T Cell Regulation as a Side Effect of Homeostasis and Competition

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Abstract

We have previously hypothesized that maintaining a balanced peripheral immune system may not be the sole responsibility of a specialized subset of T cells dedicated to immune regulation, but also a side effect of normal competition for shared resources within an intact immune system. Here we show that regulatory activity is correlated with high homeostatic expansion potential, reflecting the avidity for self-peptide:MHC complexes. Monoclonal transgenic T cells with high homeostatic expansion potential and lacking characteristics previously associated with regulatory function were able to regulate wasting disease induced by transfer of a small number of naive CD45RB^{hi} CD4 T cells into lymphopenic hosts. Self-regulatory function is also found in the naive polyclonal T cell repertoire depleted of CD25$^+$ T cells. T cells capable of preventing immune pathology, like the transgenic T cells, express higher than average levels of CD5, an indicator of avidity for self:MHC peptide complexes. We therefore propose that dysregulated expansion of potentially pathogenic T cells in a lymphopenic environment can be prevented by members of the naive T cell repertoire, irrespective of their specificity, as a side effect of their response to homeostatic and antigenic stimulation.

Key words: T cell regulation • homeostasis • lymphopenia • immune pathology • CD4 T cells

Introduction

Regulation or control of responsiveness is an intrinsic property of the immune system. Excessive T cell responses to self-antigens (autoimmune disease) or innocuous environmental antigens (allergy) are usually prevented in the vast majority of individuals. Furthermore, maximal responses to foreign antigens are also normally controlled by a number of mechanisms, including competition between responding T cells. This phenomenon is thought to be responsible for affinity maturation of the T cell response (1) and helps prevent disproportionate damage to self (immunopathology). The control mechanisms that maintain a stable and diverse repertoire of naive and memory T cells in a healthy functional immune system (2, 3) are severely compromised in lymphopenic mice. For instance, thymectomy shortly after birth or transfer of small numbers of T cells into T cell–deficient hosts are consistently associated with immunopathology and an array of autoimmune diseases (4–8). Furthermore, responses to cognate antigens are also exaggerated in lymphopenic hosts (9). Thus, lymphopenia is invariably associated not only with homeostatic expansion of the remaining T cells in response to endogenous MHC ligands and IL-7, but also with immune reactivity both to self- and foreign antigens that would not normally be apparent in T cell–replete hosts.

Suppression of immune reactivity is currently attributed to a population of regulatory T cells dedicated to maintaining peripheral tolerance to self-antigens or preventing harmful immunopathological responses (for a review, see reference 10). Regulatory T cells, obtained from unmanipulated hosts, are defined by markers such as CD25 (11) and CTLA-4 (12, 13) and more recently expression of the glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR; references 14 and 15) and may mediate their function via release of inhibitory cytokines such as TGF-β and IL-10 (16–19) and/or cell contact dependent mechanisms (14, 20). Although T cell–mediated regulation of immune pathology is amply documented, it is not well understood how many different types of regulatory T cells there might be, how they are related and how they function.

We have previously suggested that maintaining a balanced peripheral immune system may not be the sole responsibility of a specialized subset of T cells dedicated to immune regulation, but rather a side effect of normal com-

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petition for shared resources within an intact immune system (21). To demonstrate that T cell competition for shared resources, independent of specificity, or effector function can achieve T cell regulation, we employed one of the most widely used animal models for studies of regulatory T cells. In this model severe gut pathology and wasting disease are induced by transfer of a small number of naive CD45RB^{hi} CD4 T cells into lymphopenic hosts and pathology is prevented by cotransfer of cells from the reciprocal CD45RB^{lo} subset, especially those that also express CD25. To demonstrate that competition for space by T cells that do not have any of the characteristics associated with regulatory T cells, can underlie regulatory activity we employed monoclonal TCR transgenic T cells. We show here that cotransfer of AND T cells with a defined antigenic specificity unrelated to that of the pathological response can protect mice from pathology as well as CD25$^+$CD45RB^{lo} T cells. Regulatory function of AND T cells did not require acquisition of markers previously associated with regulatory function, but was correlated with exceptionally high homeostatic expansion potential. Furthermore, even monoclonal CD8 T cells from the OT-I strain which expand considerably, if less than AND T cells, in lymphopenic hosts afforded at least partial protection.

In addition, we demonstrate here that T cells with properties similar to AND T cells can also be found in the polyclonal naive CD4 T cell repertoire depleted of CD25$^+$ T cells. Such T cells, like AND T cells show increased homeostatic expansion and express high levels of CD5, reflecting their avidity for self-peptide:MHC complexes (22, 23). CD5, a negative regulator of TCR signaling (24), is up-regulated during thymic differentiation and ensures that thymocytes with high avidity for self peptide:MHC complexes are not overtly self-reactive in the periphery because they display lower responsiveness to self-antigen (25). On the other hand, CD5^{hi} T cells possess higher homeostatic expansion potential to self peptide:MHC signals than their CD5^{lo} counterparts (26). Cells that are involved in negative regulation may therefore be members of the naive T cell repertoire that fulfill this function not as their sole purpose, but rather as a side effect of their response to homeostatic and antigenic stimulation.

Materials and Methods

Mice. Mice of strain A (H-2^a) and C57BL/10 (H-2^b), wild-type and backcrossed onto a Rag-1$^{-/-}$ background, and TCR transgenic AND mice, on a Rag-1$^{-/-}$ H-2^a MHC and Rag-1$^{-/-}$ H-2^b background, as well as OT-I Rag1$^{-/-}$ mice were bred and maintained in the animal facilities of the National Institute for Medical Research in accordance with established guidelines.

Flow Cytometry. Conjugated mAb were purchased from BD Biosciences and flow cytometric analyses were performed using standard staining procedures on FACScan™ and FACSCalibur™ cytometers with CELLQuest™ software. For intracellular cytokine stainings mesenteric lymph node cell suspensions were stimulated for 4 h with PdBU and ionomycin (both at 50 ng/ml). For the last 2 h Brefeldin A (10 μg/ml) was added to the cultures. Cells were washed and stained for CD4 and Vα11, fixed in 1%

PFA, and permeabilized in 0.1% NP-40. After washing, unspecific staining was blocked with anti–FcR (2.4G2) and cells were stained with anti–IL-10 APC in conjunction with anti–IFN-γ PE or anti–IL-2 PE. Intracellular CTLA-4 staining was performed without the PdBU/ionomycin stimulation step.

Cell Sorting and Adoptive Transfer Experiments. Single cell suspensions of spleen and lymph nodes from 6- to 10-wk-old donor mice were enriched for CD4 T cells using magnetic beads conjugated with CD4 beads (Miltenyi Biotech) on an AutoMACS. Thereafter CD4 cells were sorted on a MoFlo cytometer according to CD45RB, CD25, and CD5 expression as stated in the respective experiments. Between 2 and 4 × 10^5 (unless otherwise stated) CD4 T cells were injected intravenously. In cotransfer experiments equal numbers of each T cell population were injected. Recipient mice were weighed weekly and the weight on the day of injection was set as 100%. Mice were culled when 20% weight loss occurred and/or excessive diarrhea developed. The assessment of mean survival time therefore corresponds with the time point of 20% weight loss.

In Vitro Experiments. In vitro suppression experiments were done as described previously (27). Briefly, 5 × 10^4 sorted CD45RB^{hi} CD4 T cells were cultured in 96-well flat bottom plates with 5 × 10^4 irradiated (3,000 Rad) Rag$^{-/-}$ spleen cells in the presence of 0.5 μg/ml anti CD3 (2C11) mAb. Increasing numbers of sorted ex vivo AND H-2^a Rag$^{-/-}$ CD4 T cells, or AND H-2^a Rag$^{-/-}$ CD4 T cells resorted after transfer (4 × 10^5 AND T cells transferred into Rag$^{-/-}$ H-2^a hosts 8 wk before the experiment) or sorted CD25$^+$CD45RB^{lo} CD4 T cells were added to the cultures. After 48 h, supernatants were taken and analyzed for IL-2 content in standard CTLL assay.

Results

Co-transfer of CD4 T Cells from AND Rag$^{-/-}$ Mice Prevents Immune Pathology in H-2^a, but Not H-2^b Recipients of CD45RB^{hi} CD4 T Cells. Transfer of low numbers of CD4 T cells expressing CD45RB at high levels into lymphopenic recipients results in immune pathology characterized by a wasting disease with severe weight loss, diarrhea, and development of inflammatory bowel disease (IBD; reference 5). This can be prevented by cotransfer of CD45RB^{lo} CD4 T cells and specifically those that also express CD25 (11). To test our hypothesis that competition for space between T cell populations might underlie the protective effect we chose as 'regulatory' population a monoclonal transgenic T cell population with known antigenic specificity unrelated to that involved in development of gut pathology in this system. Lymphopenic Rag$^{-/-}$ mice on an H-2^a MHC background (i.e., expressing class II A^k and E^k molecules) were injected either with 2 × 10^5 sorted CD45RB^{hi} CD4 T cells from H-2^a mice only or with a mixture of CD45RB^{hi} CD4 T cells (2 × 10^5 of each) and CD4 T cells from the AND Rag$^{-/-}$ strain expressing H-2E^k or CD45RB^{lo} CD4 T cells which contain regulatory T cells. AND T cells are known to rapidly divide upon transfer into H-2E^k containing hosts (28, 29). From ~4 wk after transfer all recipients of CD45RB^{hi} CD4 T cells alone lost weight and eventually developed severe diarrhea so that they had to be culled around 6–7 wk after transfer (Fig. 1 a, top row left). None of the animals that

received AND Rag$^{-/-}$ T cells together with CD45RB^{hi} T cells developed such symptoms (Fig. 1 a, top row right) and at termination of the experiment these mice were as healthy as mice that had received cotransfer of CD45RB^{lo} T cells (Fig. 1 a, top row middle).

Monitoring of CD4 T cell expansion in blood (Fig. 1 a, middle row) over 50 d and in lymphoid organs upon termination of the experiment (Fig. 1 a, bottom row) showed dramatic expansion of CD4 T cells in recipients of CD45RB^{hi} CD4 T cells, whereas there was no equivalent expansion over time in recipients cotransferred with AND Rag$^{-/-}$ T cells or CD45RB^{lo}. On the other hand AND T cells expanded enormously for the first 7 d after transfer as evident from the proportion of AND T cells present in blood on day 7. Although the number of AND T cells in blood decreased from day 7 after transfer, they still comprised 35% of the cells in mesenteric lymph nodes and more than 10% in spleen 50 d later (Fig. 1 a, bottom row). Expansion of polyclonal CD4 T cells was most prominent in the mesenteric lymph nodes draining the gut, but was strongly reduced in mice that had been cotransferred with AND T cells.

While AND T cells are known to have exceptionally high expansion potential in H-2E^k expressing hosts, AND T cells on an H-2^b background that also selects this T cell receptor undergo more limited expansion in lymphopenic hosts (28). We therefore performed a similar experiment transferring CD45RB^{hi} CD4 T cells from H-2^b hosts with AND T cells or CD45RB^{lo} cells from H-2^b donors into Rag$^{-/-}$ H-2^b mice. In this case cotransfer of AND T cells did not protect the recipients from weight loss and development of diarrhea, whereas CD45RB^{lo} cells prevented all symptoms of gut pathology (Fig. 1 b, top panel). Analysis of the CD4 T cell numbers revealed that AND T cells from H-2^b mice did not expand extensively and also did not prevent the expansion of the injected CD45RB^{hi} population (Fig. 1 b, bottom panel).

AND T cells did not express markers typically associated with regulatory T cells, such as CD25 or CTLA-4 at the time of transfer (Fig. 2 a), nor did they acquire a 'classical' regulatory phenotype during their expansion in vivo (Fig. 2 b). All AND T cells harvested from protected mice 8 wk after transfer have an activated phenotype judged by high levels of CD44 expression, but did not express CD25. Their cytokine potential as revealed after restimulation in vitro revealed IL-2 and γ-interferon activity, but no IL-10 production (Fig. 2 c).

A summary of experiments (endpoints 8–15 wk after transfer) shown in Table I demonstrates that 75% of mice which had received cotransfer of AND H-2^a T cells were protected from disease similar to mice that had received cotransfer of CD45RB^{lo} T cells (85% protected), whereas
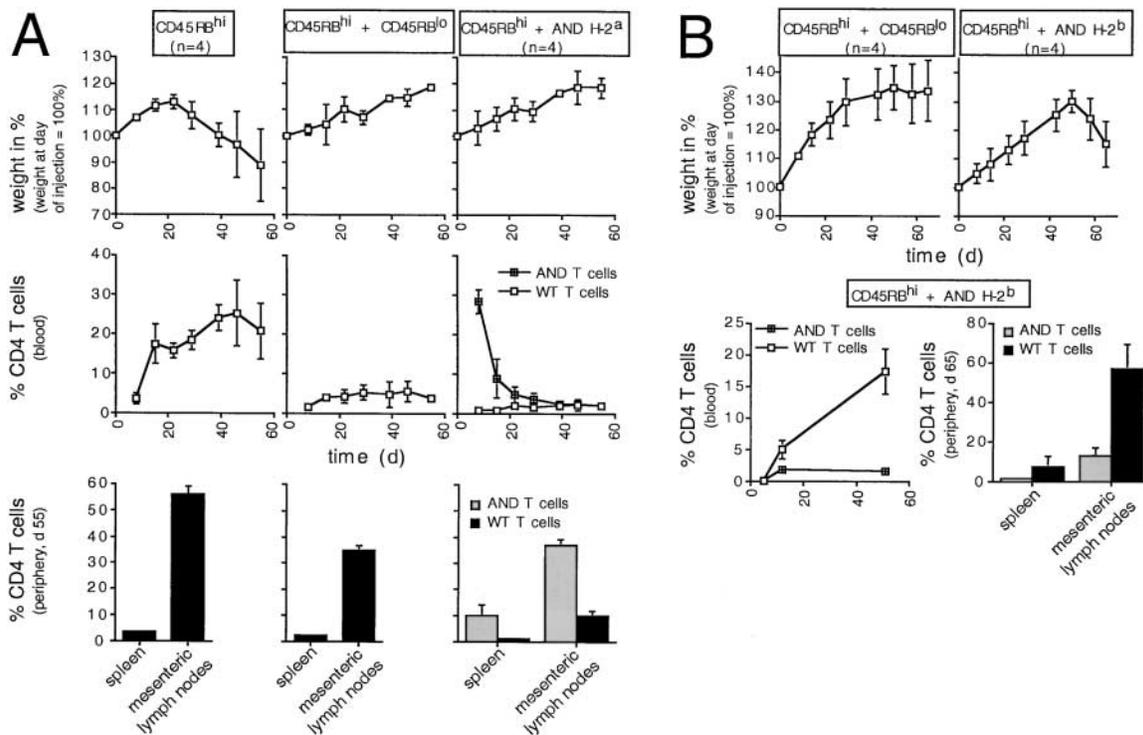
Figure 1. TCR transgenic AND Rag$^{-/-}$ CD4 T cells from H-2E^{k+}, but not H-2A^{b+} mice can regulate CD45RB^{hi} T cells in lymphopenic hosts. (A) Equal numbers (2 × 10^5) of CD45RB^{hi} and CD45RB^{lo} or AND Rag$^{-/-}$ T cells (all H-2^a) were adoptively transferred into Rag$^{-/-}$ H-2^a hosts. Body weight and percentage of T cells in the blood were monitored weekly and peripheral lymphoid organs were assessed for CD4 T cells as a proportion of total live cells at the endpoint of the experiment. AND T cells were identified based on expression of Vα11. (B) Equal numbers (2 × 10^5) of CD45RB^{hi} and CD45RB^{lo} or AND Rag$^{-/-}$ CD4 T cells (all H-2^b) were adoptively transferred into Rag$^{-/-}$ H-2^b hosts. Body weight and percentage of T cells in blood and lymphoid organs were monitored as in A.
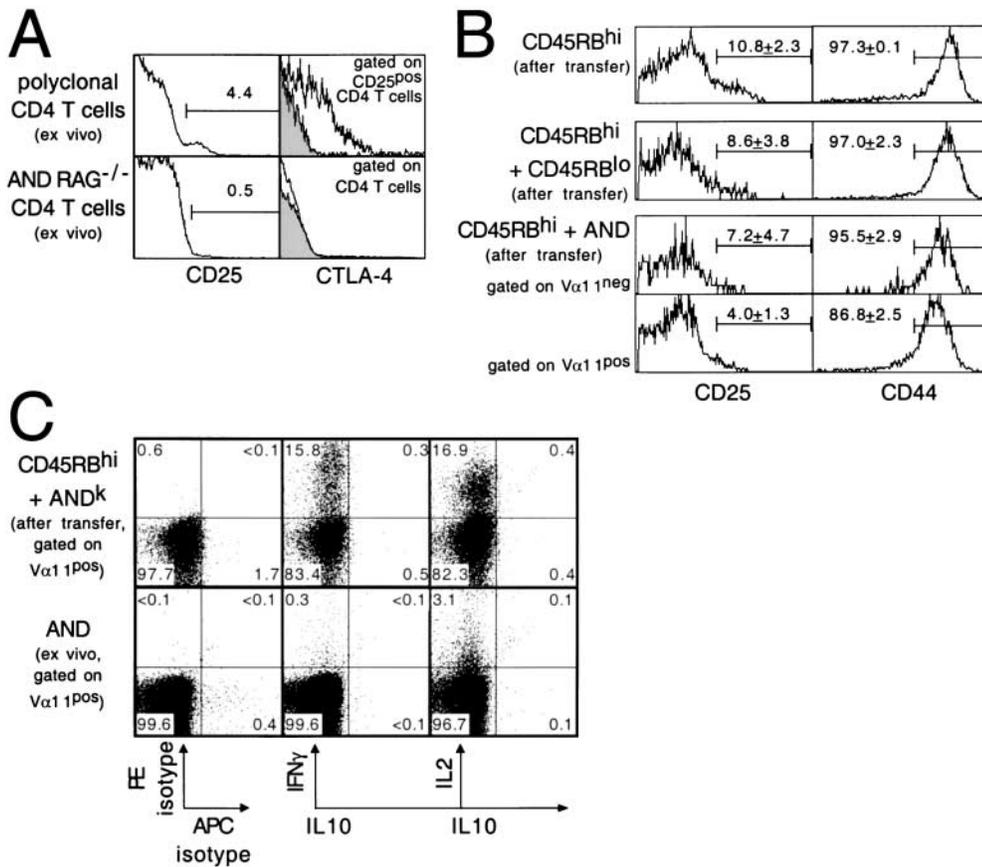
Figure 2. Activation markers and cytokine expression. (A) Polyclonal and AND Rag$^{-/-}$ CD4 T cells (all H-2^a) were stained for CD25 and intracellular CTLA-4 (shaded histograms = isotype control). (B) CD25 and CD44 expression on polyclonal and AND Rag$^{-/-}$ CD4 T cells (mesenteric lymph nodes) after adoptive transfer into Rag$^{-/-}$ H-2^a hosts (experiment from Fig. 1 A). (C) IFN-γ, IL-2, and IL-10 expression on AND Rag$^{-/-}$ CD4 T cells ex vivo and after adoptive transfer with CD45RB^{hi} CD4 T cells into Rag$^{-/-}$ hosts (all H-2^a). Percentages are indicated in the histograms and dot plots.

only 10% of mice injected with CD45RB^{hi} T cells alone did not develop pathology. On an H-2^b background AND T cells were unable to protect recipients against colitis, whereas 100% of mice that received cotransfer of CD45RB^{lo} T cells remained healthy. Thus, these experiments show an intriguing correlation between regulatory activity and the capacity for expansion in a lymphopenic environment.

AND T Cells Are Not Suppressive In Vitro. Numerous reports have shown in vitro suppressive activity of the CD25$^+$ T cell population (for a review, see reference 30). To assess whether the regulatory function of AND T cells would be detectable in vitro, we set up standard mixtures of T cells with irradiated spleen cells and anti-CD3 stimulation. Coculture of fresh CD25$^+$ T cells suppressed IL-2 production and proliferation of CD45RB^{hi} T cells as previously reported. However, AND T cells, taken either ex vivo as naive T cells or after expansion in a lymphopenic host, did not show any suppressive effect in vitro (Fig. 3, left panel). On their own, CD3 stimulated AND T cells produced IL-2, but much lower amounts than the polyclonal CD45RB^{hi} population (Fig. 3, right panel) whereas CD25$^+$ CD4 T cells did not make IL-2 at all. This indicates that AND T cells do not share the ability for in vitro suppression. On the other hand, in vivo suppression by both CD25$^+$ CD4 T cells and AND T cells led to indistinguishable outcomes. Thus, in vitro suppression is not a pre-

requisite and might be of limited predictive value for in vivo regulation.

Homeostatically Expanding CD8 T Cells Offer Partial Protection. While the AND TCR transgenic strain served as an example of a monoclonal T cell population that was able to regulate immune pathology irrespective of its unrelated antigenic specificity and phenotype, the expansion capacity of AND T cells in lymphopenic hosts is exceptional and does not resemble that of any other TCR transgenic strain. One might therefore be concerned to link protective function to homeostatic expansion potential based on this strain alone. To further substantiate our claim that homeostatic expansion of transgenic T cells in a lymphopenic host can interfere with the induction of immune pathology by CD45RB^{hi} polyclonal T cells, we used another TCR transgenic strain on a Rag$^{-/-}$ background, OT-I. CD8 T

Table I. *Summary of Adoptive Transfer Experiments with AND TCRtg CD4 T Cells*

|	Percentage of protected mice (total mice)		
	CD45RB^{hi}	CD45RB^{hi} + RB^{lo}	CD45RB^{hi} + AND
H-2^a	10 (10)	85 (7)	75 (12)
H-2^b	8 (12)	100 (8)	8 (12)
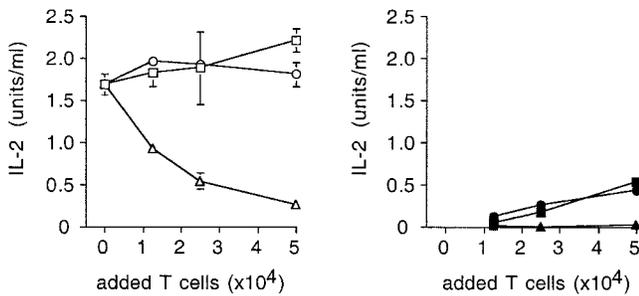
Figure 3. AND T cells do not suppress in vitro Sorted naive ex vivo AND T cells (■, □), sorted AND T cells after homeostatic expansion in a lymphopenic host (●, ○), or sorted ex vivo CD25$^+$ T cells (▲, △) were cultured in the presence of anti-CD3 with irradiated Rag$^{-/-}$ spleen cells and with (open symbols) or without (filled symbols) sorted syngeneic CD45RB^{hi} CD4 T cells. Supernatants were assessed for IL-2 content in a standard CTLL assay.

cells from the OT-I strain have been shown to undergo substantial expansion and phenotypic conversion upon transfer into lymphopenic hosts (31). As CD4 and CD8 T cells to some extent share a peripheral niche (3), homeostatic expansion of one population would be expected to affect the other. Although OT-I T cells divide extensively, their proliferation is much less pronounced than that of AND T cells, illustrated by the CFSE profile shown in Fig. 4 a. Nevertheless, cotransfer of OT-I T cells had a strong effect on immune pathology caused by CD4 CD45RB^{hi} T cells, delaying the onset of disease and protecting 50% of the mice completely.

Self-regulation by High Numbers of CD45RB^{hi} CD4 T Cells. Excessive expansion may be more pronounced with a small inoculum of T cells that encounters unlimited resources than with larger numbers of T cells that would compete amongst each other for factors controlling their expansion. To test this assumption, we compared groups of mice that received either 4×10^5, 6×10^6, or 12×10^6 sorted CD45RB^{hi}CD25$^-$ CD4 T cells (Fig. 5 a). While the recipients of the smallest cell dose had all developed symptoms of colitis by 3–4 wk (Fig. 5 b), transfer of higher numbers of T cells showed either partial protection, preventing disease in 3 out of 5 animals (Fig. 5 b, left) or in the case of the highest dose complete protection (Fig. 5 b, right). T cells in lymphoid organs from recipients of the high dose of CD45RB^{hi} cells were not all activated, as shown by retention of the CD45RB^{hi} marker in 26% of the cells compared with 9% of the cells from recipients of

the low dose (Fig. 5 c, top panel). Furthermore, while 92% of the transferred T cells found in recipients of small numbers of CD45RB^{hi} cells expressed high levels of CD44 indicating their activation, only 58% of the T cells in mice that had received high doses of CD45RB^{hi} cells showed an activated phenotype (Fig. 5 c, bottom panel). This finding is compatible with the hypothesis that competition with neighboring T cells for limited resources may restrain excessive activation and outgrowth of transferred T cells.

Immune Regulation by CD5^{hi}CD45RB^{hi} Polyclonal T Cells. Self-regulation after transfer of large numbers of CD45RB^{hi}CD25$^-$ T cells into lymphopenic hosts indicated the existence of a subset of T cells within this population which, similarly to AND T cells, could inhibit the development of immune pathology. Although AND T cells might represent an extreme example, we hypothesized that T cells with similar properties that might make them 'regulatory' are also contained in the polyclonal repertoire. Extrapolating from the behavior of AND T cells transferred into H-2E^{k+} recipients, such cells would be expected to have high avidity for self-peptide:MHC. One of the hallmarks of avidity for self peptide:MHC is the level of expression of CD5, a negative regulator of TCR signaling. As the strength of interaction with self-peptide: MHC is reflected in the levels of expression of CD5, we compared CD5 expression on AND cells and polyclonal CD45RB^{hi} T cells. As shown in Fig. 6 a, CD5 expression on AND T cells on an H-2E^{k+} background falls within the high range of expression levels on polyclonal T cells, correlating well with their reported high avidity for self peptide:MHC. CD5 expression of AND T cells on an H-2^b background, on the other hand, falls within the lower expression levels of polyclonal T cells in agreement with their reported lower avidity (32). When analyzing T cells in adoptive hosts of CD45RB^{hi} T cells that succumbed to disease (transfer of low T cell numbers middle panel) with those that remained healthy (transfer of high T cell numbers top panel), it was clear that healthy mice contained a larger proportion of CD5^{hi} T cells, whereas in sick animals CD5^{lo} T cells predominated (Fig. 6 b). We therefore hypothesized that CD5^{lo} T cells may be enriched in potentially pathogenic T cells. These may normally be kept in check by CD5^{hi} T cells in the repertoire that out compete them due to their higher avidity for self peptide:MHC complexes on APCs; their decreased responsiveness to cognate antigens, on the other hand, would make CD5^{hi} T cells less likely to cause immune pathology themselves.
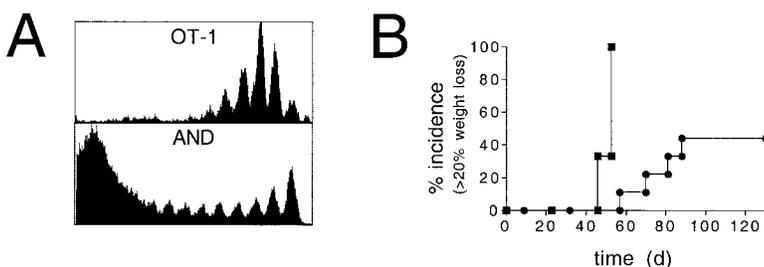

Figure 4. CD8 T cells from OT-I show regulatory activity. (A) OT-I H-2^b and AND Rag$^{-/-}$ H-2^a T cells were labeled with CFSE and transferred into corresponding syngeneic Rag$^{-/-}$ hosts. The CFSE profiles were assessed 7 d later. (B) 4×10^5 CD45RB^{hi} CD4 T cells from H-2^b B10 mice were adoptively transferred either alone ($n = 3$, ■) or together with the same number of OT-I Rag$^{-/-}$ T cells ($n = 9$, ●) into syngeneic Rag$^{-/-}$ hosts and monitored for body weight weekly. The figure shows cumulative incidence of 20% weight loss at which time mice had to be culled.
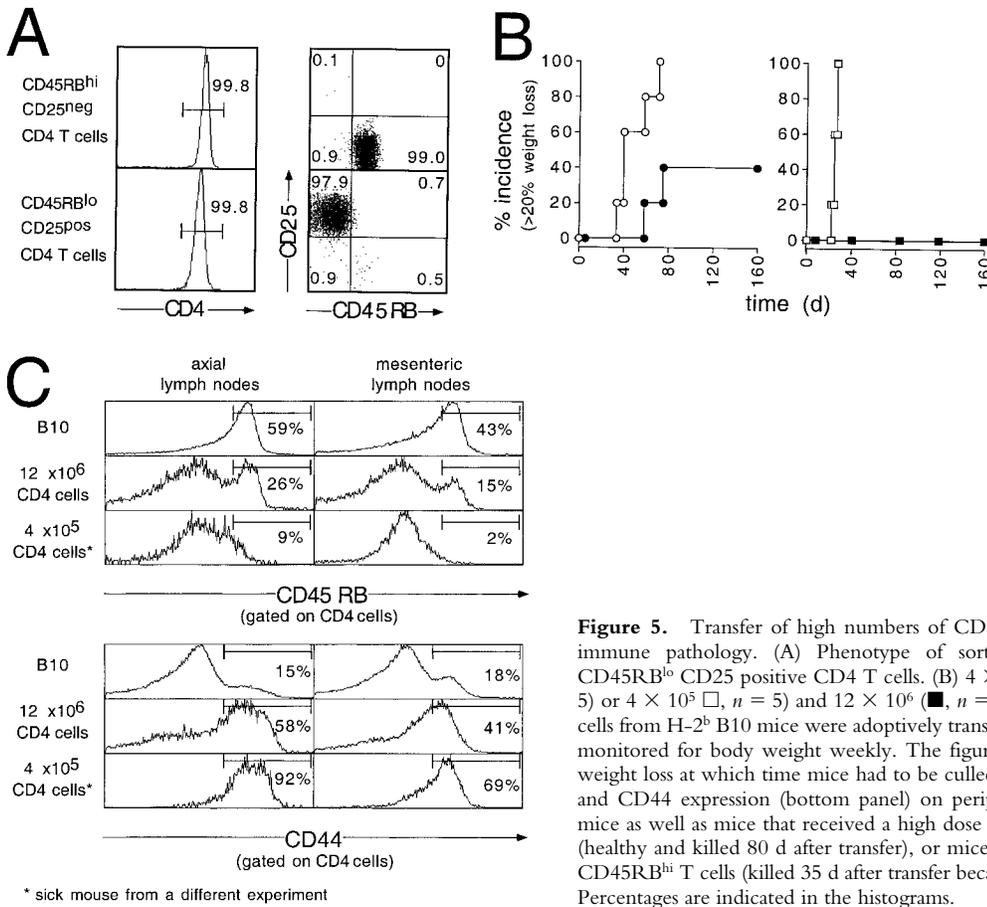
Figure 5. Transfer of high numbers of CD45RB^{hi} CD4 T cells does not lead to immune pathology. (A) Phenotype of sorted CD45RB^{hi} CD25 negative and CD45RB^{lo} CD25 positive CD4 T cells. (B) 4×10^5 (○, $n = 5$) and 6×10^6 (●, $n = 5$) or 4×10^5 (□, $n = 5$) and 12×10^6 (■, $n = 4$) CD45RB^{hi} CD25 negative CD4 T cells from H-2[b] B10 mice were adoptively transferred into syngeneic Rag^{−/−} hosts and monitored for body weight weekly. The figure shows cumulative incidence of 20% weight loss at which time mice had to be culled. (C) CD45RB expression (top panel) and CD44 expression (bottom panel) on peripheral CD4 T cells from B10 control mice as well as mice that received a high dose (12×10^6) of CD45RB^{hi} CD4 T cells (healthy and killed 80 d after transfer), or mice that received a low dose (4×10^5) of CD45RB^{hi} T cells (killed 35 d after transfer because of severe weight loss and diarrhea). Percentages are indicated in the histograms.

To test this hypothesis, CD45RB^{hi}CD25^{−} T cells were sorted for low and high CD5 expression and 3×10^5 sorted CD5^{hi} or CD5^{lo} (see Fig. 6 c) were transferred into lymphopenic hosts and compared with unseparated CD45RB^{hi}CD25^{−} cells (Fig. 6 d). Mice that received CD45RB^{hi} CD25^{−} T cells all developed disease with a

mean survival time of 44.3 ± 11.9 d after injection. Mice that received the sorted CD5^{lo} population succumbed to disease significantly earlier with a mean survival time of 30.1 ± 6.5 d ($P < 0.005$). In contrast, onset of disease in recipients of the CD5^{hi} subset was substantially delayed (mean survival time 57.1 ± 12.0 d, $P < 0.05$) and 40% of
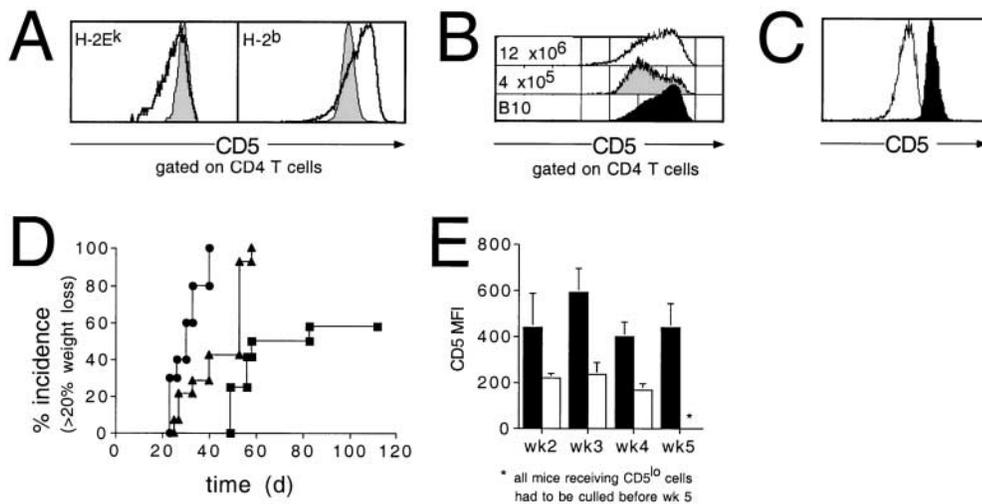
Figure 6. CD45RB^{hi} CD5^{hi} CD4 T cells act as regulatory T cells. (A) CD5 expression on AND CD4 T cells (shaded histogram) and on polyclonal CD4 T cells (open histogram) on different MHC backgrounds. (B) CD5 expression on peripheral CD4 T cells from experiment described in Fig. 3 B. B10 control, (black histogram), low dose recipient (4×10^5 CD45RB^{hi}, gray histogram), and high dose recipient (12×10^6 CD45RB^{hi}, open histogram). (C) CD5 phenotype of sorted CD45RB^{hi} CD25^{−} CD4 T cells. (D) Cumulative incidence of 20% weight loss in groups of mice receiving $3–4 \times 10^5$ CD45RB^{hi} CD4 T cells which were either unseparated (▲, $n = 14$) or sorted into CD5^{hi} (■, $n = 12$) and CD5^{lo} (●, $n = 10$). (E) Mean CD5 fluorescence on peripheral blood lymphocytes over time in mice receiving sorted CD5^{lo} (white bars, $n = 5$) or CD5^{hi} (black bars, $n = 5$) CD45RB^{hi} CD25^{−} CD4 T cells.

them remained healthy. CD5 levels on sorted populations did not change after transfer in vivo (Fig. 6 e). This suggests that the predominance of CD5^{hi} and CD5^{lo} T cells in healthy and sick animals respectively (Fig. 6 b) is the result of selection and expansion rather than of conversion. These results further emphasize that there are cells, distinct from CD25^{+} cells, in the normal T cell repertoire that can regulate the development of immune pathology. This happens irrespective of antigen specificity by virtue of their high responsiveness to homeostatic signals in lymphopenic hosts.

Discussion

Regulation of peripheral T cell responses is an essential feature in the immune system, preventing not only the development of autoimmune disease, but also excessive responses to 'foreign' antigens that could lead to immune pathology. Maintenance of tolerance to self in the periphery as well as prevention of exaggerated outgrowth of activated T cells after their stimulation by foreign antigen are fundamentally important functions of the immune system. Robustness in the mechanisms to achieve these goals is therefore expected and indeed a vast variety of peripheral mechanisms have been described to exist for this purpose. These include peripheral deletion (33, 34), differential action of cytokines, including TGF-β (35) or IL-10- production (36), self-antigen presentation by immature rather than mature dendritic cells (37) or induction of anergy after suboptimal activation (38, 39), restriction of clonal expansion by CTLA-4 (40), and Fas/Fas ligand interactions (41).

We have focused on the regulation of lymphopenia associated immune pathology induced by an excessive T cell response, presumably directed against commensal microorganisms of the gut. The mouse model used in our experiments is one of the most commonly used in vivo models for the study of regulatory T cells. However, the principles of regulation put forward in our study may also apply for autoimmune diseases, such as diabetes in the NOD mouse or BB rat model both of which exhibit a degree of lymphopenia (42, 43). We have shown here, using the extreme example of a monoclonal T cell receptor transgenic T cell populations, that T cells lacking characteristic markers ascribed to the regulatory lineage can function as regulatory T cells. It should be emphasized that neither regulation by monoclonal AND T cells nor self-regulation by high numbers of CD45RB^{hi}CD25^{-} T cells correlated with expression of CD25, although CD25^{+} T cells emerge in polyclonal T cells even if they are transferred as CD45RB^{hi}CD25^{-} cells, as also noted by Annacker et al. (44). In fact, AND T cells from H-2^{b} donors show a marked increase in CD25^{+} cells after transfer into lymphopenic hosts, yet these T cells failed to act as regulatory T cells (45). Other studies have shown that CD25^{-} cells can have regulatory capacity and it was suggested that both CD4^{+}CD25^{+} and CD4^{+}CD25^{-} regulatory cells exist (46, 47).

While it is highly unlikely that AND only regulate because they differentiate into one of the currently described types of regulatory T cells in vivo, their capacity for regulation correlated with rapid expansion potential in response to endogenous MHC–peptide ligands in lymphopenic hosts. Representative data on polyclonal CD4 T cell expansion suggest that AND reflect the behavior of a minor (1%) subset of polyclonal T cells that divide more than 8 times within 7 d after transfer into lymphopenic hosts (48). Previous studies using monoclonal transgenic T cells as a source of regulatory T cells did not observe inhibition of either EAE (49) or gastritis (50). It is not clear, however, in these different experimental models if T cells from the DO-11–10 strain used could expand early and sufficiently enough to out-compete either polyclonal or MBP-specific T cells even when subjected to immunization with ovalbumin. The expansion potential of AND T cells is exceptional and not shared by any other TCR transgenic population available so that one could rightfully be concerned about their representative value. However, we obtained similar results with a monoclonal transgenic CD8 population which divide substantially, if less than AND T cells, upon transfer into lymphopenic mice. OT-I T cells offered at least partial protection, supporting our claim that homeostatic expansion and the resulting competition for shared resources impacts on regulation of immune responses irrespective of specificity and phenotype of the protective T cell population. Their reduced efficiency compared with AND T cells could be due to less exaggerated proliferation or might also reflect differential homeostatic requirements. Although naive CD4 and CD8 T cells share the same peripheral niche (3), resources controlling the number of activated/memory CD4 and CD8 T cells, while partially overlapping (51), are not identical (52, 53) so that one might expect only partial competition between activated CD4 and CD8 T cells.

While our data do not rule out that regulatory cells function via release of inhibitory mediators such as IL-10 and/ or TGF-β, they nevertheless suggest that a certain degree of expansion is an essential prerequisite for manifestation of regulatory function in vivo under conditions of lymphopenia. It is important to stress that regulatory function in vivo need not correlate with suppressive activity in vitro, as we show in these experiments. As far as the contribution of homeostatic expansion to regulation is concerned, this is understandable, as proliferation in lymphopenic hosts takes place only in the microenvironment of lymphoid organs (54) that would not be mimicked by any in vitro suspension culture.

Lymphocyte diversity can prevent the outgrowth of potentially harmful cells by lymphocyte competition. In contrast, lymphopenia is invariably associated with homeostatic expansion of the remaining T cells, resulting in an oligoclonal repertoire of a few clones that may become irreversibly dominant and cause pathology (55). Although the rules that regulate T cell homeostasis are not entirely understood, naive T cells depend on self-peptide:MHC and IL-7 signals for expansion, whereas for T cells with an activated phenotype such as CD25^{+} T cells IL-7 signals are not essential for further expansion (56). Naive T cells exhibit variable degrees of homeostatic expansion, reflecting their

avidity for self peptide:MHC complexes and in some cases homeostatic expansion can lead to activation, such that all cells acquire characteristics typical for memory T cells. This is exemplified by AND T cells which can be selected on both H–2E^k for which they have very high affinity and H–2A^b for which they have lower affinity (32), reflected in dramatic proliferation and activation of AND T cells from H–2E^k mice transferred into an H–2E^k environment and more limited proliferation in an H–2^b background (28, 54). Similarly OT-I T cells were shown to divide in response to self peptide:MHC ligands resulting in acquisition of an activated phenotype (31).

T cell avidity for self peptide:MHC complexes not only influences their potential for homeostatic expansion, but also tunes their activation threshold in response to cognate antigen. Up-regulation of CD5, a negative regulator of TCR signaling, in the thymus ensures that thymocytes with high avidity for self peptide:MHC are not overtly self-reactive in the periphery because they display lower responsiveness to cognate antigen (23). On the other hand CD5^{hi} T cells possess higher homeostatic expansion potential in response to self peptide:MHC signals than their CD5^{lo} counterparts (26). AND T cells on an H–2E^k background fulfill these criteria, being CD5^{hi}, relatively insensitive to CD3 stimulation, with a very high homeostatic potential and the capacity to regulate expansion of other T cells. We have shown here that polyclonal CD4 T cells, depleted of putative regulatory T cells (CD45RB^{lo} CD25$^+$), exhibit self-regulatory activity, provided they are injected in higher numbers. While the higher homeostatic expansion potential of CD5^{hi} T cells is not evident in completely lymphopenic mice, presumably due to the excess of resources in that situation, they expand more than CD5^{lo} T cells in partially lymphopenic hosts (26). This would explain why CD5^{hi} T cells in the CD45RB^{hi} inoculum can prevent immune pathology in recipients of higher (12×10^6) T cell numbers, whereas they can only delay it in recipients of low T cell numbers (see Figs. 4 and 5). Therefore, T cell regulation by transgenic T cells or self-regulation by high numbers of CD45RB^{hi}CD25$^-$ CD4 T cells might simply be the result of competition for limited homeostatic signals such as IL-7 and MHC.

However, there are more levels of competition involved in immune regulation. CD25$^+$ T cells are likely to compete with any other T cell for access to antigen-carrying APCs and growth factors such as IL-2. IL-2 signaling may be involved in the regulatory function (46, 57–59) and might underlie the differential capacities of AND versus CD25$^+$ cells for in vitro regulation. Moreover, activated T cells may directly or indirectly inhibit the expansion of rival T cells by secretion of inhibitory cytokines, such as IL-10 and TGF-β, both of which have been implicated in T cell regulation (35, 36). Expression of CTLA-4, another 'marker' for regulatory T cells, represents yet another mechanism by which activated T cells control their own expansion, and presumably the expansion of other T cells via induction of TGF-β (60).

The origin of CD45RB^{lo}CD25$^+$ found in the normal T cell repertoire is currently unknown. Their functional properties however, suggest that they may represent T cells, which have acquired an activated phenotype by high avidity interactions either in the thymus (57, 61) or the periphery. Interestingly, CD25$^+$ regulatory T cells have also been reported to express high levels of CD5 (11), thus representing T cells with high avidity for self-peptide:MHC; their poor responsiveness to TCR stimulation in vitro and in vivo is well documented (20, 62, 63). The CD25$^+$ T cell population might also include effector cells transiently responding to their cognate antigen at that time. Therefore there may exist, in addition to the independently regulated pools of naive and memory T cells (3), a regulated pool of effector T cells, which employs exploitation and interference competition strategies to inhibit the expansion of rival T cells as a bystander effect of their own expansion. The existence of such a pool could also explain enhanced responses to nonimmunogenic tumors observed upon depletion of CD4$^+$CD25$^+$ cells (64, 65), suggesting that depletion of the effector pool can make otherwise limited resources available to the next wave of responding T cells.

In conclusion, our results show that T cell regulation, at least in partially or fully lymphopenic states, can be effected by members of the normal T cell repertoire. These need not be dedicated suppressor T cells, but instead regulate T cell responses as a side effect of their own response to homeostatic and antigenic stimulation. Such context dependent regulation has been proposed earlier on theoretical grounds (66). Lymphopenia per se constitutes a high-risk scenario for the development of pathological or autoimmune responses, yet so far little attention has been given to the contribution of lymphocyte homeostasis and competition in prevention of immune dysregulation. Thus, further definition of the mechanisms responsible for immune regulation will inevitably depend on our understanding of the consequences of perturbations in the control mechanisms that maintain a balanced peripheral immune system.

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