γ chain required for naïve CD4+ T cell survival but not for antigen proliferation

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Lymphoid homeostasis is required to ensure immune responsiveness and to prevent immunodeficiency. As such, the immune system must maintain distinct populations of naïve T cells that are able to respond to new antigens as well as memory T cells specific to those antigens it has already encountered. Though both naïve and memory T cells reside in and traffic through secondary lymphoid organs, there is growing evidence that the two populations may be regulated differently. We show here that naïve T cell survival and memory T cell survival have different requirements for cytokines (including the interleukins IL-2, IL-4, IL-7, IL-9 and IL-15) that use the common cytokine receptor gamma chain (γ_c). Using monoclonal populations of antigen-specific CD4+ T cells, we found that naïve T cells cannot survive without γ_c, whereas memory T cells show no such requirement. In contrast, neither naïve nor γ_c-deficient memory T cells were impaired in their ability to proliferate and produce cytokines in response to *in vivo* antigenic stimulation. These data call into question the physiological role of γ_c-dependent cytokines as T cell growth factors and show that naïve and memory CD4+ T cell survival is maintained by distinct mechanisms.

To balance the ability to generate vigorous recall responses with the need to maintain reactivity to new antigens, the peripheral T cell compartment contains populations of memory T cells that have previously responded to antigen as well as populations of naïve T cells that have not yet been stimulated. There is evidence to suggest that the two populations are independently regulated and that major histocompatibility complex (MHC) molecules may be involved[1–3]. For example, the lifespan of mouse memory T cells can be as long as 18 months in the complete absence of MHC molecules and specific antigen[4,5], whereas the lifespan of naïve T cells is no longer than 6 months in the absence of antigen[6], and considerably shorter than that in the absence of MHC molecules[3,7,8]. In addition, the naïve T cell pool depends on continuous thymic export, whereas the pool of memory T cells does not[6].

Cytokines may also be involved in lymphoid homeostasis, as these pleiotropic factors can modulate cell survival, growth, differentiation and apoptosis. In particular, the group of cytokines (including interleukin 2(IL-2), IL-4, IL-7, IL-9 and IL-15) that signal through receptors sharing the common cytokine receptor gamma chain (γ_c) has been shown to promote lymphoid proliferation *in vitro* and early lymphoid development *in vivo*[9,10]. However, despite their well established *in vitro* activities (IL-2, for example, was first known as "T cell growth factor"), the relative importance for γ_c cytokines in naïve and memory T cell survival and proliferation *in vivo* has not been clearly defined.

In this report, we analyze the requirement for γ_c cytokines during the generation and maintenance of an immune response involving CD4+ αβ T cells. Our results suggest that the mechanisms governing the homeostasis of mature peripheral naïve, activated and memory T cells are different with regards to γ_c cytokine dependency.

Results

Intrathymic selection of γ_c-deficient CD4+ T cells

To investigate the role of γ_c-dependent cytokines in the homeostasis of antigen-specific CD4+ T cells *in vivo*, we generated RAG-2-deficient T cell antigen receptor (TCR) transgenic (Tg) mice (called Marilyn) harboring monoclonal CD4+ T cell populations specific for the male antigen H-Y, and bred them onto the γ_c^+ or γ_c^- backgrounds. Male γ_c^+ or γ_c^- mice bearing this autoreactive receptor showed identical and complete negative selection in the thymus (data not shown). Wild-type Marilyn female mice, having an intact gene for γ_c, generated mature CD4+ T cells in the thymus (**Fig. 1a**), and these cells migrated into the periphery, establishing a pure population of nondividing, naïve CD4+ T cells with low CD44 and high CD62L expression (**Fig. 1b** and data not shown). Thus, as predicted[11], these H-Y–specific T cells appeared to lack cross-reactivity with self or environmental antigens.

In the absence of the γ_c chain, a completely different picture emerged. Absolute numbers of total thymocytes in γ_c^- female Marilyn mice were 5% of those in female Marilyn γ_c^+ mice, reflecting the known role of γ_c in the IL-7-mediated expansion of early thymic precursors[10]. However, the development of CD4 cells was fairly efficient, with absolute numbers of CD4+ mature thymocytes in female Marilyn γ_c^- mice at about 30% of those found in γ_c^+ littermates (**Fig. 1a** and **c**). Nevertheless, there were very few peripheral CD4+ T cells in female Marilyn γ_c^- mice (<0.4% of γ_c^+ controls; **Fig. 1b** and **c**). As with their γ_c^+ counterparts, the few splenic γ_c^- CD4+ T cells that did exist were nondividing cells with a typical naïve phenotype, low CD44 and high CD62L expression, and steady-state numbers not changing with age (**Fig. 1b** and data not shown). The difference in the absolute numbers of γ_c^- CD4+ T cells present in the thymus and the numbers present in the periphery suggested that these cells might have a limited peripheral persistence.

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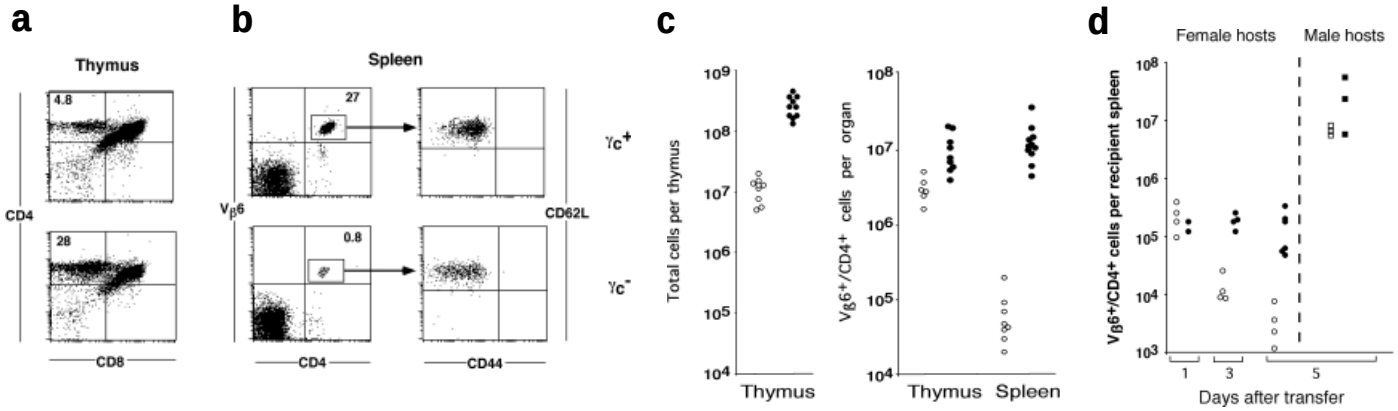

Figure 1. γ_c-dependent lymphokines are required for the peripheral survival of naïve CD4⁺ T cells. (**a**) Thymocytes and (**b**) splenocytes from either γ_c^+ or γ_c^- monoclonal (RAG-2$^{-/-}$) female Marilyn mice were studied by cytofluorometry using the indicated antibodies. (**c**) The absolute numbers of total lymphocytes or mature CD4⁺ T cells in γ_c^+ (filled circles) and γ_c^- (open circles) Tg mice in the thymus or the spleen of individual mice are shown. (**d**) 3×10^6 CD4⁺ single positive (SP) thymocytes from γ_c^+ (filled) or γ_c^- (open) Marilyn female mice were transferred into alymphoid RAG-2$^{-/-}$ $\gamma_c^{-/-}$ female (circles) or male (squares) hosts and analyzed 1, 3 and 5 days later. Data are derived from three independent experiments.

Survival of γ_c-deficient naïve CD4⁺ T cells

To assess the survival capacity afforded by the γ_c chain, we adoptively transferred mature γ_c^+ or γ_c^- Marilyn thymocytes into syngeneic H2^b alymphoid hosts (RAG-2$^{-/-}$ $\gamma_c^{-/-}$ double mutant mice which lack all T, B and NK cells and their precursors[12]). γ_c^+ Marilyn cells survived for extended periods of time in the periphery of alymphoid female hosts, retaining a naïve phenotype (**Fig. 1d** and data not shown). γ_c^+ Marilyn cells did not expand after transfer, which was consistent with their lack of cross-reactivity and inability to non-specifically proliferate in the alymphoid host in the absence of antigen[13]. In contrast, transferred γ_c^- Marilyn cells were lost within 5 days following transfer (**Fig. 1d**). These data suggest that the defect in γ_c^- Marilyn cells lies with their inability to survive in the periphery and identifies an essential role for γ_c-cytokines in providing survival signals to naïve CD4⁺ T cells *in vivo*.

To gain further insight into the survival defect in γ_c^- mice, we examined the pattern of expression of Bcl-2, which it is known to be tightly controlled in the thymus. Bcl-2 expression normally appears highest in thymocyte precursors, drops to low levels in double positive (DP) cells, and is sharply upregulated during or immediately after positive selection[14,15]. Although γ_c cytokines regulate Bcl-2 levels in early thymocytes[16,17], we found that these cytokines do not have an apparent role in the upregulation of Bcl-2 expression during positive selection: both DP (data not shown) and SP CD4⁺ thymocytes from female Marilyn γ_c^+ and γ_c^- mice contained similar amounts of intracellular Bcl-2 protein (**Fig. 2a** and **b**). In contrast, Bcl-2 levels were dramatically reduced in the few detectable peripheral T cells of γ_c^- Marilyn female mice (**Fig. 2d**), suggesting that γ_c-dependent cytokines play a pivotal role in the homeostasis of the naïve peripheral T cell pool[18].

Collectively, these results demonstrate that intrathymic and peripheral survival signals are not equivalent. During thymic positive selection, γ_c-dependent cytokines appear dispensable for the survival of SP CD4⁺ thymocytes (spontaneous apoptosis in both γ_c^+ and γ_c^- post-selection thymocytes was <2%; data not shown), although γ_c-cytokines may provide additional signals for the proliferation of these cells. Once in the periphery, however, γ_c-cytokines appear to be essential for naïve cell survival through the maintenance of anti-apoptotic pathways. Transfer of naïve Marilyn γ_c^+ T cells into mice deficient in different γ_c-dependent cytokines should help to identify which particular ligands drive this process.

γ_c-cytokine roles in proliferation of CD4⁺ T cells

Current models of antigen-driven T cell proliferation generally invoke the necessity for *de novo* cytokine synthesis (including the γ_c-dependent cytokines IL-2 and IL-4) for subsequent cell cycle entry and clonal expansion[19]. Because our γ_c^- Marilyn female mice harbored small numbers of phenotypically naïve, antigen-reactive T cells, they provided a unique opportunity to re-examine potential redundant roles for γ_c cytokines in T cell activation, proliferation and survival. First, to assess whether γ_c cytokines were essential for antigen-driven T cell responses and memory cell formation *in vivo*, we primed naïve female Marilyn mice by an intravenous (i.v.) injection of splenocytes from male CD3ε-deficient mice. Under these *in vivo* conditions, we saw clonal expansion of CD4⁺ T cells in γ_c^+ mice and, quite unexpectedly, also in γ_c^- mice (**Fig. 3a**) with similar kinetics of activation. On day 2, both γ_c^+ and γ_c^- T cells had acquired the CD25 and CD69

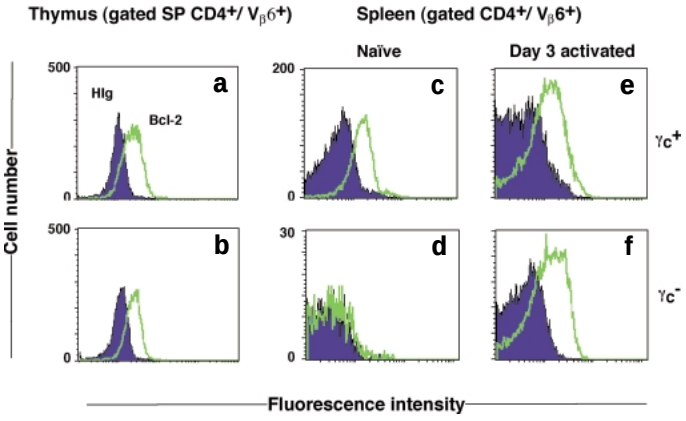

Figure 2. Bcl-2 is expressed normally in γ_c^- CD4⁺ SP thymocytes but not in naïve γ_c^- Vβ6⁺/CD4⁺ splenocytes. Bcl-2 (open histograms) and hamster immunoglobulin isotype control (filled histograms) staining are shown on gated SP CD4⁺ thymocytes from either (**a**) γ_c^+ or (**b**) γ_c^- Marilyn female mice. Bcl-2 expression in (**c**) γ_c^+ or (**d**) γ_c^- naïve Vβ6⁺CD4⁺ splenocytes. There are very few of these cells in the γ_c^- Marilyn female mice. (**e** and **f**) Bcl-2 expression is upregulated in Vβ6⁺CD4⁺ splenocytes from γ_c^- Tg Marilyn mice 3 days after *in vivo* antigenic stimulation.

activation markers. By day 4, both types of cells had undergone blastogenesis, and by day 6, they both had high CD44 and low CD62L expression (**Fig. 3c** and data not shown). TCR activation also resulted in the upregulation of Bcl-2 levels (**Fig. 2e** and **f**). After 1 week, the absolute numbers of γ_c^+ Marilyn T cells had increased five-fold, whereas the γ_c^- cells had increased over 100-fold to reach almost equivalent plateaus (**Fig. 3a**), and the frequency of cycling cells had returned to baseline levels (data not shown). After priming, both γ_c^+ and γ_c^- memory Marilyn T cells were maintained *in vivo* for at least 1 month (**Fig. 3a**). Thus, injection of cells expressing male antigen into Marilyn female mice generates a typical immune response with conversion of naïve T cells into activated T cells, and this process is independent of the presence of the γ_c chain.

To determine whether the apparently heightened ability of γ_c^- T cells to respond to antigen in comparison to their γ_c^+ counterparts was due to their low starting numbers, we transferred small numbers of naïve γ_c^+ Marilyn T cells into $H2^b$ alymphoid female hosts and challenged them with syngeneic male cells. Under these conditions, γ_c^+ Marilyn T cells were also strongly activated (**Fig. 3c**) and expanded at least 100-fold by day 7 (**Fig. 3b**). These observations suggest that γ_c^+ and γ_c^- naïve Marilyn T cells have a similar intrinsic capacity to respond to antigen *in vivo* and that the plateau of cell numbers is set by other parameters. The γ_c^- cells also have an equivalent capacity to produce IL-2, despite their inability to respond to this cytokine (**Fig. 3d**), and express normal levels of transcripts for interferon γ and tumor necrosis factor α (data not shown). Finally, whereas γ_c cytokines promote the survival of activated T cells *in vitro*[20], we found that they were not necessary *in vivo*, as primed T cells persisted in the absence of γ_c for up to a month (**Fig. 3a**).

Based on the parameters studied (the kinetics and magnitude of proliferation, and phenotypic and functional changes associated with antigen activation), the responses of γ_c^- Marilyn $CD4^+$ T cells appeared similar to normal cells. Two additional observations supported this conclusion. First, when a mixture of 80% γ_c^+ and 20% γ_c^- naïve Marilyn T cells was injected into male alymphoid mice, similar robust proliferation was observed by both cell types: 86 ± 2% γ_c^+ cells and 14 ± 1% γ_c^- activated Marilyn T cells were recovered four days later. Second, *in vivo* treatment with cyclosporin inhibited the antigen-driven proliferation of both γ_c^+ or γ_c^- Marilyn T cells by 90% (data not shown). Thus, the available evidence suggests that the same physiological processes govern the proliferative responses of γ_c^+ and γ_c^- $CD4^+$ T cells. Although we cannot formally rule out a role for γ_c cytokines in antigen-driven responses *in vivo*, our results demonstrate that this process can efficiently proceed in the absence of γ_c.

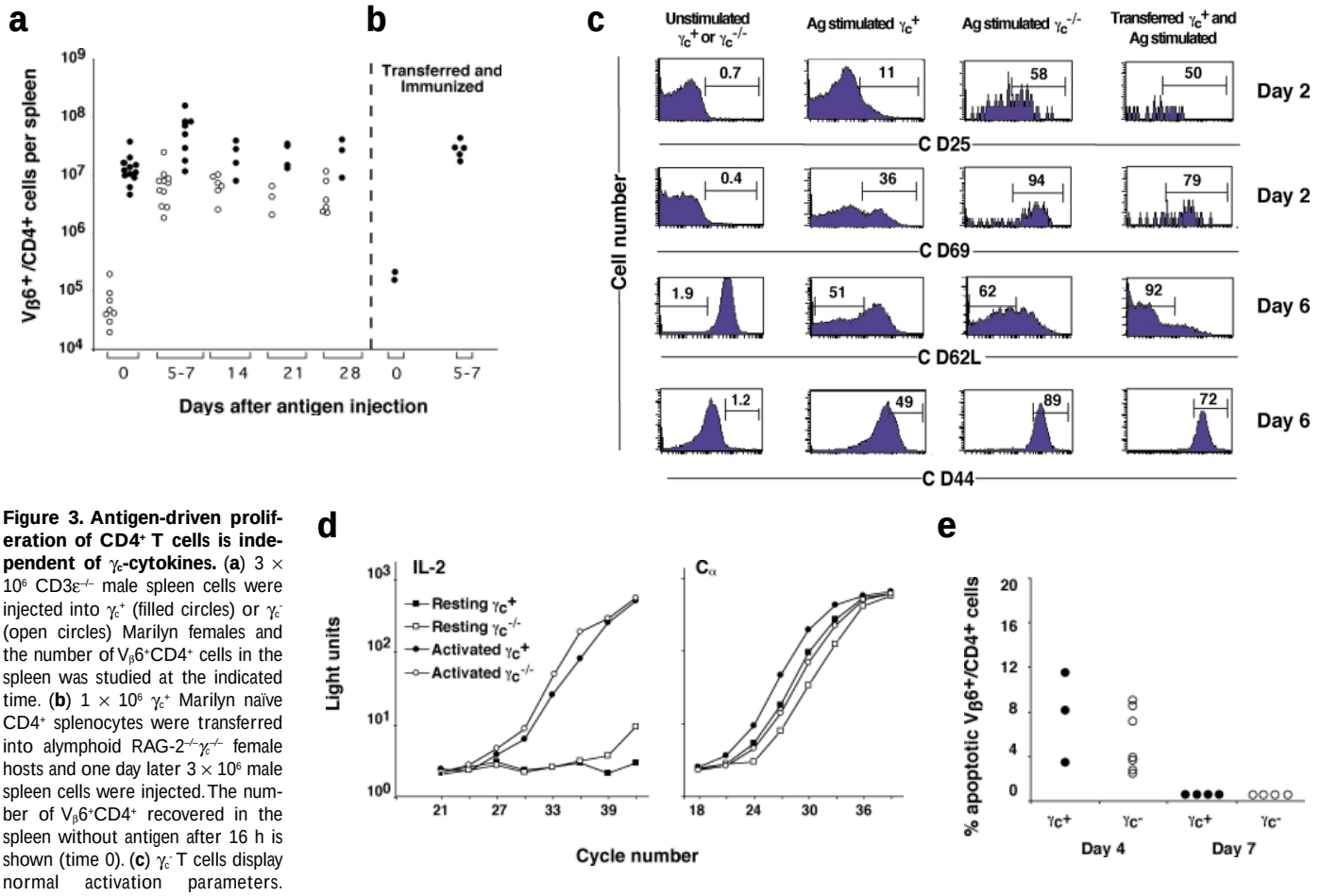

Figure 3. Antigen-driven proliferation of $CD4^+$ T cells is independent of γ_c-cytokines. (**a**) 3×10^6 $CD3\varepsilon^{-/-}$ male spleen cells were injected into γ_c^+ (filled circles) or γ_c^- (open circles) Marilyn females and the number of $V_\beta6^+CD4^+$ cells in the spleen was studied at the indicated time. (**b**) 1×10^6 γ_c^+ Marilyn naïve $CD4^+$ splenocytes were transferred into alymphoid RAG-$2^{-/-}\gamma_c^{-/-}$ female hosts and one day later 3×10^6 male spleen cells were injected. The number of $V_\beta6^+CD4^+$ recovered in the spleen without antigen after 16 h is shown (time 0). (**c**) γ_c^- T cells display normal activation parameters. Splenocytes from γ_c^+ or γ_c^- Marilyn females were studied at the indicated times after antigenic (Ag) stimulation. The right hand panel displays results obtained on 1×10^5 γ_c^+ Marilyn T cells transferred into alymphoid hosts and then challenged with male spleen cells. (**d**) Resting (squares) or 1 day antigen stimulated (circles) γ_c^+ (closed symbols) or γ_c^- (open symbols) $CD4^+$ T cells were purified and total RNA extracted. Kinetic ELISA-PCR was carried out for IL-2 (left-hand panel) or C_α (right-hand panel). C_α levels were comparable indicating similar amounts of T cell-derived RNA among the different samples analyzed.

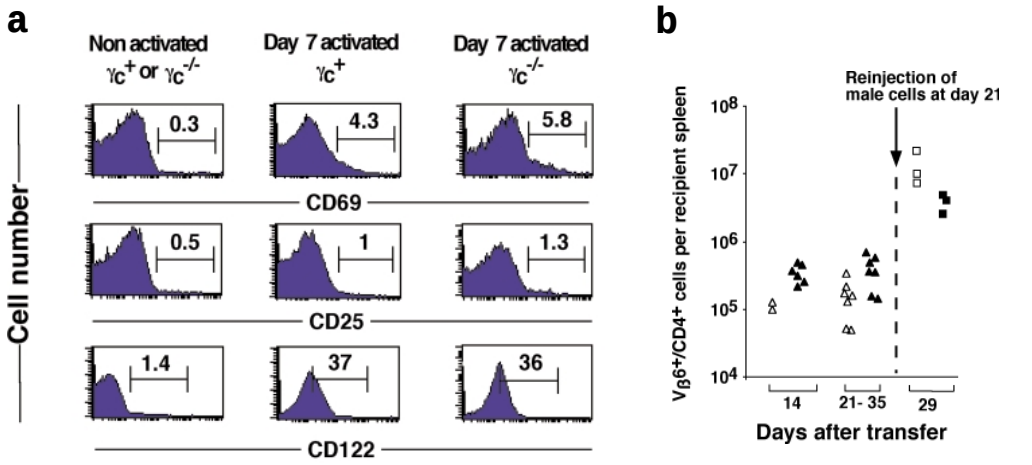

Figure 4. Memory CD4⁺ T cell survival and recall responses are independent of γ_c-cytokines. (**a**) Phenotype of day 7 activated γ_c^+ or γ_c^- Marilyn T cells prior to transfer. (**b**) Primed γ_c^+ or γ_c^- Marilyn T cells (1×10^6 cells) were transferred into alymphoid female hosts. The absolute number of $V_\beta6^+CD4^+$ T cells in the spleen was determined at the indicated times (filled triangles) γ_c^+ (open triangles) γ_c^-. For recall responses, mice were injected with 3×10^6 $CD3\varepsilon^{-/-}$ male spleen cells and the number of splenic $V_\beta6^+CD4^+$ cells evaluated 1 week later (filled squares) γ_c^+ (open squares) γ_c^-.

γ_c-deficiency and memory CD4⁺T cell homeostasis

Although these results suggested that homeostasis of γ_c^+ and γ_c^- memory T cells is similar, we needed to examine the possibility that it is maintained by different mechanisms. For example, one might argue from previously observations[21], that there could be higher proliferation and increased apoptosis in activated γ_c^- T cells. However, this was not the case as the frequency of apoptotic cells was similar during the course of activation of γ_c^+ and γ_c^- Marilyn T cells (**Fig. 3e**). To determine whether γ_c-cytokines could be required for the survival and restimulation of memory cells in the absence of antigen and thymic output, we transferred previously primed γ_c^+ or γ_c^- Marilyn T cells into $H2^b$ alymphoid female hosts. At the time of transfer, both γ_c^+ and γ_c^- T cells were CD25 and CD69 negative and contained <2 % cycling cells (**Fig. 4a** and data not shown). We found that both γ_c^+ and γ_c^- Marilyn T cells persisted for at least 5 weeks under these conditions (**Fig. 4b**). We then boosted these mice at day 21 post-transfer and found that, like naïve T cells, both γ_c^+ and γ_c^- memory Marilyn T cells proliferated extensively upon antigen re-exposure (**Fig. 4b**). These results suggest that γ_c-dependent cytokines are dispensable for the maintenance and restimulation of memory $CD4^+$ T cells.

Discussion

Numerous *in vitro* studies have demonstrated that T cells are dependent on IL-2, IL-4, IL7 or IL-15 for antigen-induced proliferation, survival of activated and memory cells and/or prevention of anergy[22]. Although rare exceptions have been reported *in vitro*[23], our data clearly demonstrate that these functions of $CD4^+$ T cells can proceed *in vivo* in the absence of all γ_c-dependent cytokines. The most likely explanation for these contradictory findings is that antigen-driven proliferation (or memory cell survival) *in vivo* is supported by soluble or membrane-bound interactions which are not provided in dissociated cell cultures *in vitro*, or which are provided only for a limited period of time by cellular components that are not maintained in culture. Consistent with this hypothesis, we found that γ_c^- Marilyn T cells can be activated following antigen stimulation *in vitro*, but their subsequent proliferation is clearly reduced compare to γ_c^+ Marilyn cells (data not shown). Our data also argue against the concept of γ_c cytokine redundancy[24] for $CD4^+$ T cell proliferation and memory responses *in vivo*. It remains to be determined if similar rules apply to $CD8^+$ T cells.

Our data shed new light on previous results obtained in normal and TCR Tg γ_c^- mice, where γ_c-dependent cytokines were implicated in intrathymic development and peripheral T cell homeostasis. In γ_c^- mice expressing a normal TCR repertoire there were almost no naïve T cells[21,25], and in the TCR Tg mice, there were few T cells expressing the Tg TCR but many expressing endogenous receptors[21,26,27]. Some studies have hypothesized that these results reflect a differential role for γ_c signals depending on TCR affinity[26]; they in fact reflect the differences in γ_c-cytokine dependency between naïve and memory cells that we observed. If naïve cells cannot survive without γ_c cytokines, but can proliferate when stimulated by antigen and become memory cells, then for γ_c^- mice, only those naïve T cells responsive to cross-reactive antigens would be found, and not surprisingly, most of them would have an activated phenotype and respond poorly to further immunization. Our observations also explain the results obtained in one report on γ_c^- TCR Tg mice[26] (which were RAG-$2^{-/-}$ and therefore had the capacity to rearrange endogenous receptors), that naïve T cells purely expressing the Tg TCR would fail to respond to environmental antigens and would soon die, whereas some of the remaining T cells, expressing either low or high levels of endogenous TCRα chains, would respond to self or environmental antigens, continue to express Bcl-2 and become activated/memory cells.

Finally, our data suggest that the varying requirements of T cells for cytokines at different stages of their development might reflect the types of signals perceived at each step. Differentiating T cells receive a myriad of signals during their lifetime, which vary depending on the stage of development (intrathymic versus peripheral) or activation status (naïve versus memory) of the T cell. Here we provide evidence that the cytokine requirements of T cells also differ with the stage of maturation and/or activation. Striking parallels can be observed: the survival and development of early pre-T cells in the thymus, for example, is γ_c-dependent prior to TCRβ expression[17], whereas later stages of repertoire positive and negative selection, which are triggered by signals through the TCR, appear γ_c-independent[27] as shown here. Likewise, naïve thymic emigrants require γ_c for their survival in the periphery, but not for antigen–antigen presenting cell (antigen-APC) stimulated activation or for subsequent survival as memory cells. We suggest that there are two possibilities for these differential susceptibilities to cytokine signaling. First, the T cells are at profoundly different stages of development. Second, in both the thymus and the periphery, these cells may reside in distinct microenvironments that provide different signals. Naïve and memory T cell "niches", for example, may harbor different types of APCs (professional versus semi-professional[11,28], and non-APC accessory cells such as follicular dendritic or other cells), which are capable of providing the necessary antigens, costimulatory

molecules and cytokines for naïve versus memory cell survival and/or activation. The next step will be to unravel how discrete populations of accessory cells provide these stimuli and how, at different stages in their development, T cells respond to them.

Methods

Mice. VJ segments encoding the TCRα (V_α1.1-J_α35) and TCRβ (V_β6-J_β2.3) chains from Marilyn, a CD4 T cell clone specific for the complex of the male antigen (H-Y) with I-A^{b}[29] were inserted into TCR shuttle vectors as described[30]. Constructs were coinjected into fertilized C57Bl/6 (B6) eggs at the NIAID transgenic facility in Frederick, MD. Founder lines were crossed to RAG-$2^{-/-}$ mice (9th backcross to B6) and then to RAG-$2^{-/-}$ $\gamma c^{-/-}$ mice (4th backcross to B6). All Marilyn Tg mice used in this study were RAG-$2^{-/-}$ and thereby expressed only monoclonal populations of Marilyn T cells.

Cytofluorometry. Four color cytometry was performed with directly conjugated antibodies (Pharmingen) according to standard techniques and analyzed using a Facscalibur flow cytometer. Intracellular Bcl-2 staining was carried out as described[31]. Cell cycle status and activation-induced apoptosis were determined using 7-amino-actinomycin D staining in saponin-permeabilized or untreated cell preparations, respectively. The following antibodies were used: anti-CD3–FITC, anti-CD4–PE and –APC, anti-CD8–APC, anti-V_β6–PE, anti-CD44–FITC, anti-CD122–FITC and biotinylated anti-V_β6, anti-CD62L, anti-CD25 and anti-CD69 revealed by strepavidin-Cychrome.

Naïve cell survival. Mature $CD4^+$ thymocytes were obtained following depletion by anti-CD8 and anti-HSA antibodies and complement, and $1–3 \times 10^6$ mature $CD4^+$ cells were injected i.v. into non-irradiated alymphoid, syngeneic alymphoid RAG-$2^{-/-}$ $\gamma c^{-/-}$ $H2^b$ hosts[12].

Antigen stimulation *in vivo*, generation of memory T cells. Splenocytes from CD3ε-deficient male mice (3×10^6) were injected i.v. into γc^+ or γc^- female Marilyn mice. At various times after antigen stimulation, cell numbers, activation markers and cell cycle status were assessed, and RNA was obtained from purified $CD4^+$ T cells. To generate memory T cells, $CD3\varepsilon^{-/-}$ male spleen cells (10^7/ml) were treated with mitomycin (70 μg/ml) for 45 min at 37 °C before injection into γc^+ or γc^- female Marilyn mice. Splenic $CD4^+$ T cells were isolated 7 days after antigen injection using anti-CD4 magnetic beads and the VarioMacs system from Miltenyi Biotech and 1×10^6 primed T cells were transferred into non-irradiated alymphoid RAG-$2^{-/-}$ $\gamma c^{-/-}$ $H2^b$ female hosts.

Polymerase chain reaction (PCR). Total RNA from $1–5 \times 10^5$ cells was extracted, reverse transcribed and a quantitative kinetic ELISA-based PCR was performed[32]. The *Il2* primers were as follows: *Il2* - 5′: TGTACAGCATGCAGCTCGCATCC; *Il2* - 3′: CATGCCGCAGAGGTCCAAGTTCA. C_α primers have been described[33].

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