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Regulatory CD4 T Cells Control the Size of the Peripheral Activated/Memory CD4 T Cell Compartment

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The mechanisms leading to stable T cell numbers in the periphery of a healthy animal are, to date, not well understood. We followed the expansion of CD45RBhigh (naive) and CD45RBlow (activated/memory) CD4 T cells transferred from normal mice into syngeneic Rag-2−/− recipients and the dynamics of peripheral reconstitution when both populations were cojected. Naive cells acquired an activated phenotype and showed a high proliferative capacity that was dependent on the environment in which the recipients were kept (specific pathogen-free vs conventional housing conditions), the age of the recipients, and the presence of CD45RBlow T cells in the injected cohort. CD45RBlow CD4 T cells protected the host from CD45RBhigh CD4 T cell-induced inflammatory bowel disease and showed a limited degree of expansion. CD45RBlow CD4 T cells isolated from GF mice also showed the ability to prevent inflammatory bowel disease, indicating that at least part of the natural regulatory T cells are self-reactive. The results indicate that 1) peripheral T cell expansion in lymphocyte-deficient recipients represent classical immune responses, which are mainly promoted by exogenous Ags and 2) natural regulatory T cells control the size of the activated/memory peripheral CD4 T cell compartment. The Journal of Immunology, 2000, 164: 3573–3580.

Total cell numbers in tissues and organs of multicellular organisms are under a relatively strict regulation (1). An unresolved question in the physiology of the immune system concerns the mechanisms that keep the number of peripheral lymphocytes constant. This implies that in healthy individuals, a balanced compromise exists between the sizes of the naive and the memory cell pools to ensure a large spectrum of Ag recognition and the maintenance of memory to previous antigenic experiences. Disregulation of this equilibrium may lead to alterations in lymphocyte population dynamics and peripheral repertoire selection which, aside from immunodeficiency, may lead to abnormal clonal expansion and the onset of autoimmune diseases (2, 3).

The peripheral T cell repertoire is maintained by thymic output of naive T cells and by self-renewal of part of the peripheral T cell pool (4). In the mouse, export of ~106 T cells/day from the thymus to the periphery (5) requires the loss of similar numbers of peripheral resident T cells. Recent observations suggest that this peripheral T cell loss is mostly due to the replacement of naive resident T cells by recent thymic emigrants (6, 7). Furthermore, studies using TCR-transgenic animal models conclude that the naive and the activated/memory CD8 T cell pools are independently regulated (8, 9).

To date, it is neither known how the naive and the naturally activated/memory CD4 T cell pools are regulated nor what the relative contribution of exogenous and endogenous Ags in promoting peripheral CD4 T cell self-renewability is. Peripheral naive CD4 T cells can be identified by high surface density of the CD45RB molecule, and the transition from a naive to an Ag-experienced state is accompanied by down-regulation of CD45RB (10).

Based on this marker, several groups reported that transfers of naive CD4 T cells into T and B cell-deficient, syngeneic recipients leads to the development of a lethal form of inflammatory bowel disease (IBD)4 (11, 12). This disease is caused by massive Th1-mediated immune responses in the gut developed against (or promoted by) local Ags which result in high levels of IFN-γ and TNF-α in the sick animals (12–15). Moreover, naive CD4 T cells were unable to induce IBD in the absence of IFN-γ (14, 16).

In this experimental system, naturally activated/memory CD4 T cells did not trigger disease, and cotransfer of both naive and activated cells revealed the ability of the latter to prevent the onset of IBD (11–14). Further studies showed that the protective effect was dependent on TGF-β and/or IL-10 (14, 17–20), but not on IL-4 (18).

These experiments demonstrated the importance of T cell-dependent regulatory mechanisms in the control of the magnitude of mucosal immune responses, but to date it is unknown at which level regulation occurs: differentiation of Th1 precursors, migration of aggressive T cells, or expansion of cells capable of inducing IBD.

In this study, we investigated the possibility that the regulatory events implicated in mucosal immune responses illustrate a general mechanism responsible for maintaining the systemic equilibrium between functional subsets of peripheral T cells.

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4 Abbreviations used in this paper: IBD, inflammatory bowel disease; SPF, specific pathogen free; GF, germfree.
To evaluate the potential for expansion of naive and activated/memory CD4 T cell subsets and their respective ability to reconstitute Rag-2<sup>−/−</sup> recipients, we used T cells from donor strains congenic for the CD45 (Ly5) marker to follow both populations in cotransfer experiments.

We show that the expansion potential of naive cells, once activated, is not limited by natural resources and that naturally activated/memory T cells regulate the size of the activated CD4 T cell pool. The results support the notion that CD4 T cell expansion in Rag-2<sup>−/−</sup> recipients is largely dependent on exogenous Ags.

### Materials and Methods

#### Mice

C57BL/6 mice were obtained from Janvier (Le Genest-St-Irse, France). C57BL/6 Ly5.1 and C57BL/6 Rag-2-deficient (Rag<sup>−/−</sup>) mice were purchased from CDBA (Orleans, France) and were bred either under specific pathogen-free (SPF) or conventional conditions in our animal facilities.

BALB<sup>scid</sup> mice were purchased from Charles River Breeding Laboratories (Saint Aubin les Elbeuf, France) and maintained under SPF conditions. BALB/c germfree (GF) mice were purchased from Centre de Développement des Technique Avancées pour L’Expérimentation Animale and used immediately upon arrival.

#### Antibodies

The following mAbs were used: anti-Ly5.1-FITC (clone A.20); anti-CD4-FITC, -PE, or -APC (L3T4); anti-CD8-PE (53-6.7); and anti-CD45RB-PE (23G2). All Abs were purchased from PharMingen (San Diego, CA).

#### T cell preparations

Splenic single-cell suspensions were first enriched for CD4<sup+</sup> cells either by depletion of B cells and macrophages using Dynabeads (Dynal, Oslo, Norway) or by positive selection on miniMacs columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturers’ instructions. In brief, for cell depletion, suspensions were incubated with anti-CD8 and anti-B220 rat IgG Ab for 20 min at 4°C followed by sheep-anti-rat-IgDynabeads (M450) for 20 min at 4°C before separation in a magnetic field. For positive enrichment of CD4<sup+</sup> cells before sorting, cells were first incubated with biotinylated anti-CD4 Abs for 20 min on ice in PBS supplemented with 0.5% FCS and then incubated in the same buffer with streptavidin-microbeads for 15 min. The magnetically labeled positive fraction was retained on a miniMACs column. After enrichment, the cells were labeled with FITC-labeled anti-CD4 (or streptavidin-FITC) and anti-CD45RB-PE Ab for 20 min on ice and then sorted on a FACSstar (Becton Dickinson, Mountain View, CA). The 40–50% of CD4<sup+</sup> cells that stained brightest and the 15–30% of CD4<sup+</sup> cells staining the lowest for CD45RB were sorted as CD4<sup>+</sup>CD45RB<sup>high</sup> and CD4<sup>+</sup>CD45RB<sup>low</sup> populations, respectively. The purity of the sorted populations was routinely >97%.

For the preparation of intestinal cell suspensions, large and small intestines were first flushed extensively to eliminate the lumen content and then opened longitudinally and cut into 1–2-cm pieces. These were incubated twice in prewarmed Optimem medium (Life Technologies, Rockville, MD) containing 5% FCS and 450 U of collagenase (Sigma, St. Louis, MO) for 20 min at 37°C. After filtering through gauze, lymphoid cells were isolated on a 40% Percoll gradient. The cells were then washed and stained for fluorocytometric analysis.

#### Cell transfers

C57BL/6 Rag-2<sup>−/−</sup> mice were injected i.v. with 3–6 × 10<sup>6</sup> CD4 T cells from either CD45RB<sup>high</sup> or CD45RB<sup>low</sup> subpopulations. When both populations were cotransferred, Ly5.1<sup+</sup> and Ly5.2<sup+</sup> donor cells were used (at a ratio of 1:1). When C.B.-17<sup>−/−</sup> recipients is largely dependent on exogenous Ags.

#### Flow cytometric analysis

Cells from spleen, lymph nodes, or intestine were incubated for 20 min at 4°C in microtiter plates with 50 µl of the appropriate Ab preparations in PBS supplemented with 3% FCS and 0.001% azide. Where possible, one million cells were stained. Alternatively, the whole organ cell suspension was used. The Ab concentrations used were tested for optimal stainings of splenic control samples before use in experiments. Dead cells were excluded from the analysis by propidium iodide. Blood samples were first stained with appropriate Abs before lysing erythrocytes with FACS lysing solution (Becton Dickinson). Flow cytometric analysis was performed on a FACScan (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

### Statistical analysis

Analysis was performed using the unpaired t test. The data were considered significantly different when p values were <0.05.

#### Results

**CD45RB<sup>low</sup> CD4 T lymphocytes regulate the peripheral expansion of CD45RB<sup>high</sup> CD4 T cells**

Splenic CD4 T cells of donor C57BL/6-Ly5.2 and C57BL/6-Ly5.1 mice were separated into CD45RB<sup>low</sup> and CD45RB<sup>high</sup> subsets (hereafter referred to as RB<sup>low</sup> and RB<sup>high</sup>, respectively). Adult Rag-2<sup>−/−</sup> mice were i.v. injected with 3 × 10<sup>6</sup> cells of either cell population alone or with both populations, distinguishable by the Ly5 allotype marker, at a ratio of 1:1. Weight was recorded during the following weeks and mice were regularly bled.

In agreement with previous studies (11, 12, 14), most mice injected with RB<sup>high</sup> CD4 T cell populations started losing weight by the end of the second week after transfer, and signs of diarrhea were apparent by the third week. Rectal prolapse occurred later and was observed in most animals. In contrast, animals injected with RB<sup>low</sup> CD4 T cells gained weight and with one exception, no signs of wasting disease were observed. The protective role of RB<sup>low</sup> CD4 T cells was efficient since only 3 of 17 mice that received this population, either alone or along with RB<sup>high</sup>, became sick (Fig. 1A).

Two to 6 mo after transfer, mice were sacrificed and the number of T cells present in the blood, spleen, mesenteric lymph nodes, and collagenase-treated small and large intestinal cell suspensions was counted.

Fig. 1C shows the absolute number of T cells in the indicated organs for each individual recipient. In mice injected with RB<sup>low</sup> CD4 T cells, analyzed mostly 6 mo after the transfers, the majority of the T cells (range, 0.5–2.5 × 10<sup>6</sup>) was found in the spleen (Fig. 1C). A small fraction (<0.5 × 10<sup>6</sup>) was present in mesenteric lymph nodes, whereas essentially no cells were recovered from the wall of both the small and large intestine which were normal in appearance. T cells were recirculating in the blood at low numbers (range, 2–10 × 10<sup>4</sup>/ml), and these values were similar to those recorded as early as 10 days posttransfer (data not shown).

In contrast, a typical IBD colon with a thickened wall was observed in every recipient of RB<sup>high</sup> CD4 T cells, which were mostly analyzed after 2 mo. Correspondingly, high (although variable) numbers of T cells were recovered from the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown). Although splenic T cell numbers were not significantly different from those in the group of RB<sup>low</sup> recipients, 3.5 times more CD4 T cells on average were found in the gut (range, 0.2–5.0 × 10<sup>6</sup> T cells). This was associated with a very high frequency (15–40%) of TNF-α- and IFN-γ-producing CD4 T cells (data not shown).
In the blood, the RB\textsuperscript{low}:RB\textsuperscript{high} ratio of the injected cohort was maintained and both populations were recirculating in low numbers.

Clearly, the presence of RB\textsuperscript{low} CD\textsuperscript{4} T lymphocytes limited the degree of expansion of the naive T cell pool ($p < 0.0042$, comparing total cell numbers for RB\textsuperscript{high} injected either alone or along with RB\textsuperscript{low}), and very markedly inhibited their migration and/or expansion at the level of the gut. These differences do not appear to reflect distinct kinetics since the same results were observed when mice were analyzed at comparable time points (data not shown\textsuperscript{5}). As pointed out by others (11), in the two groups of mice that received RB\textsuperscript{high} CD\textsuperscript{4} T cells, 90\% of these T cells acquired an activated/memory phenotype in all organs analyzed (data not shown), indicating that most of the cells that survived in the recipient encountered Ag.

In summary, the results show that 1) RB\textsuperscript{low} CD\textsuperscript{4} T cells seem to have a relatively limited potential for expansion (on average 6-fold the number of injected cells) with autonomic dynamics, i.e., independent of the presence of naive cells; 2) naive CD\textsuperscript{4} T cells, once activated, have a higher potential for expansion and the difference in the level of peripheral replenishment of the hosts is due to immune responses at the level of the gut, which are also reflected in the number of blood circulating T cells; and 3) naturally activated/memory CD\textsuperscript{4} T cells regulate the size of the peripheral pool of activated CD\textsuperscript{4} T cells in the reconstitution of Rag-2\textsuperscript{-/-} mice.

Peripheral expansion of CD\textsuperscript{45RB\textsuperscript{high}} CD\textsuperscript{4} T cells depends on the age of the recipient

In the next set of experiments, we studied the survival and/or expansion of naive CD\textsuperscript{4} T cells in an environment where stimulation by exogenous Ags, in particular those related to bacterial colonization of the gut, was reduced.

RB\textsuperscript{high} CD\textsuperscript{4} T cells were injected into Rag-2\textsuperscript{-/-} newborns within 24 h after birth. As shown in Fig. 2A, during the first 3 wk of age, injected animals developed in a similar manner as un.injected controls. From then on, all injected mice grew slower and barely gained weight. Signs of diarrhea and bleeding became apparent, although no death occurred during a period of 2.5 mo.

At the time of sacrifice (10–12 wk of age), all injected animals showed very marked enlargement of the colon. Fig. 2C compares the numbers of T cells recovered from the indicated organs of newborn recipients with those plotted in Fig. 1 which correspond to adult Rag-2\textsuperscript{-/-} recipients injected with the same number of RB\textsuperscript{high} CD\textsuperscript{4} T cells. As shown, significantly higher T cell numbers were obtained in recipients that received the T cell cohort as newborns ($p < 0.0074$), particularly from the intestines (6-fold on average; $p < 0.0088$). CD\textsuperscript{4} T cell numbers were also increased in the mesenteric lymph nodes ($p < 0.0165$) and, although not significantly, in the blood. It is noteworthy that in 5 of 10 mice injected as newborns, the total number of CD\textsuperscript{4} T cells varied between 26 and 57 million cells, representing roughly 100–200 times the number of injected cells.

Given the important contribution of intestinal T cells to the level of peripheral T cell reconstitution and the absence of clinical signs of IBD in newborn recipients before the fourth week after transfer, we addressed the possibility that the bulk of what is called peripheral T cell expansion is the result of immune responses, particularly at the level of the gut. If so, T cell expansion should be limited and the majority of the injected RB\textsuperscript{high} CD\textsuperscript{4} T cells should remain in a resting phenotype when transferred into very young recipients and analyzed before the period of weaning.

Fig. 3A shows the total number of T cells recovered 2 and 3 wk after the transfer of RB\textsuperscript{high} CD\textsuperscript{4} T cells into recipients of 4, 7, or 10 days of age. Very few cells were detected 2 wk after transfer (when the mice were 17, 20, or 23 days old) in the peripheral lymphoid organs in all groups of mice, and no cells were recovered.
from the gut (data not shown). In contrast, massive expansion had occurred after 3 wk in the 10-day-old recipients, which at the time of sacrifice were 1-mo old. As predicted, no increase in T cell numbers was yet observed in the 4-day-old recipients whereas in the intermediate group; 1.5 million cells were scored. Most T cells in the spleen of the youngest recipients still expressed high levels of the CD45RB molecule, compatible with a resting phenotype (Fig. 3B).

Altogether, these experiments indicate that natural environmental resources required to support extensive T cell proliferation are

FIGURE 2. Naive CD4 T cell-induced IBD is delayed in newborn recipients, allowing for extensive proliferation of the transferred T cells. SPF Rag-2-/- were injected as newborns (n = 10) with 3 x 10^6 splenic CD45RB^high^ CD4 T cells from normal syngenic donors. A, Body weight of the recipients and noninjected littermates. B and C, Means (±SEM) of total CD4 T cell numbers and the corresponding organ distribution (shaded bars) at the time of sacrifice (at 10–12 wk of age). For comparison, data obtained from SPF Rag-2-/- recipients injected as adults (Fig. 1) are shown (filled bars). The total cell numbers and the cell recovery from the intestine and the mesenteric lymph nodes (mes. LN) are significantly different (p < 0.05).

FIGURE 3. Peripheral expansion of CD45RB^high^ CD4 T cells depends on the age of the recipient. Four-, 7-, and 10-day-old SPF Rag-2-/- mice were injected with CD45RB^high^ CD4 T cells as in Fig. 1. A. Total CD4^+^ cell numbers recovered per mouse 2 and 3 wk after transfer. B, Expression of CD45RB on gated CD4^+^ splenocytes of the mice in A 3 wk after transfer.
not limiting in Rag-2<sup>−/−</sup> recipients. Considerable T cell expansion coincides with the postweaning period, when diversification of the bacterial flora of the intestine occurs (21).

The naturally activated splenic CD45RB<sub>low</sub> CD4 T cell pool contains T cells that are capable of inducing IBD. As described, RB<sub>low</sub> CD4 T cells contain regulatory T cells, which protect lymphocyte-deficient recipients from RB<sub>high</sub>-induced IBD. Nevertheless, one of nine RB<sub>low</sub> recipients in our experiments showed signs of disease (Fig. 1A), suggesting the presence of primed, aggressive T cells.

When the same transfer experiments done under SPF-conditions were performed in Rag-2<sup>−/−</sup> recipients kept continually under conventional animal housing conditions, all mice injected showed weight loss and diarrhea shortly after transfer, including those of the group that received RB<sub>low</sub> CD4 T cells only (Fig. 4A), whereas noninjected control Rag-2<sup>−/−</sup> mice did not show signs of disease (data not shown). The severity of the disease was much more pronounced and animals had to be sacrificed within a period of 7 wk.

At the time of sacrifice, signs of colon disease were apparent in every mouse, and 1–7 million T cells were recovered from the intestine of recipients of RB<sub>low</sub> CD4 T cells (Fig. 4C), with comparable T cell numbers between the three groups of recipients. In some cases, >10<sup>7</sup> T cells were recovered from the spleen (Fig. 4C).

The present results show that the pool of naturally activated splenic CD4 T cells contains not only regulatory cells, but also primed, potentially aggressive cells.

CD45RB<sub>low</sub> CD4 T cells of GF mice control naive CD4 T cell-induced IBD

The origin and Ag recognition of natural regulatory T cells is to date unknown. Mice kept under GF and Ag-free conditions contain almost normal numbers of naturally activated T and B cells in the spleen, including functionally differentiated effector cells (22).

Here, we studied the ability of naturally activated RB<sub>low</sub> CD4 T cells from GF mice to suppress the development of IBD if co-transferred with naive CD4 T cells. GF donor BALB/c mice were sacrificed immediately upon arrival and several lymphoid organs were analyzed to ascertain that the animals were in a GF state.

Numbers of splenic CD4 T cells were similar to those in SPF mice, and 5.5 and 14.4% of these were, respectively, low and intermediate for expression of the CD45RB molecule (Fig. 5A).

Analysis of mesenteric tissue and collagenase-treated small intestine (including Peyer’s patches) from GF mice of the same colony revealed no more than a total of 6 × 10<sup>4</sup> CD4 T cells in these tissues per mouse, of which more than 70% were CD45RB<sub>high</sub>. In normal SPF mice, at least 100–200 times more CD4 T cells were

FIGURE 4. The splenic CD45RB<sub>low</sub> CD4 T cell pool contains T cells capable of inducing IBD. Non-SPF Rag-2<sup>−/−</sup> mice were injected as in Fig. 1. A, Body weight of individual mice at the time of sacrifice (3–7 wk after transfer). B and C, Total CD4<sup>+</sup> cell numbers and recoveries from the indicated organs. The differences in cell numbers between the different groups are not significant.

FIGURE 5. GF mice contain naturally activated splenic regulatory T cells. A, CD45RB expression on gated CD4<sup>+</sup> cells from GF (bold line) or SPF (plain line) BALB/c mice. B, Body weight of individual C.B.-17-scid mice injected with the indicated splenic CD4 T cell subsets from GF BALB/c donors at the time of sacrifice (2–3 wk for recipients of RB<sub>high</sub> cells, and 7–8 wk for recipients of RB<sub>low</sub> or both populations).
recovered, of which no more than 20% expressed a resting phenotype.

Finally, serum levels of total IgG were on average 56-fold reduced, whereas three times more IgM was detected in GF mice, as compared with normal SPF animals. We conclude that the characteristics of the donor GF mice used in our studies were in agreement with previous descriptions.

Sorted RB<sup>low</sup> CD4 T cells obtained from the spleens of BALB/c GF mice were coinjected with RB<sup>high</sup> CD4 T cells from the same GF mice into C.B.-17-<sup>scid</sup> recipients. As controls, each population was transferred alone.

GF naive CD4 T cells were particularly aggressive to the recipients since most animals started losing weight 10–12 days after transfer, and all mice had to be sacrificed within the next few days. As shown in Fig. 5B, recipients of RB<sup>low</sup> CD4 T cells remained at normal weight until sacrifice. In the group cotransferred with both CD4 T cell subsets, although fluctuation of weight was observed in some mice, all animals were otherwise healthy, without signs of diarrhea or bleeding. Two months after transfer at the time of sacrifice, the large intestine was, in all recipients, macroscopically indistinguishable from that of normal uninjected hosts or from recipients of RB<sup>low</sup> CD4 T cells. As expected, essentially no T cells were recovered from the small and large intestines (data not shown).

**Discussion**

In the present study, we followed the fate of naive and activated/memory CD4 T cells upon transfer, either alone or mixed, into syngenic Rag-2<sup>−/−</sup> recipients. Our results showed that both populations contained cells capable of extensive proliferation and of inducing IBD in the recipient. However, only the naturally activated/memory CD4 T cell pool contained regulatory T cells capable of preventing disease (12–14) and limiting the expansion of activated T cells.

Total T cell recoveries in SPF recipients of RB<sup>high</sup> CD4 T cells were variable and in the range of 8–200 times the number of injected lymphocytes. Since no more than 2–10% of the injected cells survived in the host 24 h after transfer, we estimate that the population underwent 6–11 rounds of division (generating up to 10,000 times the number of surviving cells). The magnitude of expansion was dependent on the capacity of the hosts to survive IBD. Regardless of the degree of expansion, most of the recovered T cells were CD45RB<sup>low</sup>, indicating that they were activated in the recipients. Our data thus present the possibility that these expansions, which have sometimes been interpreted as the result of non-specific homeostatic mechanisms, are actually Ag driven and result from classical immune responses to environmental Ags. This is supported by the observation that activation and expansion of the RB<sup>high</sup> CD4 T cells was negligible during the first 3 wk after transfer into newborn recipients. The correlation found between the age of the recipient and significant expansion of activated cells suggests that this response is largely dependent on colonization of the intestine by diversified bacterial flora, which is known to occur after weaning (21).

In this context, it should be noted that transfer of GF-derived T cells into GF nude mice resulted in limited peripheral replenishment of the hosts, with low T cell numbers in the spleen and no T cell colonization of the gut (A. Bandeira, C. Heusser, and A. Coutinho, unpublished observations). Other explanations for the lack of T cell expansion in newborn recipients, such as the lack of differentiated or functional antigenic niches, or limited nonspecific resources during early development, cannot be excluded at this point. However, they seem unlikely to account for the present results since a number of immune responses, including antiviral responses, can be induced after natural or experimental immunization of euthymic newborn individuals (23).

Despite the relatively limited expansion (four to seven rounds of division) under SPF conditions, the naturally activated splenic CD4 T cell pool also contains cells capable of extensive proliferation in vivo. This capacity was revealed when RB<sup>low</sup> CD4 T cells were transferred into non-SPF recipients. Cells with regulatory activity thus represent a fraction of the heterogeneous RB<sup>low</sup> CD4 T cell population which also contains aggressive cells. It is likely that these cells are reactive (or cross-reactive) to environmental, presumably intestinal, Ags. The reasons for the inefficiency of regulatory T cells to prevent disease in this case were not investigated. They could be the result of exogenous antigenic load in the recipients favoring reactivation of primed aggressive cells or be related to the repertoire of regulatory T cells, since they were originally selected in a different antigenic environment.

The persistency of low numbers of peripheral T cells in SPF mice injected with naturally activated/memory CD4 T cells, or coinjected with naive cells, is remarkable. This demonstrates that the size of the activated peripheral CD4 compartment is regulated, at least partially, at the level of growth control of activated CD4 T cells. This in turn may result in regulation of the state of differentiation of aggressive cells. Examples where helper T cell differentiation is controlled by the cell cycle include the finding that the frequency of IFN-γ-expressing cells increases with successive cell divisions (24).

Despite a chronic state of lymphopenia, in an otherwise non-limiting environment for Ag and natural resources (activated T cells may divide up to 11 times), compensatory mechanisms that try to re-establish total lymphocyte numbers do not seem to operate, at least in Rag-2<sup>−/−</sup> hosts. Peripheral T cell expansions in alymphoid animals or in situations of lymphopenia (following thymectomy or consequent to certain viral infections, e.g., HIV), rather than reflecting (“reconstitution”) or (“regeneration”) of the peripheral immune system, may simply represent unbalanced immune responses of activated T cells against exogenous Ags, thus revealing a certain level of deficiency of the peripheral regulatory T cell pool.

The survival of peripheral naive T cells requires continuous signals through the TCR which are MHC restricted (25). The nature of the MHC-bound peptides in this phenomenon of peripheral positive selection was recently addressed by several groups. Since in all cases, the studies were based on T cell transfers into lymphopenic or alymphoid recipients, these studies addressed expansion rather than survival of transferred T cells.

For CD4 T lymphocytes, Bender et al. (26) concluded that the set of peptides promoting peripheral T cell expansion is distinct from that responsible for thymic selection. Our present results are in agreement with these observations and suggest that the bulk of T cell expansion is driven by exogenous ligands.

Using a similar experimental strategy, a second group reached the opposite conclusion that peripheral expansion and thymic selection involve similar peptides. Their major argument is based on the observation that H-2 M<sup>−</sup> CD4 T cells proliferate more than wild-type B6 T cells when transferred into H-2 M<sup>−</sup> recipients (27). The same authors had previously published, however, that H-2 M<sup>−</sup> CD4 T cells proliferate strongly in vitro against wild-type B6 but not H-2 M<sup>−</sup> stimulators (28). It is therefore to be expected that H-2 M<sup>−</sup> CD4 T cells, if transferred into irradiated B6 mice, will proliferate more extensively than in H-2 M<sup>−</sup> hosts, and thus rather support the opposite conclusion. It is therefore difficult to conclude that the in vivo proliferation of H-2 M<sup>−</sup> CD4 T cells in H-2 M<sup>−</sup> hosts is driven by self-ligands.
For CD8 T cells, Goldrath and Bevan (29) suggested that peripheral T cell expansion requires specific recognition of self-peptide/MHC complexes similar to those involved in thymic maturation. Thus, an antagonist peptide, capable of promoting thymic-positive selection, induced significant T cell expansion (though poor functional differentiation) when expressed in the periphery of allogeneic recipients. A marked difference was nevertheless apparent when this peptide was compared with the high avidity one. In addition, several surface markers remained unchanged upon recognition of the antagonist peptide as compared with resting T cells. It is therefore possible that the effect now observed in vivo is equivalent to the one observed in fetal thymic organ cultures that led to the designation of antagonist peptide. We think that it is a matter of speculation whether this particular peptide is representative of the selecting ligands in the thymus of normal mice. In addition, Kiepe and Jameson (30), using the same TCR-transgenic mice, report that other peptides capable of promoting positive selection seem unable to induce peripheral expansion of these transgenic T cells. Similarly, anti-H-Y TCR transgenic CD8 T cells fail to expand when transferred into female Rag-2% recipients (9).

The variability in the behavior of different TCR-transgenic mice is to be expected given the possible differences in TCR avidity and the lack of quantitative information on the density of their respective ligands in vivo. Analysis of the behavior of normal polyclonal T cell populations should provide a global and more physiological picture of the role of endogenous vs exogenous Ags in promoting peripheral T cell survival and expansion.

Our experiments did not address possible effects of natural regulatory T cells on the size of the naive CD4 T cell pool which is obviously dependent on thymic output (7, 31). For CD8 T cells, it was suggested that the pools of naive and activated/memory cells are independently regulated (8). It remains unknown whether T cell-dependent regulatory mechanisms, similar to those here described for CD4 T cells, also operate in the homeostasis of the activated/memory CD8 T cell compartment.

Regulatory CD4 T cells have also been described in experimental systems of tolerance and autoimmune disease (for review, see Ref. 32). It is not yet known whether these various populations have a common origin, development, or function. Several studies using different experimental systems have shown that CD4⁺CD8⁻ thymocytes can also exert regulatory function (33–37). The intrathymic generation of regulatory T cells (37) supports the idea that these cells are self-reactive (38).

The self-reactive nature of at least part of the peripheral regulatory T cells is supported by our present results showing that GF mice contain effector CD4 T cells capable of preventing IBD. A formal demonstration of the absence of exogenous Ag stimulation in GF mice is difficult to provide. One should note, however, that GF mice have similar numbers of activated splenic T cells as compared with SPF animals (22), whereas a 100–200-fold reduction in the number of T cells in the gut-associated lymphoid tissue is observed. If exogenous Ags were responsible for such natural activation, a similar reduction in the number of activated splenic T cells would be expected. It is nevertheless striking that GF-derived activated T cells prevent IBD when cotransferred with naive T cells into C.B-17.scid recipients, which harbor a bacterial flora different from the one responsible for the selection of the regulatory T cells in GF mice.

It is possible that the same pool of regulatory cells controls not only the magnitude of immune responses to exogenous Ags, but maintains normal tolerance to tissue-specific Ags as well. Situations of lymphopenia may thus lead to the disruption of these regulatory circuits and allow the differentiation and/or abnormal expansion of aggressive T and B cells and the onset of autoimmune disease.

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