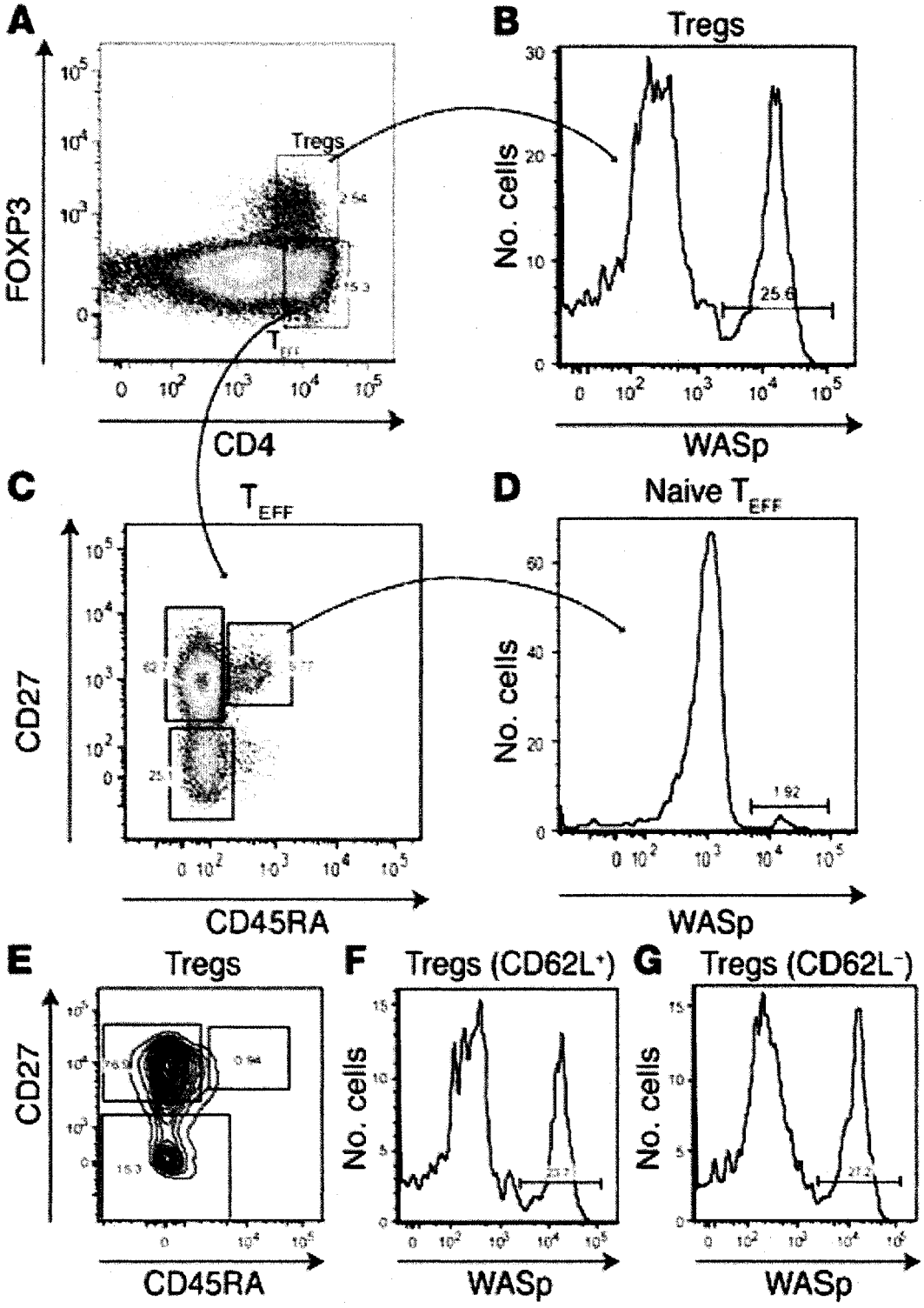
Figure 27.

WT T_R are expanded in a WAS patient following reversion of a pathogenic mutation: Peripheral blood mononuclear cells were analyzed by flow cytometry using antibodies to CD4, CD45RA, CD27, CD62L, WASp, and Foxp3. (**A**) Characterization of the $CD4^+Foxp3^+$ (T_R) and $CD4^+FOXP3^-$ (T_{eff}) cell populations within the total lymphocyte gate. (**B**) WASp expression within the $CD4^+Foxp3^+$ T_R population demonstrating that ~25% of the patient's T_R are $WASp^+$. (**C**) Identification of the naïve $CD4^+CD27^+CD45RA^+$ T cells within the $CD4^+Foxp3^-$ T_{eff} cell population. Naïve cells comprise ~7% of the T_{eff} population in this patient. (**D**) Within the naïve T cell compartment, only a small proportion of the cells (~2%) are $WASp^+$. (**E**) In comparison to the T_{eff} population, very few (<1%) $CD27^+CD45RA^+$ naïve cells are present within the$CD4^+Foxp3^+$ T_R population, $WASp^+$ cells comprise equal percentages of the $CD62L^+$ (**F**) and $CD62L^-$ (**G**) T_R subsets.


A
FOXP3
CD4
Tregs
B
Tregs
No. cells
WASp
C
T_{EFF}
CD27
CD45RA
D
Naive T_{EFF}
No. cells
WASp
E
Tregs
CD27
CD45RA
F
Tregs (CD62L$^+$)
No. cells
WASp
G
Tregs (CD62L$^-$)
No. cells
WASp

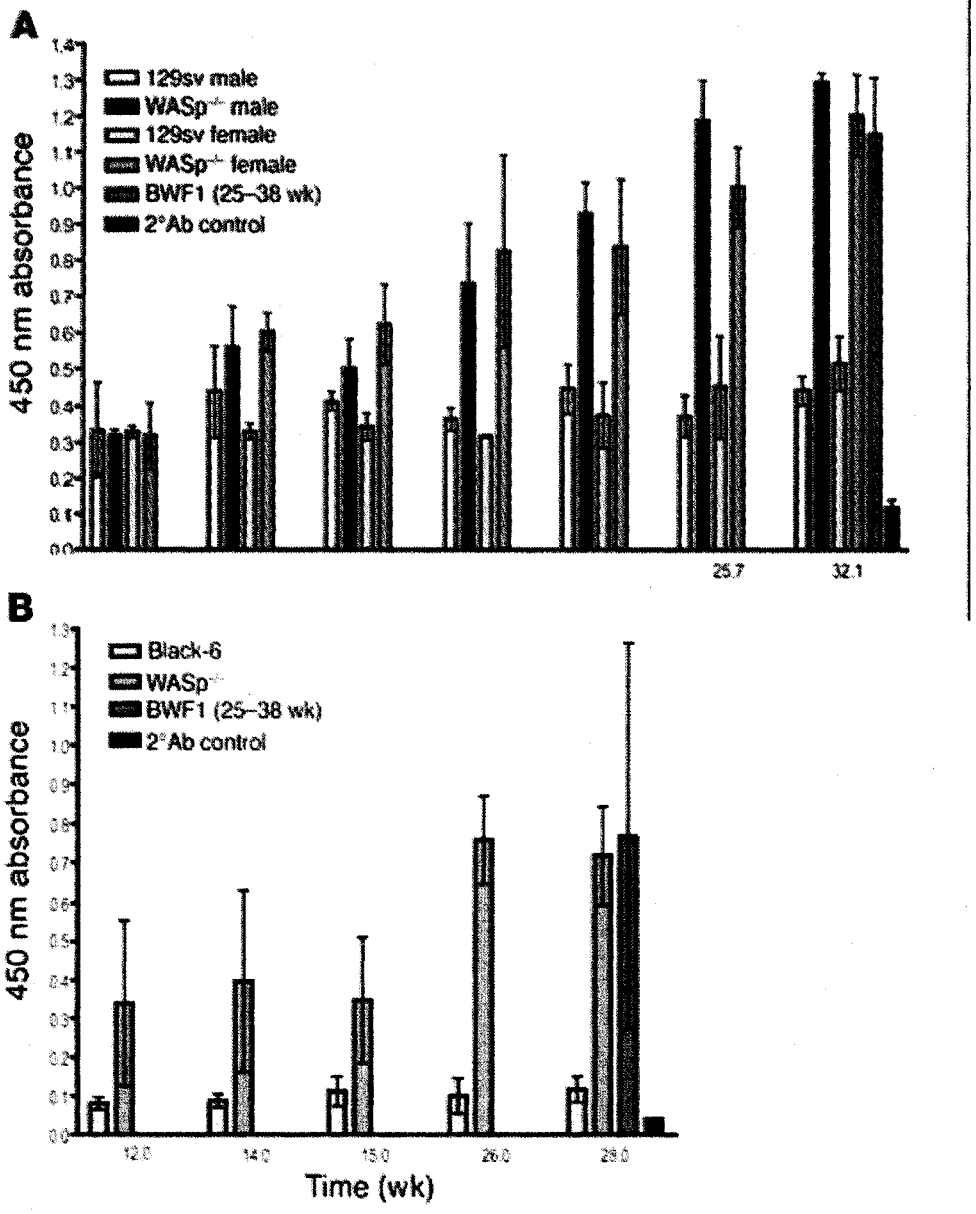


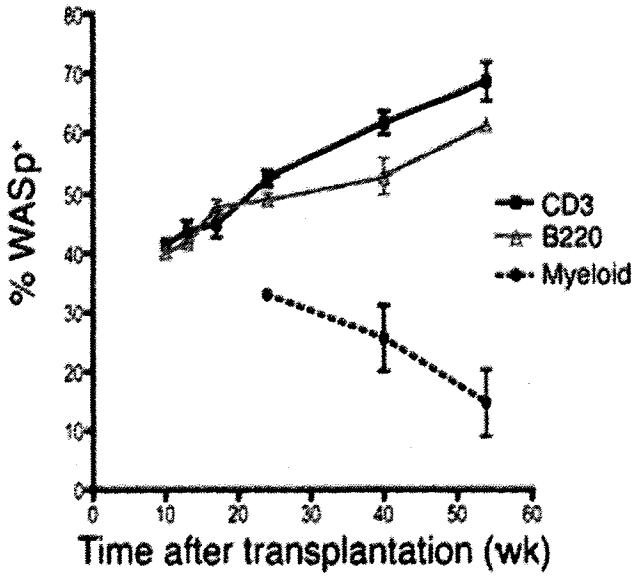
Figure 28.
WASp$^{-/-}$ mice develop high titer autoantibodies: Presence of IgG ds-DNA autoantibodies in the serum of (**A**) 129, or (**B**) Bl6 WASp$^{-/-}$ mice vs. age and sex matched WT controls; or 25-38 wk old BWF1 mice (positive control). Antibody titers were assessed by ELISA (n=3-6 animals for each strain/sex). "2°Ab" indicates background values obtained using secondary antibody alone. Error bars represent standard deviation.

Figure 29.

Chimeric BM transplantation promotes WT T_R expansion: (**A**) WASp expression within T and B cells (CD3 and B220 respectively) but not myeloid cells allows for a competitive advantage over time in lethally irradiated $WASp^{-/-}$ recipients (129 strain) transplanted with a 1:3 mixture of WT to $WASp^{-/-}$ BM cells. Peripheral blood from 5 mice was serially analyzed by flow cytometry at the indicated times post-transplant to determine the percentage of $WASp^{+}$ T, B, or myeloid cells.

(**B**) The selective advantage of WASp-expressing cells within the T cell compartment is most marked in the peripheral T_R subset. $WASp^{-/-}$ mice (129 strain) were transplanted as in (**A**) and WASp expression among the indicated T cell populations was evaluated 12 months post-transplant. $WASp^{+}$ myeloid cells remain at the same percentage as originally transplanted (~25%) indicating no selective advantage. Recipients of WT: $WASp^{-/-}$ mixed BM transplants did not develop fatal, radiation-induced colitis which occurred in all recipients of $WASp^{-/-}$ BM (data not shown). DP=$CD4^{+}CD8^{+}$ thymocytes; PLN Tregs= peripheral lymph node T_R.

A


% WASp+
Time after transplantation (wk)
CD3
B220
Myeloid
0
10
20
30
40
50
60
70
80

B

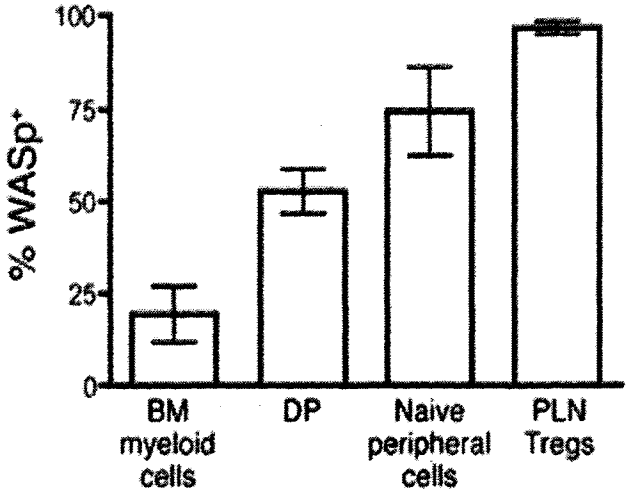
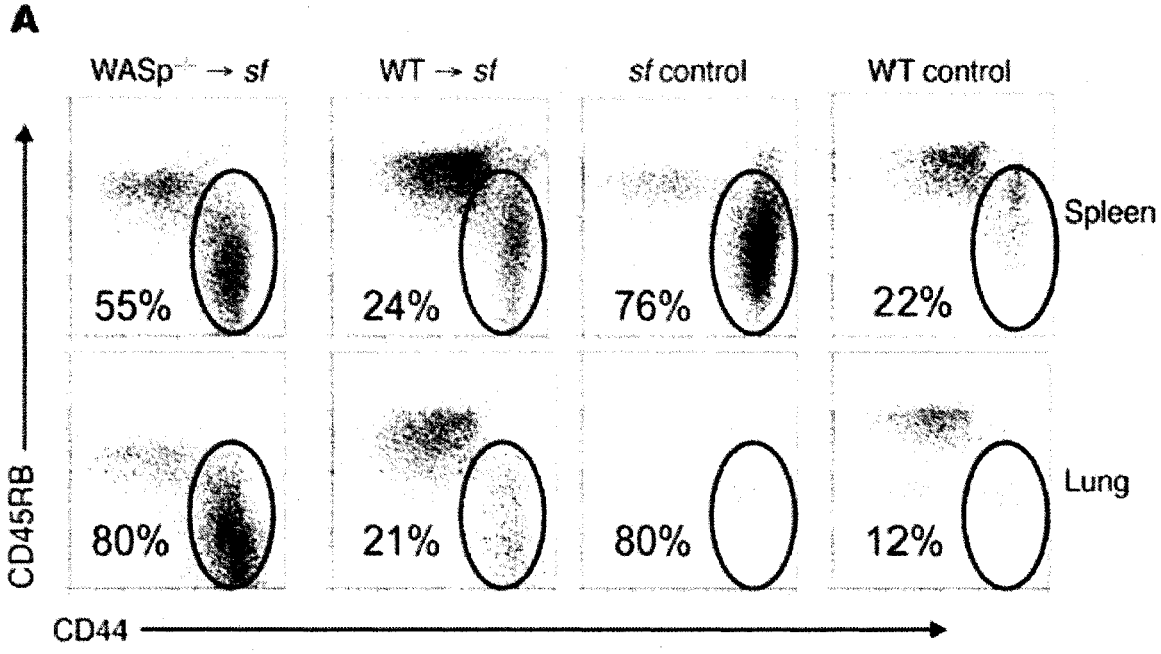

% WASp+
100
75
50
25
0
BM myeloid cells
DP
Naive peripheral cells
PLN Tregs

Figure 30.

$WASp^{-/-}$ T_R fail to control aberrant T cell activation in sf mice: Male CD45.1 *sf* neonates (>3 days of age) were injected IP with 1-2x10^6 CD4+CD25+ enriched WT or $WASp^{-/-}$ T_R (both CD45.2); sacrificed at 30-45 days post cell transfer; and evaluated for levels of T cell activation and tissue inflammation. (**A**)WT T_R but not $WASp^{-/-}$ T_R prevent development of activated *sf* lymphocytes. Lymphocytes isolated from the spleen and lung parenchyma of recipient mice were stained for CD4, CD45.2, CD44 and CD45RB. The relative percentage of activated $CD44^{hi}CD45RB^{low}$ T cells in each tissue is shown. All plots are gated on $CD4^+CD45.1^+$ cells to identify recipient-derived cells and donor cell source is indicated above each panel. Controls included age-matched, unmanipulated *sf* and WT animals. (**B**) The graph represents the percentage of $CD44^{high}CD45RB^{low}$ activated recipient-derived cells (CD4+CD45.1+) among all recipient animals. ($WASp^{-/-}$ T_R donor (n=5) or WT T_R donor (n=2).


A
WASp−/− → sf
WT → sf
sf control
WT control
55%
24%
76%
22%
80%
21%
80%
12%
Spleen
Lung
CD45RB
CD44

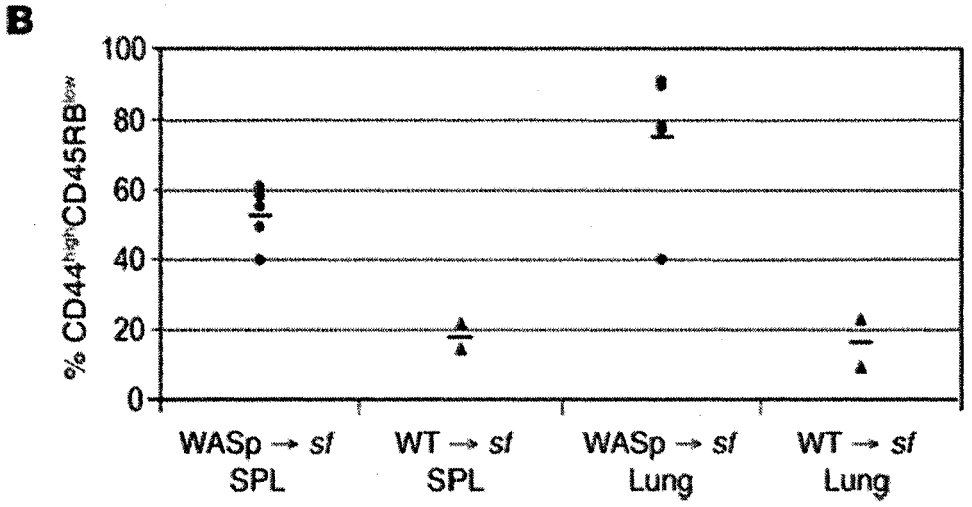

B
% CD44high CD45RBlow
100
80
60
40
20
0
WASp → sf SPL
WT → sf SPL
WASp → sf Lung
WT → sf Lung

Figure 31.

$WASp^{-/-}$ T_R fail to control autoimmune-mediated tissue damage in sf mice. Male CD45.1 *sf* neonates (>3 days of age) were injected IP with 1-2x10^6 CD4+CD25+ enriched WT or $WASp^{-/-}$ T_R (both CD45.2); sacrificed at 30-45 days post cell transfer; and evaluated for tissue inflammation. WT T_R but not $WASp^{-/-}$ T_R rescue *sf* mutant mice from development of autoimmune infiltration of major organs. Formalin fixed liver and lung tissue from *sf* mice that received WT vs. $WASp^{-/-}$ T_R were paraffin embedded, sectioned, and stained with H&E to visualize tissue structure and inflammatory cell infiltration. Liver and lung sections from unmanipulated *sf* and WT mice are shown for comparison (all photos 10X magnification).

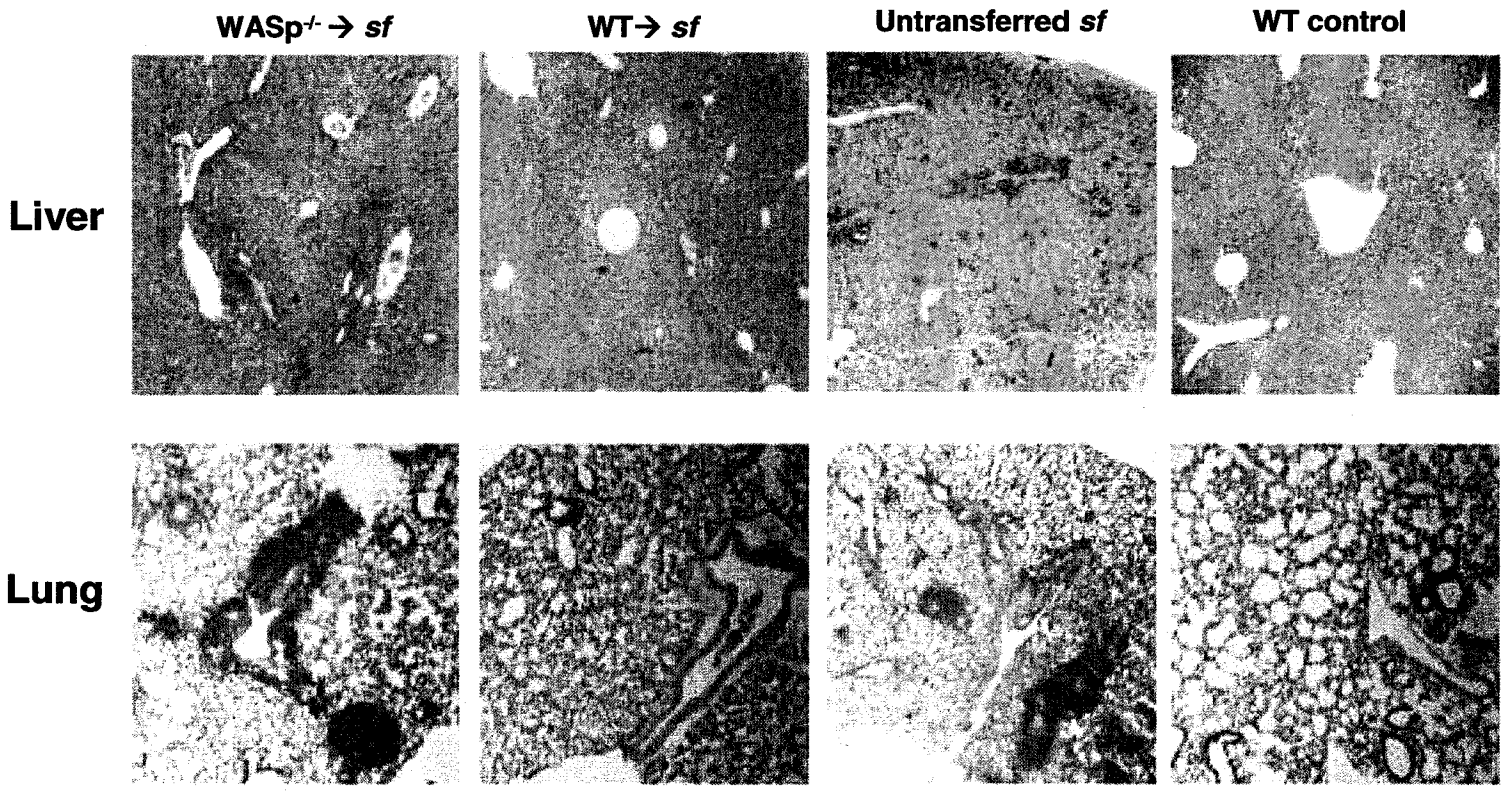

WASp-/- → sf
WT→ sf
Untransferred sf
WT control
Liver
Lung

Figure 32.

WASp is not required for generation of T_R within the thymus: (**A**) WASp is not required for the production of peripheral T_R. Peripheral lymph node cells from WT or $WASp^{-/-}$ animals were stained simultaneously for CD4, CD25, and Foxp3 and evaluated by flow cytometry. Note that $Foxp3^+$ T_R are present in $WASp^{-/-}$ mice albeit at a slightly decreased percentage.

(**B**) $WASp^{-/-}$ and WT mice have a similar percentage of T_R ($CD4^+$ $CD25^+Foxp3^+$) cells within the $CD4^+$ SP thymic population. 6 and 16 wk old WT or $WASp^{-/-}$ Bl6 mice (n=5 for each age and strain) were evaluated. (**C**) The selective advantage of $WASp^+$ T cells is not manifest in the thymus. The percentage of $WASp^+$ cells was evaluated within various thymic cell subsets in 6-8 wk old $WASp^{+/-}$ heterozygote female carriers (Bl6 strain) (n=5). Error bars show standard deviations. Relative WASp expression was not significantly different among any subset evaluated. DN=$CD4^-CD8^-$, DP=$CD4^+CD8^+$ T cells. Representative data from 1 of at least 3 experiments are shown.

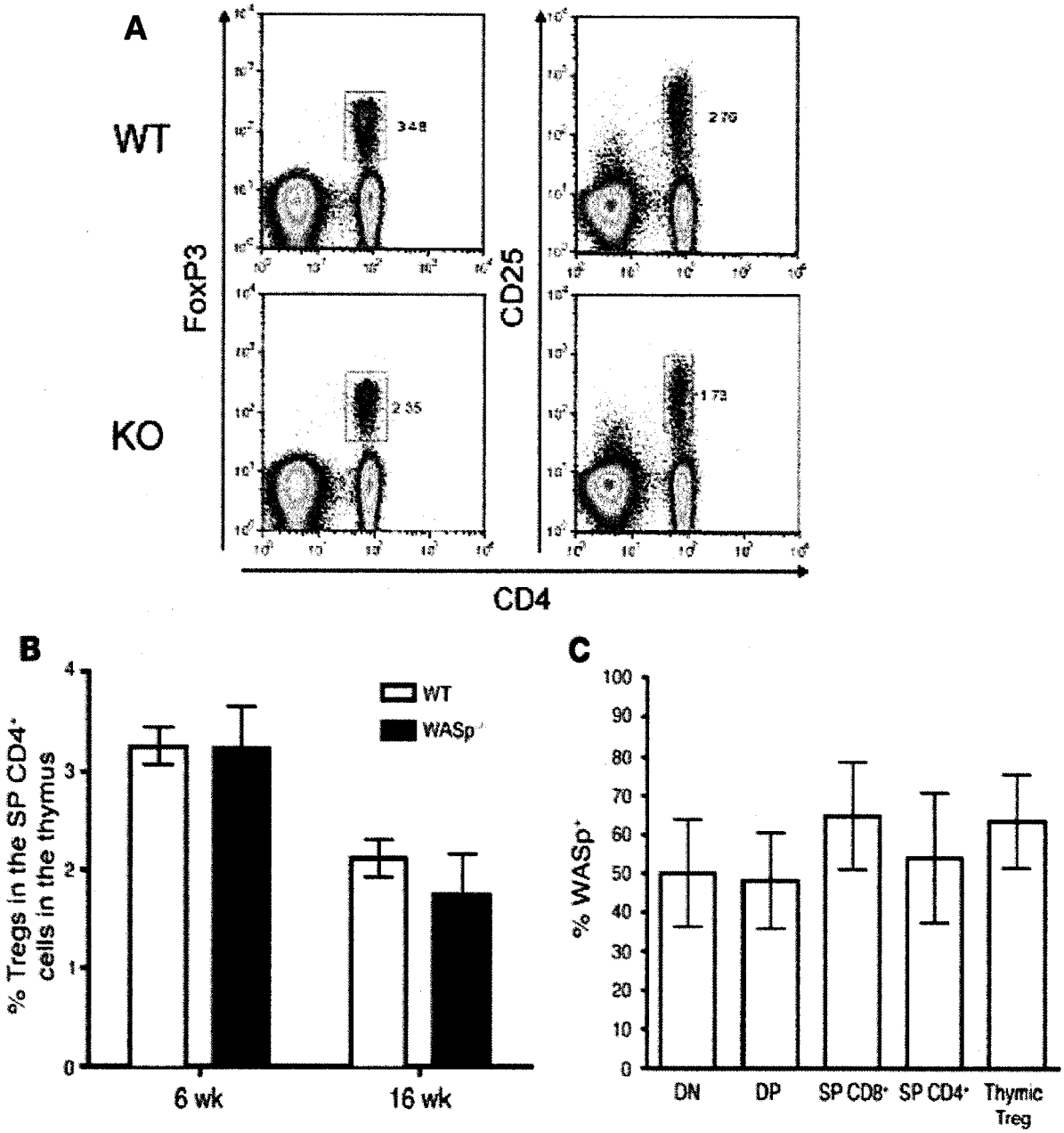
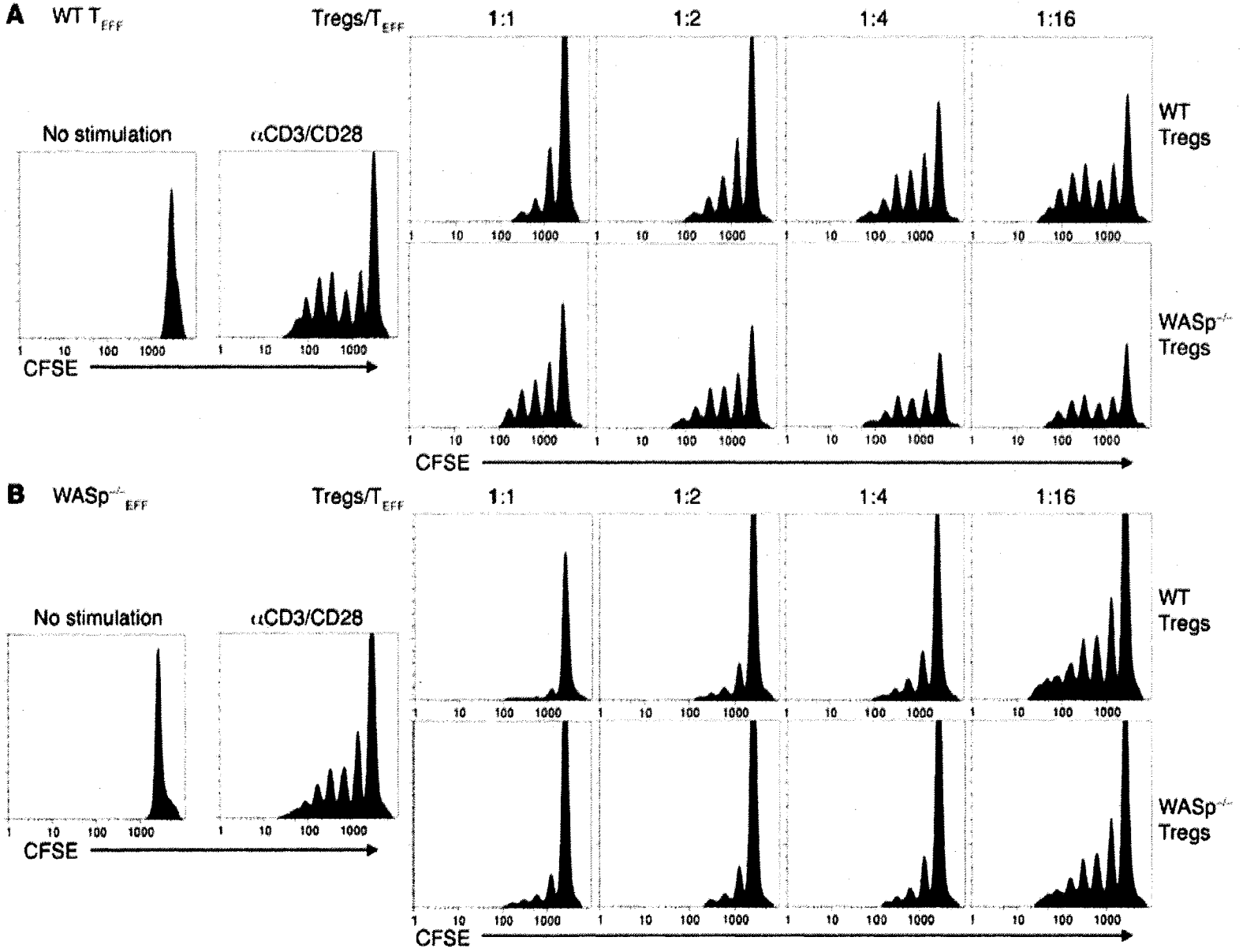

A
WT
KO
FoxP3
CD25
CD4
3.48
2.79
2.35
1.73
B
% Tregs in the SP CD4+ cells in the thymus
WT
WASp-/-
6 wk
16 wk
C
% WASp+
DN
DP
SP CD8+
SP CD4+
Thymic Treg

Figure 33.

$WASp^{-/-}$ T_R exhibit in vitro suppressive activity: $CD4^+CD25^-$effector T cells (T_{Eff}) and $CD4^+CD25^+$ (T_R) cells were isolated from WT or $WASp^{-/-}$ mice (129 strain). WT or $WASp^{-/-}$ T_{Eff} were labeled with CSFE and plated as targets with WT or $WASp^{-/-}$ T_R at the T_R : T_{Eff}(target) ratios noted in the presence of irradiated APC. Cultures were stimulated with 3μg/ml αCD3 and 1ug/ml αCD28 for 110 hours. Relative CFSE dilution was measured in cultures containing WT (**A**) or $WASp^{-/-}$ (**B**) targets. Unstimulated and control stimulated (αCD3/CD28 without T_R) cells are shown in the left panels.


A
WT T_{EFF}
Tregs/T_{EFF}
1:1
1:2
1:4
1:16
No stimulation
αCD3/CD28
WT Tregs
WASp$^{-/-}$ Tregs
CFSE
1
10
100
1000
B
WASp$^{-/-}$ $_{EFF}$
Tregs/T_{EFF}
1:1
1:2
1:4
1:16
No stimulation
αCD3/CD28
WT Tregs
WASp$^{-/-}$ Tregs
CFSE

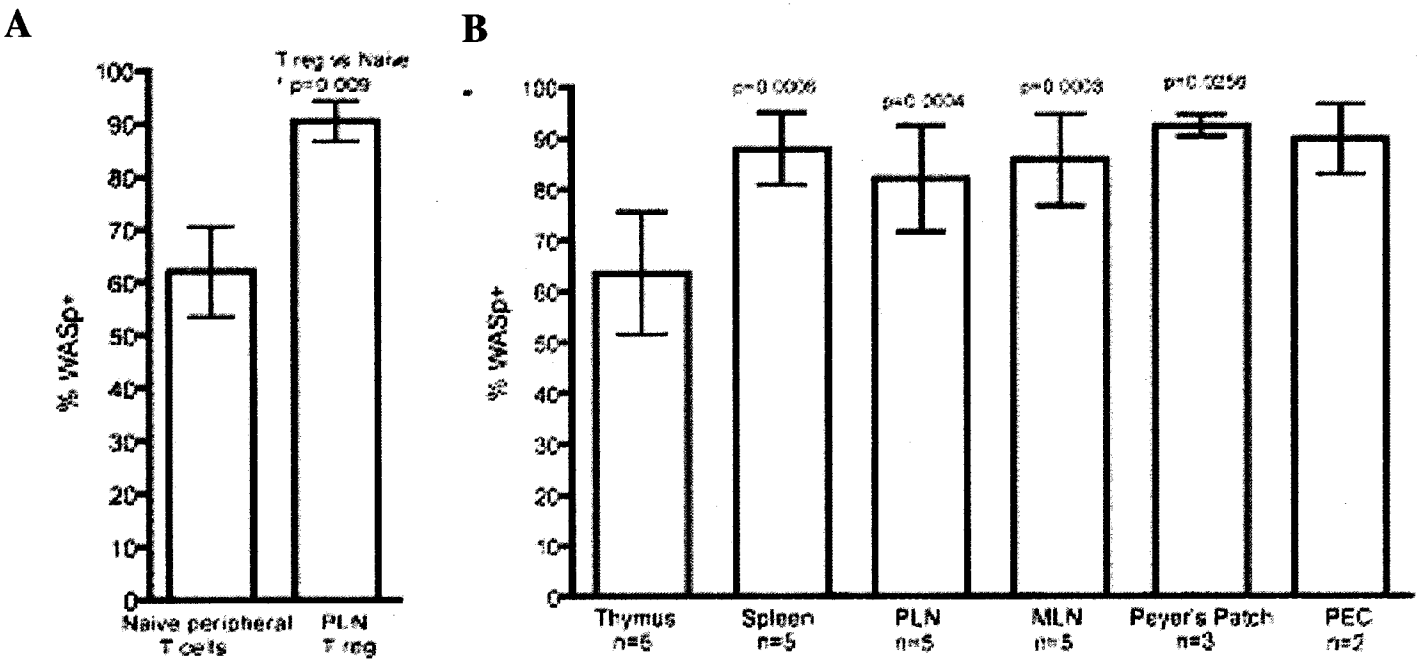


Figure 34.

WASp$^{-/-}$ T_R demonstrate a competitive disadvantage in vivo: (**A**) Heterozygote female carriers (6 mo) demonstrate marked skewing within the T_R population. Naïve T cells from peripheral blood ($CD3^+/CD62L^+/CD44^-$) and T_R cells from peripheral lymph node ($CD4^+/CD25^+/CD69^-$) were analyzed for relative WASp expression by flow cytometry. The mean with standard deviation is shown with the results of the paired t test. Identical results were obtained using $CD4^+Foxp3^+$ staining to identify T_R . (**B**) WASp confers selective advantage during peripheral maturation/expansion of T_R cells. The T_R population in different tissues from 6-8 wk old $WASp^{+/-}$ heterozygote mice (Bl6 strain) was evaluated for WASp expression by flow cytometry gated on the $CD4^+Foxp3^+$ population. Paired t test results indicate significant differences between peripheral lymphoid and thymic T_R .

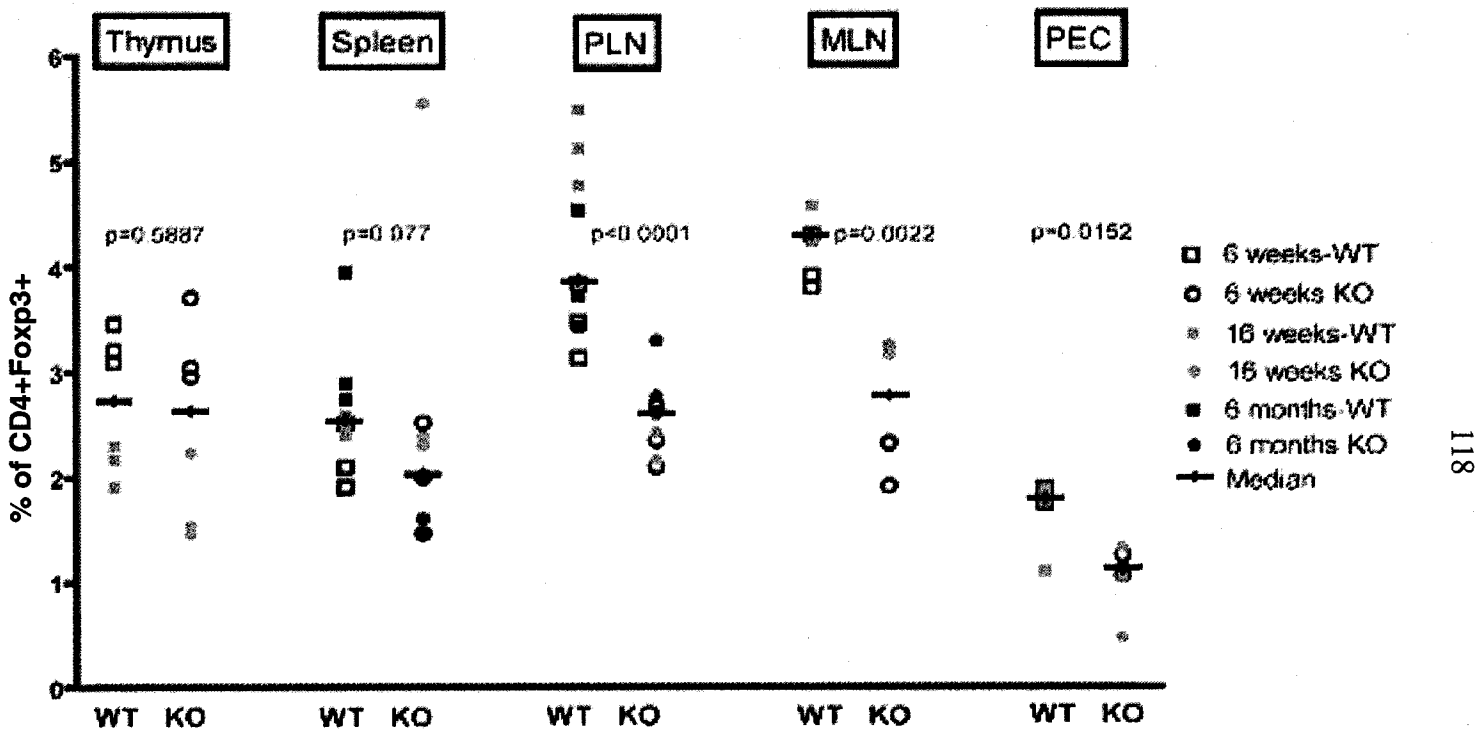
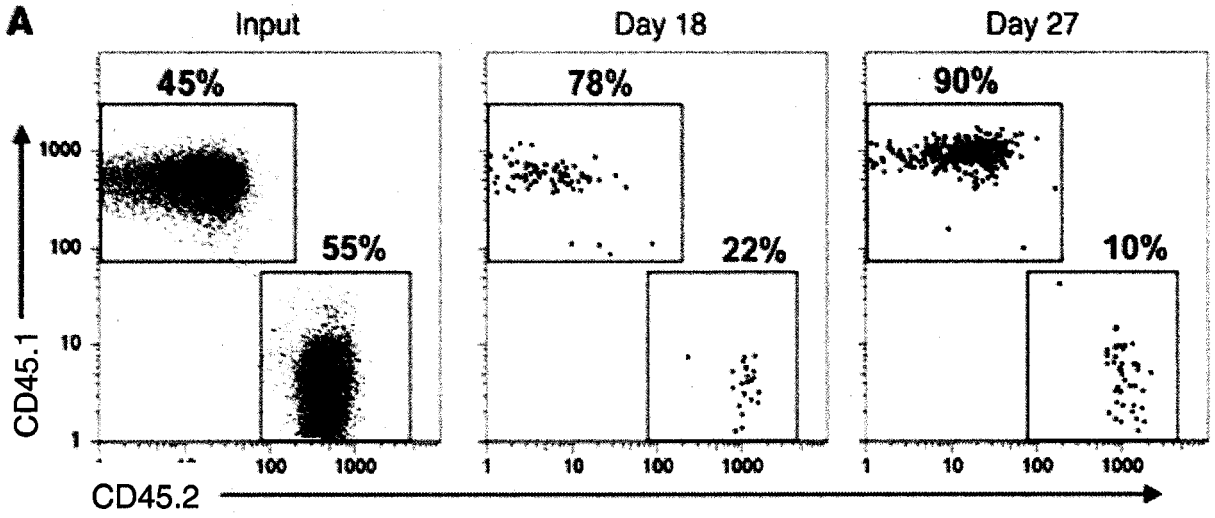


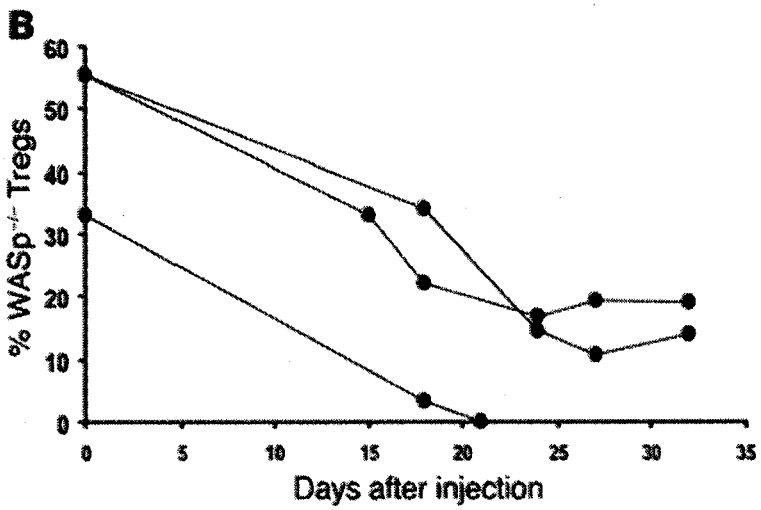
Figure 35.

$WASp^{-/-}$ T_R are not sustained efficiently in the periphery: $WASp^{-/-}$ mice have lower T_R numbers than WT mice. $CD4^+Foxp3^+$ T_R were evaluated in different tissues in WT vs. $WASp^{-/-}$ Bl6 mice (ages 6 wk to 6mo) and displayed as relative percentage of T_R within the live cell gate. Pooled data were compared using the Mann-Whitney test.

Figure 36.

Purified WASp$^{-/-}$ T_R fail to expand and compete effectively in vivo: Male *sf* neonates (>4-5 days of age; CD45.1/CD45.1 heterozygotes) were injected IP with a 50:50 mixture of WT (CD45.1) and WASp$^{-/-}$ (CD45.2) CD4$^+$CD25$^+$ T_R. PBL samples were analyzed at bi-weekly intervals starting at 14 days of age to measure the relative levels of donor T_R cells. (**A**) Representative temporal analysis of the relative numbers of WT vs. WASp$^{-/-}$ T_R . (*Left panel*) FACS analysis of input CD4+CD25+ enriched WT: WASp$^{-/-}$ T_R cell mixture stained for CD45.1 and CD45.2 (*Middle and right panels*) Analysis of the relative level for each donor population within the CD4$^+$Foxp3$^+$ gate at time-points indicated. PBL samples were co-stained for CD4, FoxP3, CD45.1 and CD45.2.(**B**) Graphic depiction of the relative percentage of WASp$^{-/-}$ T_R remaining at each time-point in 3 animals based upon phenotypic analysis as described in (**A**).


A
Input
Day 18
Day 27
45%
55%
78%
22%
90%
10%
CD45.1
CD45.2
1000
100
10
1
100
1000
1
10
100
1000


B
% WASp-/- Tregs
Days after injection
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35

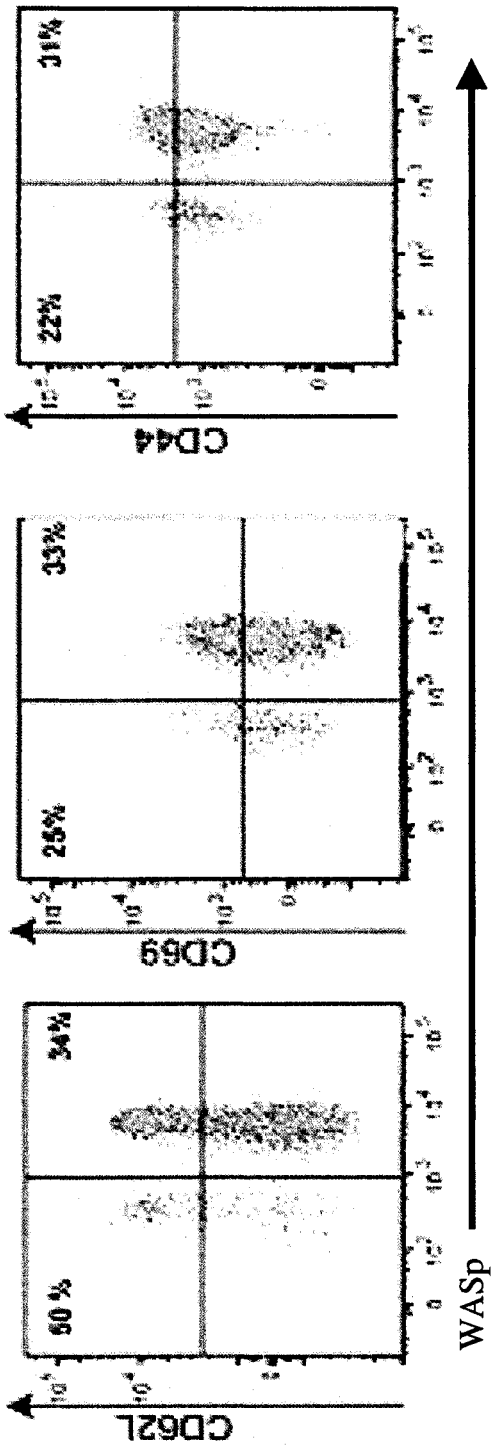


Figure 37.
WASp$^{-/-}$ T_R *consistently show a less activated phenotype:* Lymphocytes were isolated from the peripheral lymph nodes of 6 wk old WASp$^{+/-}$ heterozygous female mice.The cells were stained for CD4, Foxp3, WASp and the activation markers CD62L, CD69 and CD44. Percentages shown are of the total WASp^{+} or WASp^{-} population in each plot.

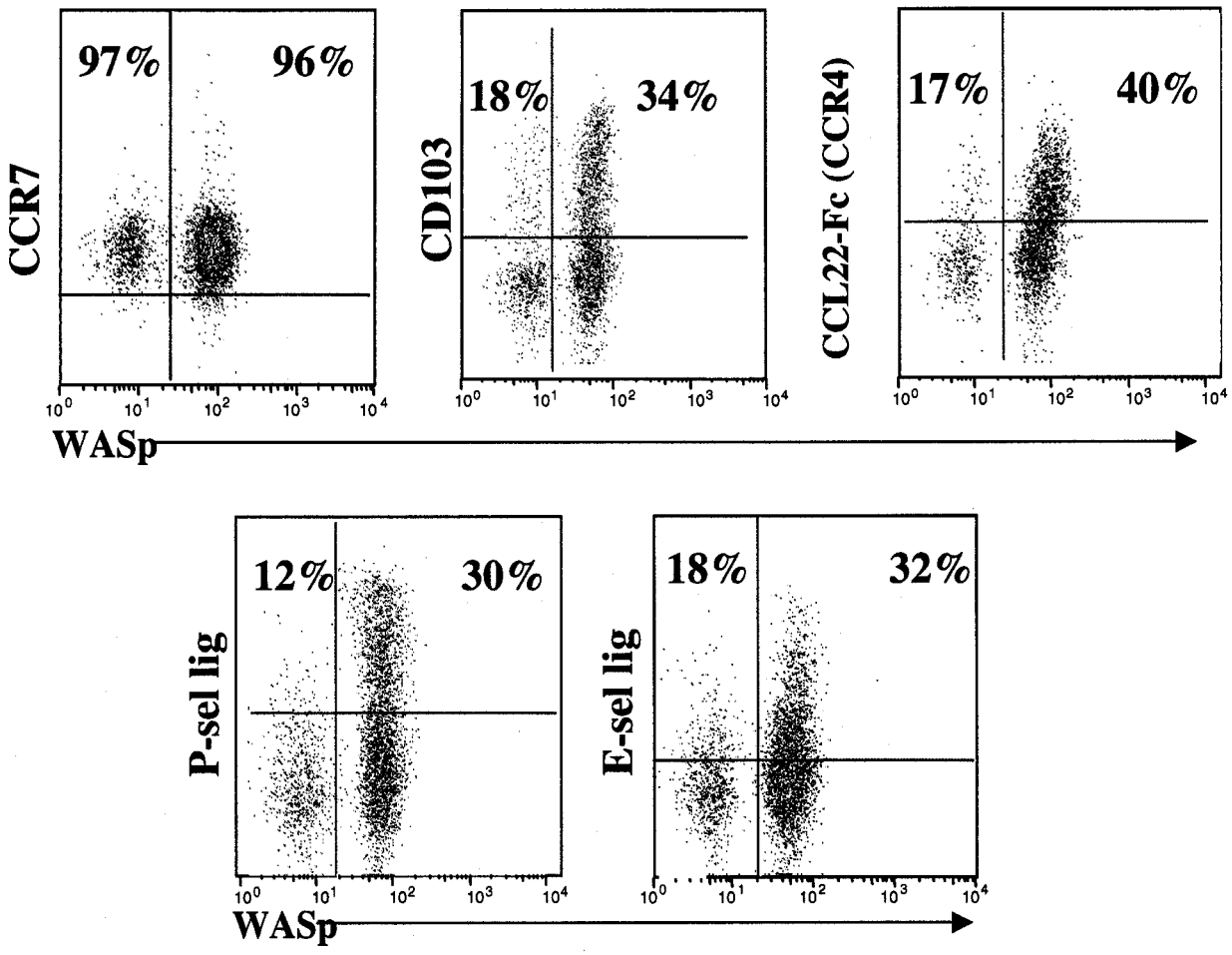


Figure 38.
WASp$^{-/-}$ T_R show a less differentiated phenotype: Lymphocytes were isolated from the peripheral lymph nodes of 6 wk old WASp$^{+/-}$ heterozygous female mice.The cells were stained for CD4, Foxp3, WASp and the homing and adhesion receptors CCR7, CD103, CCR4 (CCL22-Fc), P-selectin ligand and E-selectin ligand. Cells were gated on CD4+Foxp3+ cells and percentages shown are of the total WASp^{+} or WASp^{-} population in each plot.

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Curriculum Vitae

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Education

1993-1997	University of Colorado: Boulder, CO Bachelor of Arts: Molecular, Cellular and Developmental Biology.
2001-Present	University of Washington School of Medicine Graduate Student: Department of Immunology PhD program

Research and Work Experience

Fall 2003 –Present	PhD Candidate: Laboratory of Dr. Daniel Campbell Benaroya Research Institute at Virginia Mason Hospital *Thesis focus* - Exploring the homing properties of CD4+Foxp3+ T_R cells - Specifically the role of CCR4 expression on the localization of T_R to the skin and lungs
2002-2003	Graduate student: Laboratory of Dr. Gerald Nepom Benaroya Research Institute at Virginia Mason Hospital *Research Focus* – Studying T cells specific for pancreatic antigens and their role in Type I diabetes progression
Fall 2003	Teaching Assistant: Undergraduate Immunology at University of Washington Medical School Weekly lecture to 40 students, in addition to writing and grading exams
Winter 2002	Rotating Graduate Student: Laboratory of Dr. Alexander Rudensky University of Washington Department of Immunology
Fall 2001	Rotating Graduate Student: Laboratory of Dr. Phil Greenberg University of Washington Department of Immunology
1999-2001	Research Technician II: Laboratory of Dr. Joan Goverman University of Washington Department of Immunology
1998-1999	Marketing Representative: Restorative Care of America – medical brace company Territory: Washington and Oregon
1997-1998	Laboratory Technician and Phlebotomist: Boulder Community Hospital Boulder, CO
1997-1998	Undergraduate Researcher: Laboratory of Dr. David Prescott University of Colorado Department of Molecular, Cellular and Developmental Biology Boulder, CO *Project 1:* Actin gene evolution in ciliated protozoa *Project 2:* Study of cellular DNA structures using transmission electron microscopy

1995-1998 Research Assistant: Boulder Laboratory for Three-Dimensional Fine Structure
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Publications

Brabb T, von Dassow P, Ordonez N, Schnabel B, **Duke B** and J. Goverman. In Situ tolerance within the central nervous system as a mechanism for preventing autoimmunity. *J. Exp Med.* 2000; 192:871-880.

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Awards and Fellowships

2002-2003 Awarded pre-doctoral funding on the Department of Immunology Training Grant through the Benaroya Research Fund

2004-2006 Awarded pre-doctoral funding on the Department of Immunology Training Grant through the National Cancer Institute

Winter 2005	Awarded $1000 pre-doctoral scholarship to attend Keystone Conference in Taos, NM
August 2005	Awarded $1500 Sandra Clarke Travel Fund Scholarship from University of Washington Department of Immunology to attend Gordon Conference in Immunology and Immunochemistry in Oxford, England
Sept. 2006	Awarded travel scholarship to attend RIKEN Research Center for Allergy and Immunology summer program in Tsurami, Japan
Sept. 2006	Awarded best poster award at RIKEN Research Center for Allergy and Immunology summer program in Tsurami, Japan
Nov 2006	Awarded $1000 pre-doctoral scholarship to attend Keystone Conference in Big Sky, MT
Jan. 2007	Awarded the "Ray Owen Best Young Investigator Award" at the Midwinter Conference of Immunologists in Asilomar, CA