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Conversion of Naive T Cells to a Memory-Like Phenotype in Lymphopenic Hosts Is Not Related to a Homeostatic Mechanism That Fills the Peripheral Naive T Cell Pool

Corinne Tanchot,* Armelle Le Campion,* Bruno Martin,* Sandrine Léaument,† Nicole Dautigny,* and Bruno Lucas2*

To examine directly whether a limited number of naive T cells transferred to lymphopenic hosts can truly fill the peripheral naive T cell pool, we compared the expansion and phenotype of naive T cells transferred to three different hosts, namely recombination-activating gene-deficient mice, CD3ε-deficient mice, and irradiated normal mice. In all three recipients, the absolute number of recovered cells was much smaller than in normal mice. In addition, transferred naive T cells acquired a memory-like phenotype that remained stable with time. Finally, injected cells were rapidly replaced by host thymic migrants in irradiated normal mice. Only continuous output of naive T cells by the thymus can generate a full compartment of truly naive T cells. Thus, conversion of naive T cells to a memory-like phenotype in lymphopenic hosts is not related to a homeostatic mechanism that fills the peripheral naive T cell pool. The Journal of Immunology, 2002, 168: 5042–5046.

Peripheral naive T cells do not cycle under normal conditions (1–5). Nevertheless, using monoclonal naive T cells from TCR-transgenic mouse strains and purified polyclonal naive T cells from normal mice, it has recently been shown that most but not all tested naive T cells proliferate when transferred to lymphopenic hosts (5–21). This proliferation does not involve Ag recognition but nevertheless requires interactions with self-MHC molecules. From these observations, most authors have inferred that the proliferation of naive T cells in lymphopenic recipients might reveal the existence of a homeostatic mechanism for filling the peripheral naive T cell pool. To confirm the existence of this homeostatic mechanism, one needs to establish 1) whether this proliferation/expansion completely fills the peripheral naive T cell pool, and 2) that the phenotype and functional capacities of proliferating naive T cells are not modified, or at least that the cells revert to normal once they have returned to a resting state.

Recent studies have followed the phenotypic and functional characteristics of naive T cells over a relatively long period (>1 mo) (14–17, 21). They all showed that, during proliferation, naive T cells converted to a memory T lymphocyte phenotype. In all but one case, the phenotypic and functional characteristics of transferred T cells remained stable over time. Indeed, only Goldrath et al. (14), using irradiated normal mice rather than recombination-activating gene (rag)3-deficient mice as lymphopenic recipients, observed that the transferred cells stopped cycling and reverted to a naive phenotype after filling the peripheral T cell pool. Moreover, they showed that the same monoclonal naive T cells continued to divide in rag-deficient mice, in which the lymphocyte compartment was never reconstituted. Thus, they proposed that, in rag-deficient hosts, the absence of fully developed secondary lymphoid organs or the absence of B cells might explain the discrepancies between their data and results published by other groups (15, 16). Our recent results showing that homeostasis is not restored in CD3ε-deficient mice rule out both hypotheses (21).

In this study, to directly examine whether a limited number of naive T cells transferred to a lymphopenic host can truly fill the peripheral naive T cell pool, we compared the expansion and phenotype of naive T cells transferred to rag-deficient mice, CD3ε-deficient mice, and irradiated normal mice.

Materials and Methods

Mice

H-2b/5-AND TCR-transgenic rag-2−/− mice (21), H-2b/5-AND TCR-transgenic rag-2−/− mice (21), H-2b/5-C57BR/6 mice (21), B10.A 5CC7 TCR-transgenic rag-2−/− mice (21), B10.A CD3ε-deficient mice (22), and B10.A mice were maintained in our animal facilities. B10BR mice, C57BL/6 mice, C57BL/6 Ba (Thy1.1) mice, H-2b/5 CD3ε-deficient mice, and C57BL/6 rag-2−/− mice were obtained from Centre de Développement des Techniques Avancées pour l’ Expérimentation Animale (Orléans, France).

Adoptive transfer of naive T cells

One million CD4+ T cells from lymph nodes or spleen of AND TCR-transgenic rag-2−/− mice were injected i.v. into rag-2−/− mice, CD3ε-deficient mice, and irradiated normal mice of the same haplotype. Spleens and lymph nodes were recovered and pooled at various times after transfer.

Three million CD4+ T cells from lymph nodes of B10.A 5CC7 TCR-transgenic rag-2−/− mice were injected i.v. into B10.A CD3ε-deficient mice and irradiated B10.A mice. Spleens and lymph nodes were recovered and pooled 14 days after transfer.

Normal mice were sublethally irradiated (650 rad) 2 days before transfer.

Flow cytometry

Abs were purchased from BD PharMingen (San Diego, CA). The following Ab combinations were used: PE-conjugated anti-CD8, FITC-conjugated anti-Vα11, PerCP-conjugated anti-CD4, and biotinylated anti-Vβ3 revealed by allophycocyanin streptavidin (BD PharMingen); PE-conju-
gated anti-Vα11. FITC-conjugated anti-Vβ3, PerCP-conjugated anti-CD4, and biotinylated anti-CD25 or CD44 revealed by allophycocyanin streptavidin; PE-conjugated anti-Thy1.2, FITC-conjugated anti-CD8, PerCP-conjugated anti-CD4, and biotinylated anti-Vβ3 revealed by allophycocyanin streptavidin; PE-conjugated anti-Thy1.2, FITC-conjugated anti-Vα11, PerCP-conjugated anti-CD4, and biotinylated anti-CD25 or CD44, revealed by allophycocyanin streptavidin; PE-conjugated anti-lineage (Lin) markers (Lin = CD91 + GR.1 + MAC.1 + TER119 + NK.1), FITC-conjugated anti-CD45, PerCP-conjugated anti-CD4, and biotinylated anti-Vβ3 revealed by allophycocyanin streptavidin.

Calculations

Absolute numbers of recovered CD4⁺ T cells and recovered Vβ3⁺ Vα11⁺ CD4⁺ T cells were calculated at various times after transfer of AND CD4⁺ T cells to irradiated C57BL/6 and B10BR mice. In normal C57BL/6 and B10BR mice, some CD4⁺ T cells coexpress a TCR Vα11 chain and a TCR Vβ chain (C57BL/6: p = 0.24%; B10BR: p = 0.55%). To precisely estimate the number of donor (AND)-derived CD4⁺ T cells among all recovered Vβ3⁺ Vα11⁺ CD4⁺ T cells, the following calculations were performed: (Vβ3⁺ Vα11⁺ CD4⁺) recovered = (Vβ3⁺ Vα11⁺ CD4⁺)host + (Vβ3⁺ Vα11⁺ CD4⁺)AND and (CD4⁺) recovered = (CD4⁺)host + (Vβ3⁺ Vα11⁺ CD4⁺)AND, and all AND CD4⁺ T cells coexpress a Vα11 chain and a Vβ3 chain.

Moreover, (Vβ3⁺ Vα11⁺ CD4⁺)host = P × (CD4⁺)host, where P represents the proportion of host-derived CD4⁺ T cells coexpressing a Vα11 chain and a Vβ3 chain. Therefore, (Vβ3⁺ Vα11⁺ CD4⁺)AND = (Vβ3⁺ Vα11⁺ CD4⁺)host - P × (CD4⁺)host and (Vβ3⁺ Vα11⁺ CD4⁺)AND = ((Vβ3⁺ Vα11⁺ CD4⁺)host - P × (CD4⁺)host)/(1 - P).

Results and Discussion

Naive CD4⁺ T cells converting to a memory phenotype in lymphopenic hosts are not maintained in irradiated recipients

One million lymph node CD4⁺ T cells from AND TCR-transgenic rag-2⁻/⁻ mice (H-2⁺b/b or H-2⁺k/k) were transferred to H-2⁺b/b CD3e-deficient mice and irradiated normal B10BR mice. A. At various times after transfer, peripheral T cells were recovered, counted, and stained for CD4, CD8, Vα11, and Vβ3 surface expression. Absolute numbers of recovered Vβ3⁺ Vα11⁺ CD4⁺ T lymphocytes are shown. B. Absolute numbers of recovered Vβ3⁺ Vα11⁺ CD4⁺ T lymphocytes (○) and estimated numbers of host (X) and AND (△) Vβ3⁺ Vα11⁺ CD4⁺ T cells at various times after transfer to irradiated C57BL/6 mice. C. One week after transfer, peripheral T cells were recovered and stained for CD4, Vβ3, Vα11, and CD25 or CD44 surface expression. Shown are CD25 and CD44 fluorescence histograms of recovered Vβ3⁺ Vα11⁺ CD4⁺ T cells (thick line) in comparison with CD25 and CD44 expression on naive H-2⁺b/b AND CD4⁺ T cells before transfer (thin line).

Table 1. SCC7 CD4⁺ T cells converting to a memory phenotype in lymphopenic hosts are not maintained in irradiated hosts

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Absolute number of Vβ3⁺ Vα11⁺ CD4⁺ T cells (× 10⁶)</th>
<th>CD4⁺ T cells in Vβ3⁺ Vα11⁺ CD4⁺ T cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10A CD3eKO</td>
<td>3.8 ± 0.8</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>Irradiated B10A</td>
<td>62.4 ± 3.2</td>
<td>1.4 ± 0.6</td>
</tr>
</tbody>
</table>

*Three million CD4⁺ T cells from lymph nodes of B10.A SCC7 TCR-transgenic rag-2⁻/⁻ mice were injected i.v. into B10.A CD3e-deficient mice and irradiated B10.A mice. Fourteen days after transfer, peripheral T cells were recovered and stained for CD4, Vβ3, Vα11, and CD44 surface expression.
expression. CD4/Vβ/H9252/ peripheral T cells were recovered, counted, and stained for CD4, CD8, CD19/CD4/CD110/CD3 and TCR-transgenic rag-2−/− mice were harvested and injected lymph node cells (thin line) in comparison with CD25 and CD44/CD25 surface expression. Shown are CD25 and CD44 histograms of recovered Thy1.2/Vβ3/CD8/CD44 T lymphocytes were calculated at each time point. C, Eight weeks after transfer, peripheral T cells were stained for CD4, Thy1.2, Vo/11, and CD25 or CD44 surface expression. Shown are CD25 and CD44 fluorescence histograms of recovered Thy1.2/Vo/11/CD4 T cells derived from injected lymph node cells (thin line) in comparison with CD25 and CD44 expression by recovered Thy1.2/Vo/11/CD4 T cells derived from injected spleen cells (thick line).

were transferred to H-2b/k CD3e-deficient mice (Fig. 1A): the absolute numbers of recovered AND CD4+ T cells remained low, but constant. Similar results were found after transfer to rag-2-deficient mice, except that cell recovery was 3-fold lower than in CD3e-deficient mice.

The results obtained after transfer to irradiated normal mice were totally different. Two weeks after transfer to irradiated H-2b/k normal mice (C57BL/6), the number of recovered Vb3+/Vo/11/CD4 T lymphocytes was intermediate between values obtained in rag-deficient mice and CD3e-deficient mice, and this number fell strongly with time thereafter (Fig. 1A). The absolute numbers of host and AND-derived Vb3+/Vo/11/CD4+ T cells among all recovered Vb3+/Vo/11/CD4+ T cells were estimated in these chimeras (see Materials and Methods) (Fig. 1B). Calculation showed that virtually no injected AND CD4+ T cells could be recovered 12 wk after transfer to irradiated normal mice. Indeed, all Vb3+/Vo/11/CD4+ T cells were of host origin 12 wk post-transfer. These data were further confirmed by transferring AND CD4+ T cells (Thy1.2) to congenic irradiated C57BL/6 Ba mice (Thy1.1) (see Fig. 3). Expression of activation markers (CD25, CD44) by AND CD4+ T cells was studied 2 wk after transfer, at which time nearly all Vb3+/Vo/11/CD4+ T cells were derived from injected AND CD4+ T cells in irradiated normal mice (Fig. 1B). CD25 and CD44 expression was strongly up-regulated after transfer to rag- and CD3e-deficient mice (Fig. 1C). No CD25 expression and slight up-regulation of CD44 expression by Vb3+/Vo/11/CD4+ T cells were observed after transfer to irradiated C57BL/6 mice, suggesting that injected AND CD4+ T cells had undergone less marked activation than in the other two recipients.

Contrary to most naive CD4+ and CD8+ T cells, H-2b/k AND CD4+ T cells proliferated but did not expand after transfer to rag-2- and CD3e-deficient mice (21) (Fig. 1). We thus performed similar experiments with H-2b/k AND CD4+ T cells, as we have previously shown that these cells expand strongly when transferred to H-2b/k CD3e-deficient mice (Ref. 21 and Fig. 2A). Surprisingly, these cells did not expand when transferred to irradiated B10BR mice (Fig. 2A). By estimating the numbers of AND CD4+ T cells in these transfers, we found that, as after transfer of H-2b/k AND CD4+ T cells, H-2b/k AND CD4+ T cells disappeared after transfer to irradiated mice (Fig. 2B). Similarly, 1 wk after transfer, CD44 up-regulation on H-2b/k AND CD4+ T cells was less marked in irradiated mice than in CD3e-deficient mice (Fig. 2C). Together with the observed lack of expansion, this suggested that naive AND CD4+ T cells were submitted to a far less marked activation phase in irradiated mice than in CD3e-deficient mice. Therefore, the activation and subsequent expansion of injected naive T cells seemed to be inversely proportional to the absolute number of preexisting T cells in the host.

![FIGURE 3](https://www.jimmunol.org/) Splen but not lymph node naive AND CD4+ T cell constitute the peripheral naive T cell pool after injection into irradiated hosts. One million lymph node (LNs) or spleen CD4+ T cells from H-2b/k AND TCR-transgenic rag-2−/− mice (Thy1.2) were transferred to irradiated normal C57BL/6 Ba mice (Thy1.1). At various times after transfer, peripheral T cells were recovered, counted, and stained for CD4, CD8, Thy1.2, and Vβ3 surface expression. A, Vβ3/Thy1.2 fluorescence dot plots are presented for gated peripheral CD8+CD4+ T cells, 2 and 8 wk after transfer. B, Absolute numbers of recovered Thy1.2/Vβ3/CD8 -CD4+ T lymphocytes were calculated at each time point. C, Eight weeks after transfer, peripheral T cells were stained for CD4, Thy1.2, Vo/11, and CD25 or CD44 surface expression. Shown are CD25 and CD44 fluorescence histograms of recovered Thy1.2/Vo/11/CD4+ T cells derived from injected lymph node cells (thin line) in comparison with CD25 and CD44 expression by recovered Thy1.2/Vo/11/CD4+ T cells derived from injected spleen cells (thick line).

![FIGURE 4](https://www.jimmunol.org/) Fifteenfold more hematopoietic precursors are coinfected together with 1 × 10⁶ spleen AND CD4+ T cells than with 1 × 10⁶ lymph node AND CD4+ T cells. Lymph nodes and spleen from H-2b/k AND TCR-transgenic rag-2−/− mice were harvested and stained for CD4, Vβ3, CD45, and Lin marker (Lin = CD19 + GR.1 + MAC.1 + TER119 + NK1.1) surface expression. CD4/Vβ3 fluorescence dot plots are presented for all cells and Lin/CD45 fluorescence dot plots are presented for Vβ3+ cells. The percentage of Vβ3+ Lin/CD45+ cells are given, as well as the absolute number of such cells coinfected together with 1 × 10⁶ AND CD4+ T cells.
Similar results were obtained after transfer of lymph node CD4\(^+\) T cells from B10.A 5C7 TCR-transgenic rag-2\(^{−/−}\) mice. Indeed, when naive 5C7 CD4\(^+\) T cells were transferred to syngeneic CD3e-deficient mice they proliferated, resulting in their expansion and conversion to a memory-like phenotype that remained stable with time (22), whereas they did not expand or convert to a memory-like phenotype after transfer to irradiated normal B10.A mice (Table I). Therefore, independently of the hosts used, activation of naive CD4\(^+\) T cells did not lead to filling of the peripheral naive T cell pool. Furthermore, absolute numbers of recovered injected cells fell rapidly after transfer to irradiated normal mice, probably reflecting competition for space between injected T cells and newly produced host T cells (23–25). Contrary to Goldrath et al. (14), we did not find that naive T cells expanded better in irradiated mice than in T cell-deficient recipients (rag-deficient or CD3e-deficient mice). One major methodological difference was that we injected lymph node naive T cells instead of a pool of spleen and lymph node cells (14).

**Spleen but not lymph node naive CD4\(^+\) T cells reconstitute the peripheral naive T cell pool of irradiated hosts**

One million lymph node or spleen CD4\(^+\) T cells from H-2\(^{b/b}\) AND TCR-transgenic rag-2\(^{−/−}\) mice (Thy1.2) were transferred to irradiated normal C57BL/6 Ba mice (Thy1.1). For 2 wk after transfer, recovery of AND CD4\(^+\) T cells (Thy1.2 \(V\beta3^+\) CD4\(^+\) cells) was similar with spleen and lymph node cells (A and B). Moreover, AND CD4\(^+\) T cells converted to a memory phenotype in both cases (data not shown). Four weeks after spleen cell transfer, the absolute number of recovered AND CD4\(^+\) T cells started to increase, reaching a plateau at 8 wk, whereas, as shown above (Fig. 1), the absolute number of AND CD4\(^+\) T cells in mice injected with lymph node cells fell rapidly (Fig. 3, A and B). Moreover, 8 wk after transfer, AND CD4\(^+\) T cells recovered after spleen cell injection exhibited a true naive phenotype, which contrasted with the stable memory-like phenotype of AND CD4\(^+\) T cells recovered after lymph node cell injection (Fig. 3C).

It is unlikely that intrinsic differences between lymph node- and spleen-derived naive T cells would affect their behavior after transfer to irradiated animals to such an extent. A more logical explanation would be that the hematopoietic precursors contained in the spleen of adult mice (26, 27) reconstituted the thymuses of irradiated hosts, thereby permitting the generation of large numbers of naive CD4\(^+\) T cells. Indeed, 15-fold more hematopoietic precursors (defined by the absence of expression of Lin markers and the expression of CD45) are coinfused together with \(1 \times 10^6\) spleen AND CD4\(^+\) T cells than with \(1 \times 10^6\) lymph node AND CD4\(^+\) T cells (Fig. 4).

**Reconstitution of the host thymus by spleen hematopoietic precursors, rather than peripheral homeostasis, explains peripheral naive T cell pool reconstitution by CD4\(^+\) splenocytes**

At various times after transfer of lymph node or spleen AND CD4\(^+\) T cells to irradiated normal mice, thymocytes were recovered, counted, and stained for CD4, CD8, Thy1.2, and V\(\beta3\) surface expression. As observed in the periphery of these mice (see above), no difference between spleen and lymph node cell transfer was noted for 2 wk after transfer; at these early time points the thymus was reconstituted only by host-derived precursors (Fig. 5, A and B). Later after spleen cell transfer, but not after lymph node cell transfer, thymocytes comprised large numbers of donor cells. These results could not be explained by reentry of activated peripheral T cells into the thymus, as all thymic subsets comprised donor-derived cells (Fig. 5, A and B). Indeed, 4 wk after transfer and onwards, a large proportion of immature double-positive thymocytes were found to derive from injected splenocytes (Fig. 5B).

By contrast, no immature double-positive thymocytes were found to derive from injected lymph node cells at any time point studied.

**FIGURE 5.** Reconstitution of the host thymus by spleen hematopoietic precursors, rather than peripheral homeostasis, explains the reconstitution of the peripheral naive T cell pool by AND CD4\(^+\) splenocytes. One million lymph node (LNs) or spleen CD4\(^+\) T cells from H-2\(^{b/b}\) AND TCR-transgenic rag-2\(^{−/−}\) (Thy1.2) mice were transferred to irradiated normal C57BL/6 Ba (Thy1.1) mice (A and B) or to irradiated or nonirradiated CD3\(\epsilon\)-deficient C57BL/6 mice (C). At various times after transfer, thymocytes were recovered, counted, and stained for CD4, CD8, Thy1.2, and V\(\beta3\) surface expression. A. At various time points after transfer to irradiated C57BL/6 mice, absolute numbers of recovered CD8\(^+\) CD4\(^+\) (○) and Thy1.2\(^+\) V\(\beta3^+\) CD8\(^+\) CD4\(^+\) (□) thymocytes were calculated. B. At various time points after transfer to irradiated C57BL/6 mice, absolute numbers of recovered double-positive (○) and Thy1.2\(^+\) V\(\beta3^+\) double-positive (□) thymocytes were calculated. C. CD4/CD8 fluorescence dot plots are presented for all thymocytes 8 wk after transfer to irradiated or nonirradiated CD3\(\epsilon\)-deficient mice.
Most mature CD4+ thymocytes produced 4 wk after spleen cell transfer were AND CD4+ T cells, and this production was stable with time (Fig. 5A). These results suggested that the hematopoietic precursors injected together with spleen AND CD4+ T cells possessed self-renewal capacities. Thus, our results confirm that spleen cell transfer to irradiated animals permits thymus reconstitution by donor cells (26, 27).

Therefore, the different behavior of lymph node and spleen AND CD4+ T cells after transfer to irradiated mice would be due to de novo generation of naive CD4+ T cells by the thymus rather than to homeostatic proliferation restricted to spleen naive CD4+ T cells. Our results suggest that the observation of Goldrath et al (14) that naive T cells transiently acquire a memory-like phenotype after transfer to irradiated hosts would reflect replacement of memory-like OT-1 CD8+ T cells by newly generated OT-1 CD8+ thymic migrants rather than a phenotypic and functional reversion of these memory-like cells to a naive state after peripheral T cell pool filling.

Finally, to explain the different behavior of spleen naive T cells in rag-deficient recipients and irradiated normal hosts (14), we transferred one million lymph node or spleen CD4+ T cells to CD3- CD4-deficient recipients and irradiated normal mice (14), which we termed ‘naive T cell-deprived recipients.’ In naive T cell-deprived recipients, Thymus reconstitution by donor cells was studied 8 wk after transfer (Fig. 5C). Our results clearly show that both irradiation and subsequent injection of spleen cells are required for host thymus reconstitution by donor cells, in agreement with early papers describing thymic reconstitution after irradiation and injection of hematopoietic precursors (28–32). Thus, these observations, together with the finding that cell recovery was much lower in rag-2-decient mice than in CD3-deficient mice (Fig. 1), provide a highly plausible explanation for the different behavior of spleen naive T cells after transfer to rag-deficient recipients and irradiated hosts.

Naive T cells transferred to lymphopenic recipients (rag-deficient mice, CD3-deficient mice, and irradiated normal mice) failed to fill the peripheral naive T cell pool (15–17, 21). Indeed, absolute numbers of recovered T cells were far below those in the full peripheral naive T cell pool of normal mice. Moreover, injected naive T cells acquired a memory-like phenotype that remained stable with time, despite the absence of foreign antigenic stimulation, and their functional capacities were modified, enhanced, or abolished (15–17, 21). Finally, injected cells were rapidly replaced by host thymic migrants after transfer to irradiated normal mice. These data argue against the view that the proliferation of naive T cells in lymphopenic mice represents a homeostatic mechanism regenerating the naive T cell pool. Further studies should focus on the mechanisms underlying this proliferation and the relevance of this process to disease-induced lymphopenia.

Note. During the processing of this manuscript, an article on the same field reaching similar conclusions was published (33).

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