The Common Cytokine Receptor γ Chain Plays an Essential Role in Regulating Lymphoid Homeostasis

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Summary
In the immune system, there is a careful regulation not only of lymphoid development and proliferation, but also of the fate of activated and proliferating cells. Although the manner in which these diverse events are coordinated is incompletely understood, cytokines are known to play major roles. Whereas IL-7 is essential for lymphoid development, IL-2 and IL-4 are vital for lymphocyte proliferation. The receptors for each of these cytokines contain the common cytokine receptor γ chain (γc), and it was previously shown that γc-deficient mice exhibit severely compromised development and responsiveness to IL-2, IL-4, and IL-7. Nevertheless, these mice exhibit an age-dependent accumulation of splenic CD4+ T cells, the majority of which have a phenotype typical of memory/activated cells. When γc-deficient mice were mated to DO11.10 T cell receptor (TCR) transgenic mice, only the T cells bearing endogenous TCRs had this phenotype, suggesting that its acquisition was TCR dependent. Not only do the CD4+ T cells from γc-deficient mice exhibit an activated phenotype and greatly enhanced incorporation of bromodeoxyuridine but, consistent with the lack of γc-dependent survival signals, they also exhibit an augmented rate of apoptosis. However, because the CD4+ T cells accumulate, it is clear that the rate of proliferation exceeds the rate of cell death. Thus, surprisingly, although γc-independent signals are sufficient to mediate expansion of CD4+ T cells in these mice, γc-dependent signals are required to regulate the fate of activated CD4+ T cells, underscoring the importance of γc-dependent signals in controlling lymphoid homeostasis.

The common cytokine receptor γ chain (γc) is the genetic defect in X-linked severe combined immunodeficiency (XSCID) (1) and is a shared component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (2–5). In humans with XSCID, the lack of functional γc results in profoundly diminished T cell development and an absence of natural killer (NK) cells. In contrast, B cell numbers are normal, although the B cells are nonfunctional with concomitant hypogammaglobulinemia. Mice in which the γc gene has been targeted by homologous recombination exhibit a related, but somewhat different phenotype (6–8). In humans with XSCID, they have hypoplastic thymuses and lack NK cells. Unlike humans with XSCID, conventional B cells are greatly diminished in the bone marrow and the spleens of both young and adult γc-deficient mice, likely reflecting the key role of IL-7 as a pre-B cell growth factor in mice, but not in humans (5). In addition, these mice lacked visible gut-associated lymphoid tissue and peripheral lymph nodes were essentially absent. Although splenic T cells are diminished in number at 3 wk of age, CD4+ T cells, but not CD8+ T cells, dramatically accumulate in the spleen in an age-dependent manner. Moreover, the finding of CD4+ T cell infiltrates, particularly in the gut in association with colitis, suggested that the CD4+ T cells might be activated and involved in mediating the pathological changes found in these mice. Given that the mice are compromised in their ability to respond to γc-dependent cytokines including IL-2, IL-4, and IL-7 (6–8), this accumulation of CD4+ T cells was unexpected. We now have performed studies to characterize further these CD4+ T cells to understand better the role of γc in development of the immune response in vivo.

Materials and Methods

Mouse and Genetic Analysis. The γc-deficient mice (7) used in these studies were back-crossed to either C57BL/6 (B6) (H-2b/b) or BALB/c mice (H-2d/d) (Jackson Laboratory, Bar Harbor, ME) for three/four generations. Since the γc gene is localized on X chro-

Abbreviations used in this paper: γc, common cytokine receptor γ chain; PI, propidium iodide; TUNEL, terminal deoxynucleotide transferase (TdT)-dUTP nick end-labeling; XSCID, X-linked severe combined immunodeficiency.
mosome, DO10 TCR transgenic male mice (H-2b/d), specific for ovalbumin (9), were mated with BALB/c \(\gamma_c^{+/+}\) heterozygous female mice. These matings yielded four genotypes of male mice (DO10\(\gamma_c^{+/+}\), DO10\(\gamma_c^{+/+}\), DO10\(\gamma_c^{-/-}\), and DO10\(\gamma_c^{-/+}\)). Similarly, AND TCR transgenic male mice (H-2b/d), specific for cytchrome c (10, 11), were mated with B6 \(\gamma_c^{+/+}\) female mice. Mice were housed in microisolator cages under pathogen-free conditions. The mice were genotyped by PCR of tail DNA using Mingen) and anti-CD16/CD32 mAb (FcγRIII/II receptor, 2.4G2, PharMingen) and PE-conjugated anti-erythroid mAb (TER-119, PharMingen). Cells from spleen were treated with ACK lysing buffer to remove red blood cells, stained with PE-conjugated anti-CD8 mAb (53-6.7, PharMingen), washed twice with PBS containing 1% BSA and analyzed on a FACSort®.

In Vivo Bromodeoxyuridine (BrDU) Uptake and BrDU Staining. 8 to 12-wk-old \(\gamma_c^{-/-}\)-deficient or littermate control mice were injected i.p. with 750 \(\mu\)g of BrdU (Sigma) in 250 \(\mu\)l of PBS at time 0 and again at 4 h. Control mice were injected with PBS. 16 h after the second injection, thymocytes and purified splenic CD4+ T cells were fixed with 70% ethanol for 30 min on ice, denatured with 2 N HCl containing 0.5% Triton X-100 for 30 min to produce single-stranded DNA, and neutralized with 0.1 M sodium tetraborate, pH 9.0 (Sigma). Cells were resuspended in PBS, 1% BSA containing 0.5% Tween-20, stained with anti-BrDU FITC (Becton Dickinson) for 30 min at 25°C, washed once with PBS and once with PBS, 1% BSA, then stained with anti-CD62L PE/and anti-CD4 Cy-Chrome, and analyzed on a FACSort®.

Appendant Rates of Replication and Survival. Assuming that cells that have taken up BrdU at 20 h have divided once, the apparent replication rate/day, \(r\), can be calculated from the equation: percent BrdU uptake/20 h \(\times\) 24 h/\(d\) = 2\(r\)/(100 + \(r\)). Based on the data in Fig. 1 D, this yields values for \(r\) of 21.43%/d for \(\gamma_c^{-/-}\) mice and 6.32%/d for wild-type mice. Assuming that no death occurs and that there is no new migration of cells from thymus to periphery (neither assumption is likely to be correct), we can estimate the number of cells that might exist after 63 d of growth (from 3–12 wk of age) as \(1.3 \times 10^8 \times (1.2143)^{63} = 2.66 \times 10^{11}\) splenic T cells instead of 5.28 \(\times\) 10^9 cells for \(\gamma_c^{-/-}\) mice and 9.1 \(\times\) 10^8 \(\times\) (1.0632)^{63} = 4.31 \(\times\) 10^10 cells instead of 1.67 \(\times\) 10^7 for wild-type mice. Although not accurate, these calculations suggest that survival rates for the cells differ between the wild-type and \(\gamma_c^{-/-}\)-deficient mice. Again, assuming no new migration from the thymus to periphery and a constant replication rate, the survival rate/day can be estimated as \(\sim\)87% for \(\gamma_c^{-/-}\) mice and 95% for wild-type mice, suggesting that cells from the \(\gamma_c^{-/-}\)-deficient mice had an increased rate of cell death (see below).

**Results and Discussion**

Like humans with XSCID, \(\gamma_c^{-/-}\)-deficient mice have hypoplastic thymus and lack NK cells (5–8). However, peripheral T cells are generally not found in humans with XSCID (4), whereas peripheral CD4+ T cells are found in the \(\gamma_c^{-/-}\)-deficient mice (5–8). Although these cells are diminished in numbers in \(\gamma_c^{-/-}\)-deficient mice at 3 wk of age (6, 7), in most mice there is a dramatic age-dependent accumulation of these cells (7) (Fig. 1 A), even though the thymus remains small (7). Analysis using a series of different TCR Vβ-specific mAbs (Vβ2, Vβ3, Vβ4, Vβ5.1.5.2, Vβ6, Vβ8.1.8.2,
Vβ10, and Vβ11) indicated that the CD4+ T cell accumulation was polyclonal (unpublished data). Interestingly, the splenic CD4+ T cells in γc-deficient mice were increased in size (Fig. 1 B). The large size of the cells, coupled with the finding of CD4+ T cell infiltrates, particularly in the gut in association with colitis (5, 7), suggested that the CD4+ T cells might be activated and involved in mediating the pathological changes found in these mice. Therefore, we investigated the basis for the CD4+ T cell expansion.

A striking difference between splenic CD4+ T cells from γc-deficient and wild type mice was that the cells from the γc-deficient mice exhibited a CD62LlowCD69high phenotype typical of memory/activated cells, whereas those from wild-type mice exhibited a CD62LhighCD69low phenotype typical of naive cells (Fig. 1 C) (15). Interestingly, CD4+ T cells in mice deficient in IL-2 (16, 17), IL-2Rα (18), and IL-2Rβ (19) also exhibited activated phenotypes. Expression of CD49d (VLA-4 α chain) and CD44 was slightly increased in γc-deficient CD4+ T cells (data not shown), whereas no significant difference was seen in expression of IL-2Rα (CD25) (Fig. 1 C), or in CD40L, CD28, CTLA-4, or in Fas (data not shown). The lack of increase in these activation markers suggested that these cells were partially rather than fully activated and that γc-dependent signals are required for completing T cell activation. In contrast with the different phenotypes in splenic T cells, CD4+CD8− thymocytes from γc-deficient mice and wild type mice expressed similar levels of CD62L, CD69, and Qa-2α (data not shown).

The accumulation and activation of the CD4+ T cells appeared to result from a defect in T cells, because the defect was corrected in mice in which γc expression was selectively reconstituted in T cells (transgenic mice in which γc is under control of the CD3ε enhancer and promoter) but not in mice in which γc expression was selectively reconstituted in B cells (transgenic mice in which γc is under control of the mb-1 promoter and Eμ heavy chain enhancer) (data not shown). A similar activated phenotype in Jak3-deficient mice was corrected when these mice were reconstituted with Jak3 under control of the Lck promoter (19a), a finding consistent with the similar phenotypes in splenic T cells, CD4+CD8− thymocytes from γc-deficient mice and wild type mice expressed similar levels of CD62L, CD69, and Qa-2α (data not shown).

Next, we investigated whether the CD62LlowCD69high phenotype of the γc-deficient splenic CD4+ T cells reflected active proliferation and found that bromodeoxyuridine (BrdU) uptake in these cells was greater than in wild-type mice (Fig. 1 D, left). In contrast, γc-deficient thymocytes exhibited lower BrdU uptake than wild-type thymocytes (Fig. 1 D, right). Thus, there was more active cell division in the spleens than in the thymi of adult γc-deficient mice, correlating with the activated phenotype of the γc-deficient splenocytes. It is important to recognize that because these mice were not subjected to thymectomy, some splenic BrdU uptake could have occurred in thymocytes before CD4+ T cell migration to the periphery; however, because the thymi in γc-deficient mice are very small (0.5–5% of littermate controls) and the BrdU uptake in thymocytes in these mice was lower than in wild-type mice, it is likely that the majority of BrdU uptake occurred in the periphery.

Based on the BrdU uptake studies, the γc-deficient mice...
Annexin V binding to CD4 increased rate for the wild-type mice; see Materials and Methods). Such an increase in splenic CD4 T cells from 6-wk-old mice was 21.43%/d for wild-type mice (21.43%/d for wild-type mice) had an apparent replication rate much greater than wild-type mice, suggesting an increased rate of cell death (see Materials and Methods). In AND mice, we compared the relative frequency of memory (CD62L high) and naive peripheral CD4 T cells. Approximately 70% of CD4 T cells from DO10+/Y mice express the transgenic TCR. (Fig. 3 A), suggesting that CD4 T cells from the γc-deficient mice had an increased rate of cell death (see Materials and Methods). Indeed, when cultured without stimulation, purified splenic CD4 T cells from γc-deficient mice exhibited a higher level of death than cells from wild-type mice, with the greatest difference occurring in the first 8 h (Fig. 2 A). Increased apoptosis was confirmed both by TUNEL analysis (data not shown) and by staining with annexin V (Fig. 2 B), which allows the detection of apoptotic cells at an early stage because of its selective affinity for phospholipids, especially phosphatidylserine (PS), which is exposed on the cell surface in apoptotic cells, while normally PS is restricted to the inner cell membrane (20–22). The rapid increase in cell death seen at 8 h in γc-deficient mice (Fig. 2 A) presumably is accounted for by the large number of cells that stained with annexin V at time 0 (Fig. 2 B), suggesting that splenic CD4 T cells of γc-deficient mice may be primed to die by apoptosis in vivo. Bcl-2 levels correlate with the survival of lymphoid cells (23, 24) and are induced by γc-dependent cytokines that protect against apoptosis (25–28). Therefore, it was interesting that γc-deficient splenic CD4 T cells expressed very low levels of Bcl-2 (Fig. 2 C) (including both CD62Lhigh and CD62Llow subpopulations; data not shown), suggesting that γc is required for maintaining the normal levels of Bcl-2 expression in both activated and naive peripheral CD4 T cells.

There are at least two possible explanations for why peripheral CD4 T cells in γc-deficient mice were already activated without exogenous stimulation. In the first model, the activated phenotype does not require TCR stimulation (TCR-independent activation model); instead, γc-dependent signals are required for keeping mature CD4 T cells in a naive stage and, that without such signals, these cells are activated nonspecifically. In the second model, peripheral CD4 T cells in γc-deficient mice respond to self- or environmental antigens (TCR-dependent activation model). We investigated these possibilities using mice expressing MHC class II–restricted transgenic TCRs specific for ovalbumin (DO10 mice) or cytochrome c (AND mice). In these experiments, the mice were not exposed to the specific antigens. In DO10 male mice deficient in γc (DO10γc−/− mice), far fewer splenocytes were stained with annexin V at time 0 (Fig. 2 A) in γc-deficient mice (Fig. 2 C) (including both CD62Lhigh and CD62Llow subpopulations; data not shown), suggesting that γc is required for maintaining the normal levels of Bcl-2 expression in both activated and naive peripheral CD4 T cells.

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93% of transgenic TCR+ (Vα11+) CD4+ T cells were CD62Lhigh, similar to ANDγc/Y mice (94%). These results support the TCR-dependent activation model and suggested that TCR(s) expressed on expanding peripheral CD4+ T cells in γc-deficient mice are specific for self-antigen or environmental antigens. The finding that over 80% of adult γc-deficient mice (C57BL/6 background) develop an inflammatory bowel disease (data not shown) is consistent with the possibility of self-reactive CD4+ T cells in these mice.

In the immune response, there is a careful balance between clonal expansion and clonal deletion (29–31). Clonal expansion is believed to be regulated by mitogenic cytokines, especially IL-2. In γc-deficient mice, it was therefore surprising that although splenic T cells exhibit diminished in vitro responsiveness to mitogens (5), there was TCR-dependent activation and a marked expansion of CD4+ T cells in vivo. In addition to this in vivo proliferation, we also detected an augmented rate of apoptosis in γc-deficient CD4+ T cells, a finding consistent with the known action of γc-dependent cytokines as survival factors (25–28); nevertheless, it was evident that the overall rate of death was inadequate for the degree of proliferation. Although the CD4+ T cells were activated, they expressed only low levels of CD25, CTLA-4, and Fas. Therefore, it is conceivable that γc-dependent signals are required to achieve induction of pathways that are required to negatively regulate immune responses. Thus, γc-dependent signals are essential components of a carefully controlled homeostatic mechanism that regulates the fate of activated cells.
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