Regulation of Interleukin (IL)-18 Receptor α Chain Expression on CD4$^+$ T Cells during T Helper (Th)1/Th2 Differentiation: Critical Downregulatory Role of IL-4

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Abstract

Interleukin (IL)-18 has been well characterized as a costimulatory factor for the induction of IL-12–mediated interferon (IFN)-γ production by T helper (Th)1 cells, but also can induce IL-4 production and thus facilitate the differentiation of Th2 cells. To determine the mechanisms by which IL-18 might regulate these diametrically distinct immune responses, we have analyzed the role of cytokines in the regulation of IL-18 receptor α chain (IL-18Rα) expression. The majority of peripheral CD4$^+$ T cells constitutively expressed the IL-18Rα. Upon antigen stimulation in the presence of IL-12, marked enhancement of IL-18Rα expression was observed. IL-12–mediated upregulation of IL-18Rα required IFN-γ. Activated CD4$^+$ T cells that expressed low levels of IL-18Rα could produce IFN-γ when stimulated with the combination of IL-12 and IL-18, while CD4$^+$ cells which expressed high levels of IL-18Rα could respond to IL-18 alone. In contrast, T cell stimulation in the presence of IL-4 resulted in a downregulation of IL-18Rα expression. Both IL-4$^{-/-}$ and signal transducer and activator of transcription (Stat)6$^{-/-}$ T cells expressed higher levels of IL-18Rα after TCR stimulation. Furthermore, activated T cells from Stat6$^{-/-}$ mice produced more IFN-γ in response to IL-18 than wild-type controls. Thus, positive/negative regulation of the IL-18Rα by the major inductive cytokines (IL-12 and IL-4) determines the capacity of IL-18 to polarize an immune response.

Key words: IFN-γ–inducing factor • IL-12 • IL-1R–related protein • Stat • IFN-γ

Introduction

IL-18 is an 18-kD cytokine originally described as IFN-γ–inducing factor for its ability to augment IFN-γ production by activated T cells (1). Subsequently, IL-18 has been regarded as a proinflammatory cytokine that can synergize with IL-12 to increase IFN-γ production, NK cell cytotoxicity, and Th1 responses (2–5). While IL-18 clearly enhances Th1 responses, it is less clear whether IL-18 is capable of initiating Th1 differentiation in the absence of IL-12 (6–10). Although a majority of published reports link IL-18 with Th1-associated functions, IL-18 has recently been shown to augment Th2 responses (11–14). Administration of IL-18 to mice led to an increase in IL-4 production and elevated serum IgE. The mechanisms whereby IL-18 can influence both Th1 and Th2 responses are unclear at this time.

The IL-18R complex is composed of two known chains, IL-18 receptor α chain (IL-18Rα)* and IL-18Rβ (15–20). IL-18Rα (IL-1R5) was originally described as IL-1 receptor-related protein (IL-1Rrp) because of its homology with the IL-1/Toll receptor family and is the extracellular binding domain of the IL-18R complex. IL-18Rβ (also known as IL-18RAcPL or IL-1R7) is the signal transducing chain of the IL-18R complex and is also a member of the IL-1R family. It has been widely believed that the IL-18Rα is not expressed on naive T cells, but is induced during differentiation of Th1, but not Th2, cells. Here, we have used a recently developed polyclonal antibody to IL-18Rα to investigate regulation of its expression on differentiating CD4$^+$ T cells. In contrast to previous studies, we demonstrate that resting peripheral CD4$^+$ and CD8$^+$ T

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**Abbreviations used in this paper:* IL-18Rα, IL-18 receptor α chain; MFI, mean fluorescence intensity; NMS, normal mouse serum; RAG, recombination activating gene; Stat, signal transducer and activator of transcription.

cells express IL-18Rα. Although it has been proposed that IL-12 alone may upregulate expression of the IL-18Rα, culture of naive $CD4^+$ T cells in IL-12 or IL-18 alone had no effect on IL-18Rα expression. However, activation of T cells via the TCR in the presence of IL-12, but not IL-18, resulted in marked upregulation of IL-18Rα expression. The combination of IL-18 and IL-12 further enhanced IL-18Rα expression during TCR activation. Although IFN-γ did not directly regulate IL-18Rα expression, IL-12–mediated upregulation of IL-18Rα was not observed in the absence of IFN-γ. After activation in the presence of IL-12, the capacity of $CD4^+$ T cells to produce IFN-γ in response to IL-18 alone correlated with their expression of high levels of the IL-18Rα. In sharp contrast, the combination of TCR ligation and IL-4 led to a marked downregulation of IL-18Rα on unprimed $CD4^+$ T cells. The downregulatory effects of IL-4 on IL-18Rα were completely dependent on expression of signal transducer and activator of transcription (Stat)6. Collectively, these results demonstrate that IL-18Rα expression is finely regulated during the course of $CD4^+$ T cell differentiation along either the Th1 or Th2 pathways. They are most consistent with a model in which IL-12/IFN-γ play a critical function in upregulation of IL-18Rα expression during differentiation along the Th1 pathway, while IL-4 plays a downregulatory role on IL-18Rα during Th2 differentiation. These data are critical to furthering our understanding of the proposed dual role of IL-18 as a differentiation factor for both Th1 and Th2 responses as well as its possible therapeutic use as an adjuvant.

Materials and Methods

Animals. Female C57BL/6, C57BL/6 IFN-$\gamma^{-/-}$, C57BL/6 IL-$4^{-/-}$ mice, and 5CC7 B10.A/Ai recombination activating gene (RAG)-$2^{-/-}$ mice expressing a TCR transgene specific for cytochrome C were obtained from Taconic Farms and used at 4–8 wk of age. Female BALB/c and Stat$6^{-/-}$ (C129S2-Stat6) mice were obtained from Jackson ImmunoResearch Laboratories. All animals were housed under specific pathogen-free conditions and provided food and water ad libitum. Animals were maintained according to National Institutes of Health Animal Care Guidelines.

Cell Lines. Short-term polarized T cell lines were established as follows: $CD4^+$ T cells were isolated from lymph nodes of 5CC7 RAG-$2^{-/-}$ mice by negative selection using FITC-labeled anti-B220, anti-CD8, and anti–I-A^k, followed by anti-FITC magnetic separation. Purity of $CD4^+$ population was confirmed by FACS® analysis. $CD4^+$ T cells (10^5 cells per milliliter) were stimulated in vitro for 4–5 d with T cell–depleted, irradiated B10.A APCs (10^6 cells per milliliter) and 1 μM cytochrome C peptide in IL-2–supplemented media containing 10% fetal bovine serum, L-glutamine, antibiotics, and β-mercaptoethanol. To establish Th1 lines, IL-12 (10 ng/ml; R&D Systems) and anti–IL-4 (10 μg/ml 11B11) were added to cultures. For Th2 lines, IL-4 (1,000 U/ml), anti–IL-12 (10 μg/ml C17.8), and anti–IFN-γ (10 μg/ml XMG 1.2) were added. Th "null" cells were established by the addition of anti–IL-12, anti–IL-4, and anti–IFN-γ antibodies. After 4–5 d, cells were washed and resuspended in fresh IL-2 media for an additional 48 h (21).

In some experiments, splenic $CD4^+$ T cells from wild-type C57BL/6 or IFN-$\gamma^{-/-}$ mice were cultured (4×10^6 cells per milliliter) with plate-bound anti-CD3 (5 μg/ml 2C11; BD PharMingen) and IL-2 (with or without rIL-12, rIL-18, rIL-4, rIL-10, rhTGF-β) for 48 h. Cells were washed, then resuspended in fresh IL-2 media (with or without fresh cytokine added) for an additional 48 h before FACS® analysis.

Flow Cytometry. Cells were washed twice in PBS, resuspended in PBS, and placed ($1–2 \times 10^6$ cells) into individual wells of a 96-well V-bottomed Costar plate. The cells were first incubated for 5 min at 4°C with 1 μg of rat anti–mouse CD16/CD32 (Fc block; BD PharMingen) to block nonspecific binding of goat Ig/anti–IL-18Rα antibody to Fc receptors. Cells were then incubated with either 1 μg of goat IgG or 1 μg of goat anti-murine IL-18Rα (R&D Systems) diluted in PBS containing 5% normal mouse serum (PBS-NMS). Anti–IL-18Rα is an affinity-purified, polyclonal goat-specific IgG antibody that recognizes the extracellular domain (peptide 19–326) of recombinant murine IL-18Rα. Cells were incubated with the respective antibody for 30 min, 4°C, washed two times with PBS, incubated in PBS-NMS for 10 min, and then stained with SP-biotinylated mouse anti–goat IgG (H plus L) diluted in PBS-NMS (Jackson ImmunoResearch Laboratories) for 20 min. Cells were subsequently washed two times in PBS and then resuspended in streptavidin-PE (BD PharMingen) diluted in PBS. After a 10-min incubation at 4°C, FITC-labeled anti-CD4 (BD PharMingen) was added directly to wells for an additional 20-min incubation at 4°C. Cells were again washed two times in PBS and resuspended in PBS. 7-AAD was added immediately before FACS® analysis to exclude dead cells from analysis. Analysis was performed using CELLQuest™ software (Becton Dickinson).

Data are presented as the difference (Δ) between the mean fluorescence intensity (MFI) of the positive stain (anti–IL-18Rα) and the MFI of the negative control (goat Ig).

Cytokine Assays. T cells that had been primed in vitro for 4–7 d under various polarizing conditions were resuspended in fresh IL-2 medium. Cells (10^6 cells per milliliter) were cultured in 24-well plates for 48 h with 10 ng/ml IL-12, 30 ng/ml IL-18, or both. Cell-free supernatants were collected and levels of IFN-γ determined by ELISA (R&D Systems). Alternatively, primed cells were restimulated with either immobilized anti-CD3 (5 μg/ml 2C11; BD PharMingen) or IL-12 and IL-18 (as above) for 6–8 h, and monensin was added for the final 4 h of culture. Cells were harvested, permeabilized with saponin/PBS buffer, then stained for intracellular IFN-γ and IL-4.

Results

Naive T Cells Express the IL-18Rα. In contrast to previous studies which only detected the IL-18Rα on fully differentiated Th1 cells (22), a majority (82 ± 2%) of both $CD4^+$ and $CD8^+$ splenic T cells from C57BL/6 mice were reactive by FACS® analysis with the polyclonal antibody to recombinant IL-18Rα (Fig. 1, A and C). In multiple experiments ($n = 5$) of this type the ΔMFI of IL-18Rα expression on freshly explanted $CD4^+$ T cells was 143 ± 11. $CD4^+$ and $CD8^+$ T cells in lymph node expressed levels of IL-18Rα similar to that seen in spleen (data not shown). We also detected high levels of IL-18Rα on mature $CD4^+$ and $CD8^+$ single positive T cells in the thymus, while $CD4^+CD8^+$ double positive thymocytes expressed rela-

tively low levels (data not shown). Thus, expression of the IL-18Rα appears to be acquired during the process of T cell differentiation in the thymus. Constitutive expression of the IL-18Rα was independent of either IL-12 or IFN-γ as the level of expression of the IL-18Rα on $CD4^+$ cells from IL-$12^{-/-}$ or IFN-$\gamma^{-/-}$ mice was identical to $CD4^+$ T cells from wild-type mice (data not shown). Analysis of other cell types revealed low levels of IL-18Rα expression on $B220^+$ splenic B cells (Fig. 1 D), while the highest level of IL-18Rα expression was found on $NK1.1^+$ cells (Fig. 1 E). We consistently identified a small population (~6–10%) of splenic $CD4^+$ cells that expressed high levels of the IL-18Rα (Fig. 1 A, M2, MFI 1,072 ± 286). This IL-18Rα^{hi} population was absent in the spleens of 5CC7 TCR transgenic mice on a RAG-$2^{-/-}$ background (Fig. 1 B, arrow), suggesting the $CD4^+$ IL-18Rα^{hi} population in the former could be a population of previously activated/memory T cells or $CD4^+NK1.1^+$ T cells. Further analyses revealed the majority (~90%) of $CD4^+$ IL-18Rα^{hi} cells are $NK1.1^-$ and express markers typically associated with memory/activated T cells (data not shown). Thus, two distinct levels of IL-18Rα expression (IL-18Rα^{int} and IL-18Rα^{hi}) can be detected on $CD4^+$ T cells in spleens from normal animals.

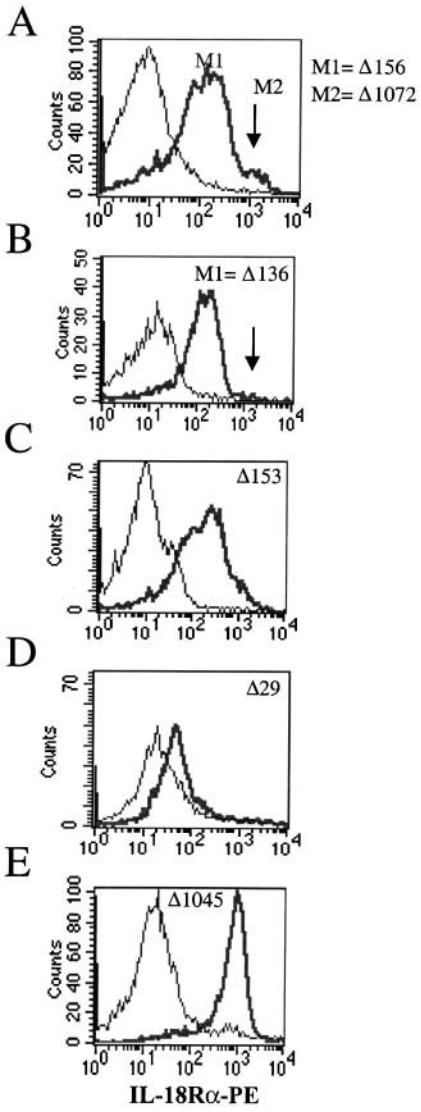

Figure 1. Expression of IL-18Rα on cells from naive mice: splenic $CD4^+$ (A) and $CD8^+$ (C) T cells from C57BL/6 mice, $CD4^+$ T cells from 5CC7 TCR transgenic mice on RAG-$2^{-/-}$ background (B), $B220^+$ cells (D), and $NK1.1^+$ cells (E) from spleens of C57BL/6 mice. Spleen cells were pooled from naive mice and analyzed for IL-18Rα expression by FACS®. Analysis was gated on 7-AAD^- cells only (i.e. viable cells). Numbers in parentheses refer to the difference (Δ) between the MFI of the positive stain and the MFI of the isotype (negative) stain.

Stimulation with IL-12 or IL-18 Alone Does Not Lead to Upregulation of IL-18Rα on $CD4^+$ IL-18Rα^{int} T Cells. Previous reports have suggested that IL-12 in the absence of TCR stimulation was capable of inducing IL-18Rα expression, thus enabling IL-18 to synergize with IL-12 to enhance IFN-γ production (23). To evaluate the effects of cytokines on IL-18Rα expression, we cultured spleen cells from normal C57BL/6 mice for 96 h in IL-2–containing media alone or in IL-2–containing media supplemented with either IL-12 or IL-18. Preliminary studies demonstrated that IL-2 was required to maintain adequate viability of the cells. Culture with IL-2 alone led to a decrease in IL-18Rα expression on $CD4^+$ IL-18Rα^{int} cells (Fig. 1 A, M1, MFI 143 ± 21; and Fig. 2 A, M1, MFI 94 ± 15), but did not modify the level of expression of IL-18Rα on the subpopulation of IL-18Rα^{hi} cells (Fig. 1 A, M2, MFI 1,088 ± 109; and Fig. 2 A, M2, MFI 1,072 ± 286). However, it should be noted that culture in IL-2 alone did result in a marked increase in the percentage of the IL-18Rα^{hi} population from 6–10% to ~20–40% of $CD4^+$ T cells during the 96-h culture (Figs. 1 A and 2 A). This increase presumably reflects the preferential expansion of previously activated/memory T cells in the presence of IL-2. Slight upregulation of IL-18Rα expression on the $CD4^+$ IL-18Rα^{int} cells was observed in the presence of IL-12 (MFI 102 ± 12), but not IL-18 (Fig. 2, B–D, M1, MFI 88 ± 13). In striking contrast, the minor population of $CD4^+$ IL-18Rα^{hi} cells upregulated IL-18Rα expression in response to IL-12 (Fig. 2 B, M2, MFI 2,425 ± 1,074), but not IL-18 (Fig. 2 C, M2, MFI 1,029 ± 171). Thus, the primary effects of IL-12 on freshly explanted $CD4^+$ T cells from normal animals appear to be via upregulation of the IL-18Rα on the minor population of $CD4^+$ T cells that already express high levels of this receptor and which appear to be memory/activated T cells.

IL-12 in Concert with TCR Stimulation Induces Upregulation of IL-18Rα Expression on $CD4^+$ T Cells. To examine the effects of TCR stimulation on IL-18Rα expression, $CD4^+$ T cells from naive C57BL/6 mice were stimulated for 2 d with immobilized anti-CD3 in IL-2–containing media, with or without IL-12/IL-18. After 2 d in culture, the cells were harvested, washed, and placed into fresh IL-2 media with fresh cytokines for an additional 2 d. This two-step culture system was used because significant apoptosis was observed when T cells were cultured for 4 d on plate-bound anti-CD3. IL-18Rα expression was detected on $CD4^+$ T cells stimulated with anti-CD3 and IL-2, but the

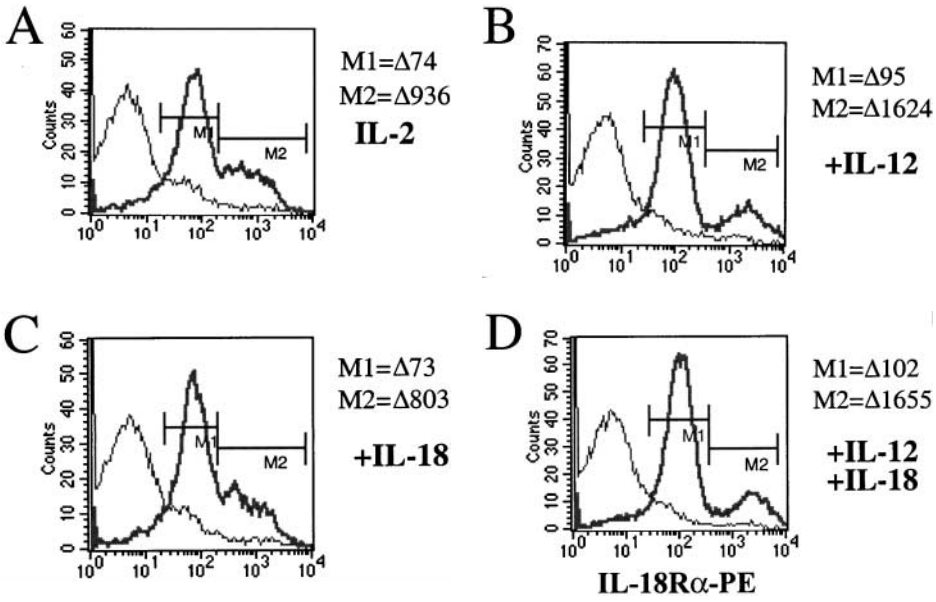

Figure 2. Stimulation with IL-12 or IL-18 alone does not lead to upregulation of IL-18Rα expression on $CD4^+$ $IL\text{-}18R\alpha^{int}$ cells. Spleen cells from C57BL/6 mice were cultured for 96 h in IL-2 media (A) supplemented with 20 ng/ml IL-12 (B), 20 ng/ml IL-18 (C), or both IL-12 and IL-18 (D). Cells were subsequently harvested and analyzed for IL-18Rα expression by FACS®.

level of expression was consistently less than that observed on freshly explanted $CD4^+$ T cells (compare Fig. 3 A with Fig. 1 A) and $CD4^+$ T cells cultured in IL-2 alone (compare Fig. 2 A, M1 with Figs. 3 A and 1 A). However, the addition of IL-12 to anti–CD3-stimulated cultures led to marked upregulation of IL-18Rα expression (Fig. 3 B), while the addition of IL-18 alone did not result in enhanced IL-18Rα expression (Fig. 3 C). IL-18 did synergize with IL-12 to enhance IL-18Rα expression (Fig. 3 D).

Resting $CD4^+$ T cells failed to produce IFN-γ on exposure to IL-12, IL-18, or the combination of IL-12/IL-18 (data not shown). However, after anti-CD3 induced T cell activation, large amounts of IFN-γ were produced in response to IL-12/IL-18 irrespective of the priming conditions (Fig. 3, E–H). More importantly, the upregulation of IL-18Rα expression seen when T cells were cultured with IL-12 correlated with the ability of IL-18 to induce IFN-γ production in the absence of IL-12 (Fig. 3, F and H). T cells primed with IL-2 (Fig. 3 E) or IL-18 (Fig. 3 G) did not produce detectable IFN-γ when cultured with IL-18 alone.

Regulation of IL-18Rα Expression by IFN-γ. As IFN-γ is induced by IL-12, it was possible that IL-12–mediated upregulation of the IL-18Rα was secondary to IFN-γ production. To test this, we stimulated T cells from $IFN\text{-}\gamma^{-/-}$ mice with anti-CD3 and IL-2. After 4 d of culture, $CD4^+$ T cells from $IFN\text{-}\gamma^{-/-}$ mice expressed levels of IL-18Rα that were similar to wild-type C57BL/6 mice (Fig. 4, A and B). Therefore, the mere absence of IFN-γ does not lead to downregulation of IL-18Rα expression. However, IL-12–dependent upregulation of IL-18Rα expression was severely impaired in the absence of IFN-γ. A majority of $CD4^+$ T cells from $IFN\text{-}\gamma^{-/-}$ mice failed to upregulate IL-18Rα expression when cultured with anti-CD3 and IL-12 (M1; Fig. 4, C and D). Addition of IFN-γ to these cultures restored IL-12–driven upregulation of

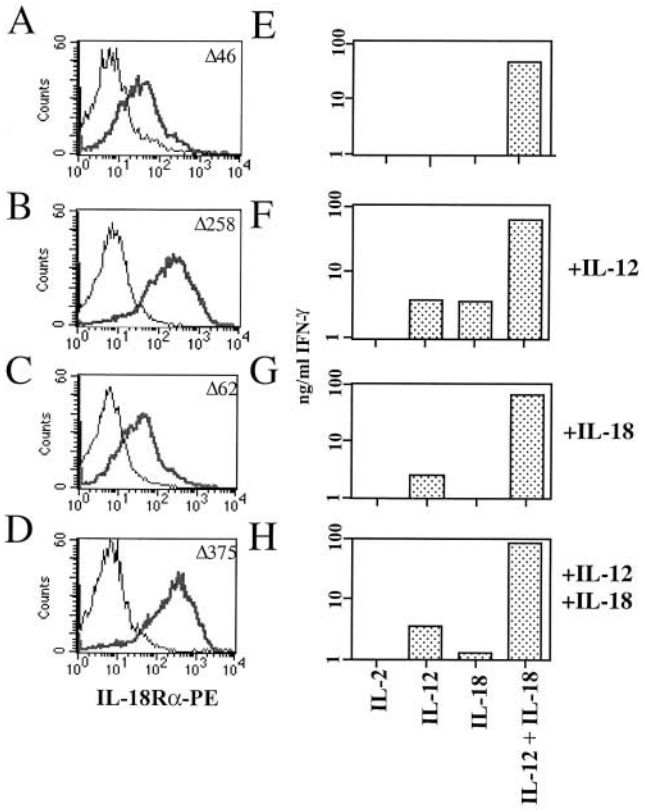

Figure 3. IL-12 in concert with TCR stimulation results in upregulation of IL-18Rα expression on $CD4^+$ T cells. $CD4^+$ T cells from C57BL/6 mice were stimulated with plate-bound anti-CD3 for 2 d in IL-2 alone (A and E), or with IL-2 and IL-12 (B and F), IL-18 (C and G), or IL-12 and IL-18 (D and H). Cells were then washed and placed into fresh IL-2 media with or without cytokines for an additional 2 d. Cells were then harvested and stained for IL-18Rα expression (A–D). Alternatively, the cells were restimulated with either IL-12, IL-18, or both IL-12 and IL-18 for 48 h and supernatants tested for IFN-γ production (E–H).

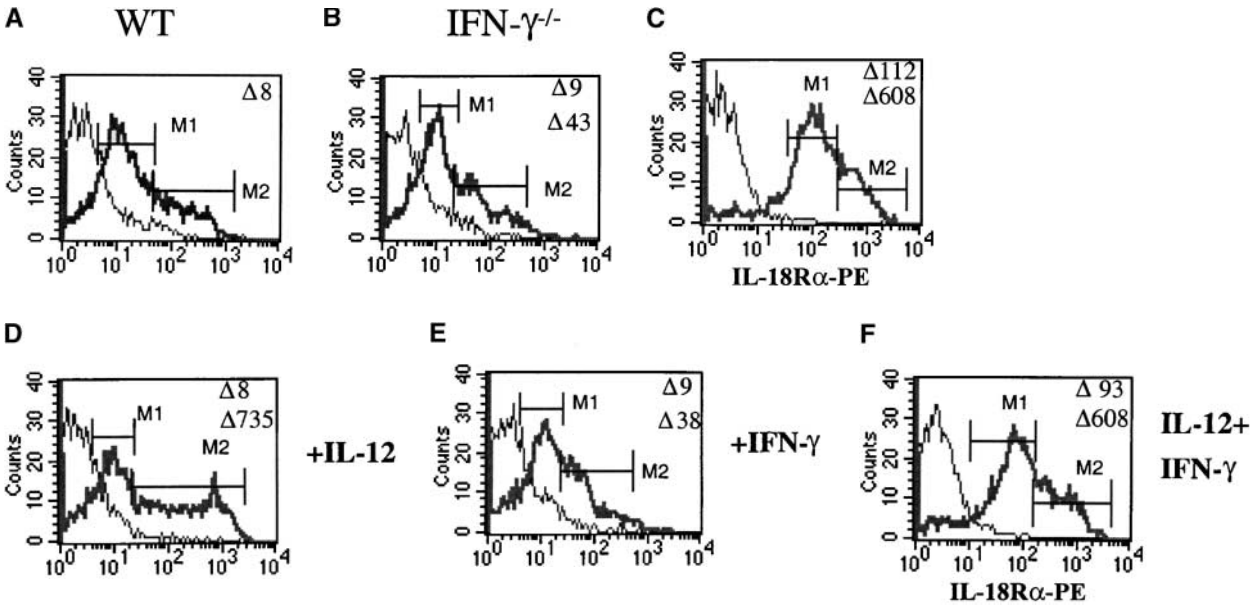

Figure 4. IL-12–mediated enhancement of IL-18Rα expression is IFN-γ dependent. Spleen cells from wild-type C57BL/6 (A and C) and IFN-$\gamma^{-/-}$ (B–F) mice were stimulated for 2 d with plate-bound anti-CD3 and IL-2 (A and B) alone, or with IL-2 and IL-12 (C and D), IFN-γ (E), or IL-12 and IFN-γ (F). After 2 d, cells were washed and placed into fresh IL-2 media with fresh cytokine for an additional 2 d. Cells were then stained for IL-18Rα expression.

IL-18Rα; however, IFN-γ itself did not enhance IL-18Rα expression in the absence of IL-12 (M1; Fig. 4, E and F). These data demonstrate that IFN-γ is required for IL-12–mediated upregulation of IL-18Rα expression and most likely functions by inducing/maintaining IL-12Rβ2 expression. However, we consistently observed a minor population of T cells that could upregulate their IL-18Rα in an IL-12–dependent but IFN-γ–independent fashion (M2; Fig. 4, B and D–F). We suspect these are memory T cells, based on our observations that memory cells can upregulate their IL-18Rα expression in response to IL-12 alone (Fig. 2, A–D).

IL-4 in Concert with TCR Stimulation Induces Downregulation of IL-18Rα. While previous studies have focused on IL-12 and its ability to augment IL-18 responses, very little is known about the cytokines which may negatively regulate IL-18Rα expression. Therefore, we stimulated C57BL/6 $CD4^+$ T cells with anti-CD3/IL-2 in the presence of IL-4, IL-10, or TGF-β. IL-4 markedly enhanced the downregulation of IL-18Rα expression observed when T cells were cultured with anti-CD3 and IL-2 (Fig. 5, A, B, I, and K). While the addition of IL-10 had no effect (Fig. 5 C), the addition of TGF-β augmented IL-18Rα expression (Fig. 5 D). Although T cells primed in the presence of IL-4 or IL-10 expressed detectable levels of the IL-18Rα, they failed to secrete IFN-γ in response to IL-18 alone (Fig. 5, F and G). However, these cells were capable of producing significant quantities of IFN-γ when cultured with both IL-12 and IL-18 (Fig. 5, F–H). Because these studies were performed without concomitant neutralization of IL-12 and/or IFN-γ, it is possible that the inability of IL-4 to completely inhibit IFN-γ induction by IL-12 and IL-18 is due to endogenous IL-12/IFN-γ.

Because the addition of exogenous IL-4 was capable of downregulating IL-18Rα, we suspected that endogenous IL-4 was partially responsible for the downregulation of IL-18Rα observed with anti-CD3 stimulation in the absence of any added cytokine (Fig. 5 A). To test this hypothesis, we stimulated T cells from IL-$4^{-/-}$ mice and measured IL-18Rα expression. When compared with wild-type C57BL/6 mice, IL-$4^{-/-}$ T cells expressed significantly higher levels of IL-18Rα after anti-CD3 stimulation (Fig. 5, I and J). Levels of IL-18Rα expression before stimulation were comparable for both C57BL/6 and IL-$4^{-/-}$ mice (data not shown). However, the addition of exogenous IL-4 resulted in a downregulation of IL-18Rα that was comparable to that seen with wild-type cells (Fig. 5, K and L). These results confirm a critical role for IL-4 in downregulation of IL-18Rα.

Requirement for Stat6 in Regulation of IL-18Rα Expression/Function on $CD4^+$ T Cells. Because IL-4 signals mainly via Stat6, we determined if Stat6 signaling was required for IL-4–induced downregulation of IL-18Rα after T cell activation. After stimulation with anti-CD3 and IL-2, $CD4^+$ T cells from Stat$6^{-/-}$ mice expressed higher levels of IL-18Rα (ΔMFI = 208) compared with wild-type T cells (ΔMFI = 93; Fig. 6, A and D). This suggests that endogenous Stat6 signaling partially contributes to anti-CD3–induced downregulation of IL-18Rα, which is consistent with the results obtained with IL-$4^{-/-}$ mice (Fig. 5, I–K). In contrast to the downregulatory effects of IL-4 seen with wild-type T cells (Fig. 6 B), the addition of IL-4 to cultures of $CD4^+$ T cells from Stat$6^{-/-}$ mice had no effect on IL-18Rα expression (Fig. 6 E) indicating that Stat6 is required for IL-4–mediated downregulation of IL-18Rα expression. Interestingly, the ability of IL-12 to augment IL-18Rα ex-

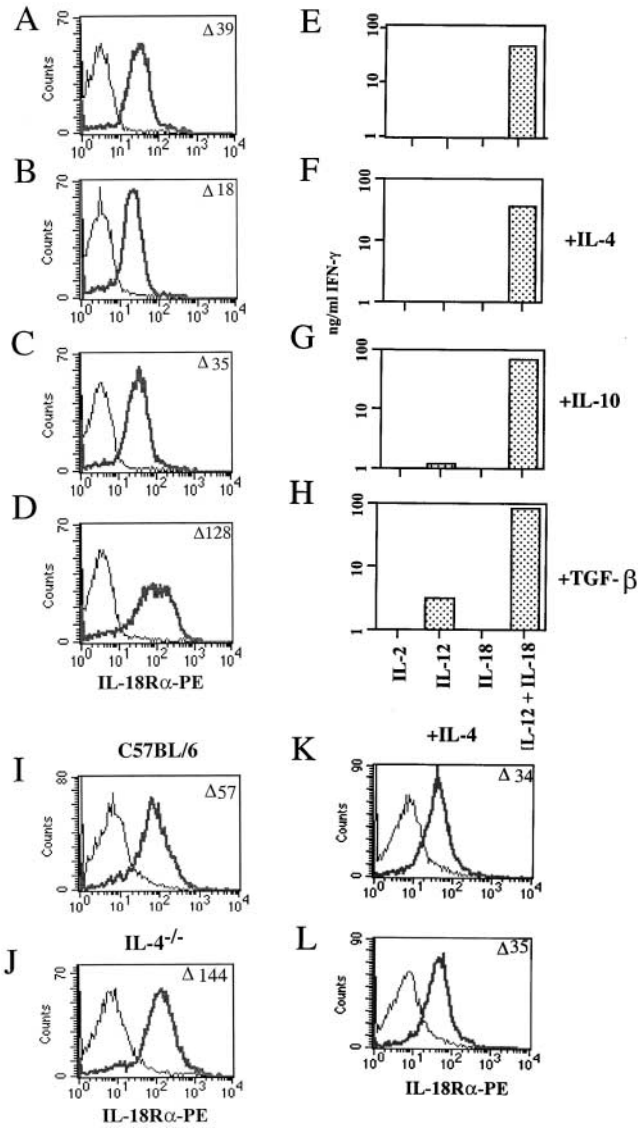

Figure 5. IL-4 induces downregulation of IL-18Rα in response to stimulation with anti-CD3. $CD4^+$ T cells from wild-type C57BL/6 (A–I and K) or IL-$4^{-/-}$ (J and L) mice were stimulated for 2 d with plate-bound anti-CD3 and IL-2 alone (A, E, I, and J), or with IL-2 and IL-4 (B, F, K, and L), IL-10 (C and G), or TGF-β (D and H). The cells were then washed and placed into fresh IL-2 media with fresh cytokine for an additional 2 d. Cells were then stained for IL-18Rα expression (A–D and I–L). Alternatively, cells were restimulated with IL-12, IL-18, or both IL-12 and IL-18, and supernatants were tested for IFN-γ production.

pression was significantly enhanced in the absence of Stat6 (Fig. 6, C and F). To correlate the differences in receptor expression with the ability of IL-18 to induce IFN-γ production, anti-CD3–primed T cells from both BALB/c and Stat$6^{-/-}$ mice were restimulated with either immobilized anti-CD3 or cytokine and subsequently stained for intracellular IFN-γ. T cells from Stat$6^{-/-}$mice produced more IFN-γ in response to anti-CD3 stimulation, as well as the combination of IL-12 and IL-18 (Fig. 6, G–K). Importantly, IL-18–induced IFN-γ production was significantly enhanced in Stat$6^{-/-}$ mice (13 vs. 7%, Fig. 6 J), which is consistent with the higher levels of IL-18Rα expression observed on Stat$6^{-/-}$ mice (Fig. 6 D). These results suggest that IL-4 and Stat6 are critical regulators of both IL-18Rα expression and IL-18 responsiveness.

$CD4^+$ T Cells Primed under Th1 Conditions Upregulate IL-18Rα. Since we found that all naive $CD4^+$ T cells expressed the IL-18Rα, it was of interest to examine the kinetics of regulation of expression during the differentiation of naive $CD4^+$ T cells into Th1 and Th2 cells. For this purpose, we used T cells from 5CC7 transgenic mice on the RAG-$2^{-/-}$ background to minimize the number of previously activated T cells in the starting population. Th1 cells were generated by culturing purified $CD4^+$ T cells with APCs, antigen, IL-12, and anti–IL-4 as described previously (21). After 1 wk in culture, the cells were restimulated with antigen and APCs. Intracellular cytokine staining revealed that 45% of 1-wk (1×) Th1 cells expressed IFN-γ (Fig. 7 A). No IL-4 could be detected in this population. 1-wk Th1 cells expressed high levels of IL-18Rα before restimulation (Fig. 7 D), but these levels increased upon stimulation with APCs and antigen (Fig. 7 G). Upon restimulation, Th1 cells secreted large amounts of IFN-γ after culture with IL-12 and IL-18, but also secreted significant amounts of IFN-γ when cultured with IL-18 alone (Fig. 7 J). The null cells were generated by priming with peptide, APCs, and IL-2 in the presence of anti–IL-4, anti–IL-12, and anti–IFN-γ antibodies. Although these cells proliferated vigorously when restimulated with peptide and APCs, they failed to differentiate into Th1 or Th2 cells as judged by the lack of intracellular staining for IFN-γ or IL-4 (Fig. 7 B). The level of IL-18Rα on Th null cells after 1 wk of culture and restimulation was similar to that seen on unstimulated $CD4^+$ T cells (Figs. 7, E and H, and 1 A). Thus, neither IL-12 nor IFN-γ is required for maintenance of the basal level of IL-18Rα expression during the 1-wk priming period. These results are consistent with those obtained with anti-CD3–stimulated T cells from IFN-$\gamma^{-/-}$ (Fig. 4) and IL-$12^{-/-}$ (data not shown) mice. Interestingly, Th null cells failed to produce IFN-γ when restimulated with IL-12 or IL-18 alone, but did respond to the combination of IL-12 and IL-18 (Fig. 7 K).

$CD4^+$ T Cells Primed under Th2 Conditions Rapidly Downregulate IL-18Rα Expression. The results obtained with Th1 cells (Fig. 7) are consistent with the observation that terminally differentiated Th1 cells express high levels of IL-18Rα. To understand the dynamics of IL-18Rα during Th2 differentiation, TCR transgenic $CD4^+$ T cells were cultured in the presence of antigen, APCs, IL-4, anti–IL-12, and anti–IFN-γ. After 1 wk of priming, 22% of the cells expressed intracellular IL-4, but not IFN-γ, when restimulated with antigen and APCs (Fig. 7 C). However, expression of the IL-18Rα was markedly downregulated after 1 wk of priming under Th2 conditions (Fig. 7, F and I). More impressively, Th2 cells failed to secrete any detectable IFN-γ when cultured with IL-12, IL-18, or both IL-12 and IL-18 (Fig. 7 L). Thus, signaling via IL-12 and IL-18 receptors is extinguished even after 1 wk of priming under Th2 conditions.

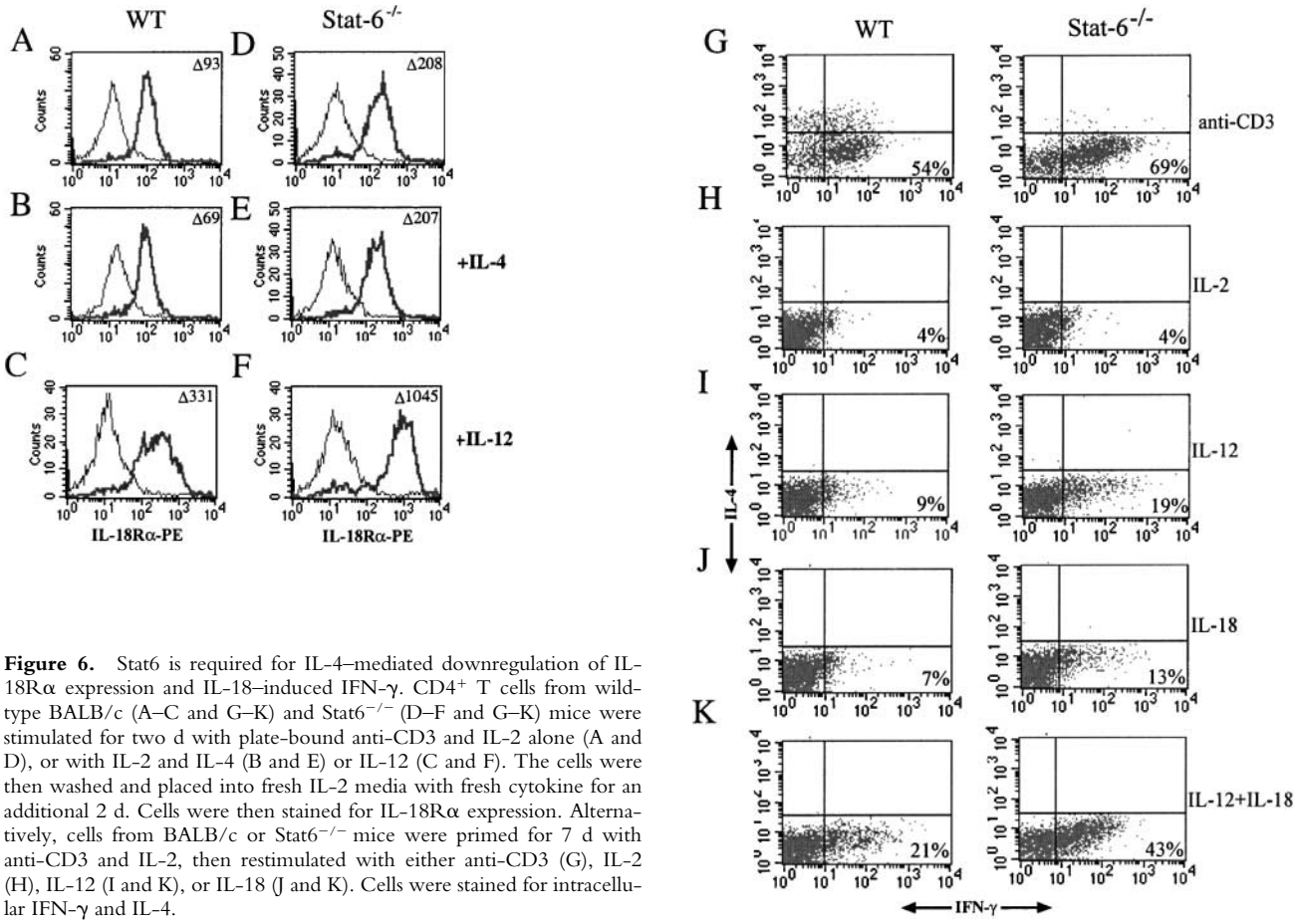

Figure 6. Stat6 is required for IL-4–mediated downregulation of IL-18Rα expression and IL-18–induced IFN-γ. $CD4^+$ T cells from wild-type BALB/c (A–C and G–K) and $Stat6^{-/-}$ (D–F and G–K) mice were stimulated for two d with plate-bound anti-CD3 and IL-2 alone (A and D), or with IL-2 and IL-4 (B and E) or IL-12 (C and F). The cells were then washed and placed into fresh IL-2 media with fresh cytokine for an additional 2 d. Cells were then stained for IL-18Rα expression. Alternatively, cells from BALB/c or $Stat6^{-/-}$ mice were primed for 7 d with anti-CD3 and IL-2, then restimulated with either anti-CD3 (G), IL-2 (H), IL-12 (I and K), or IL-18 (J and K). Cells were stained for intracellular IFN-γ and IL-4.

Discussion

IL-18 has now been well characterized as a costimulatory factor for IL-12–mediated Th1 differentiation. IL-18 may mediate its effects directly by augmentation of IFN-γ production (6, 10, 23, 24) or by enhancement of the expression of the IL-12R complex, thereby potentiating the effects of IL-12 (25). While many studies have focused attention on the regulation of the IL-12R during Th cell differentiation, fewer have focused on regulation of the IL-18R (24, 26–29). It has been widely assumed that the IL-18Rα is expressed only after T cell activation as the IL-18Rα could readily be detected on fully differentiated murine Th1 cells, but not on Th2 cells, either at the mRNA level or at the cell surface (22–24, 26). We have used a polyclonal antibody prepared against residues 19–346 of the extracellular domain of the IL-18Rα and were readily able to detect IL-18Rα on almost all $CD4^+$ and $CD8^+$ T cells. It is highly likely that our ability to detect the IL-18Rα on naive cells is related to both the specificity of the antibody we have used as well as to our staining protocol.

Although the IL-18Rα was expressed at relatively uniform levels on the majority of $CD4^+$ T cells, we detected a subpopulation of unstimulated $CD4^+$ T cells that expressed ~5–10-fold higher levels of receptor than the majority of the $CD4^+$ population. These cells appeared to be activated/memory T cells based on their coexpression of other markers ($CD62L^{low}$, $CD44^{hi}$, and data not shown). In contrast to the majority of $CD4^+$ IL-$18R\alpha^{int}$ cells, the $CD4^+$ IL-$18R\alpha^{hi}$ cells upregulated expression of the IL-18Rα on culture with IL-12 alone. It is very likely that the effects of IL-12 alone on IL-18Rα that have been reported by others (23) are secondary to stimulation of this memory T cell population. Indeed, in our hands fully differentiated Th1 clones will also upregulate IL-18Rα in response to IL-12 and produce IFN-γ in the absence of TCR ligation. Therefore, it is unlikely that truly naive cells can respond to IL-12 by upregulating the IL-18Rα, especially since these cells do not express the IL-12Rβ2 subunit which is required for IL-12 signaling. We have not yet studied the regulation of the IL-18Rα on $CD8^+$ T cells in response to IL-12 and it also remains possible that the IL-18Rα is differentially regulated on $CD8^+$ T cells. In the studies of Yoshimoto et al. (23), no effort was made to separate $CD4^+$ cells and $CD8^+$ cells.

Short-term culture of $CD4^+$ T cells in IL-2 alone led to downregulation of IL-18Rα on the $CD4^+$ IL-$18R\alpha^{int}$ population, and this downregulatory effect was further potentiated by stimulation via the TCR. These data are consistent with a previous report showing anti-CD3–triggered downregulation of IL-18Rα expression (23) and with the observations of Ahn et al. (30) that culture of a Th1 clone

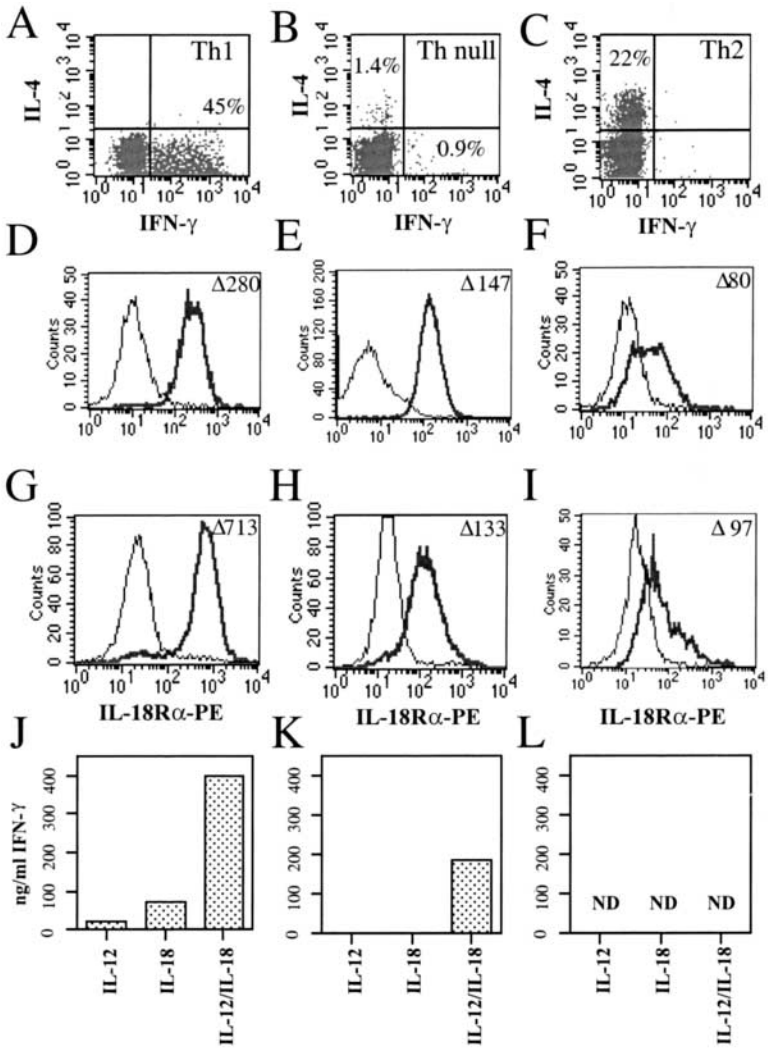

Figure 7. Regulation of IL-18Rα expression during Th1/Th2 differentiation. $CD4^+$ T cells from 5CC7 transgenic mice were primed with irradiated APCs and cytochrome C under Th1, Th null, or Th2 conditions. After 1 wk in culture, T cells were rechallenged for 6 h with PMA and ionomycin and then stained for intracellular IFN-γ and IL-4 to confirm polarization of T cells into Th1 (A), Th null (B), and Th-2 (C) phenotypes. Alternatively, T cells were rechallenged for 2 d with APCs and antigen, but in the absence of cytokines. Cells were analyzed for IL-18Rα expression both before (D–F) and after (G–I) rechallenge. Also, Th1 (J), Th null (K), or Th2 (L) cells were restimulated with the indicated cytokine and levels of IFN-γ determined by ELISA.

with IL-2 alone also led to downregulation of IL-18Rα expression. In contrast to the findings of Yoshimoto et al. (23), we found that culture of $CD4^+$ T cells with the combination of anti-CD3 and IL-12 led to a pronounced increase in IL-18Rα expression on the $CD4^+$ IL-18Rα^{int} population. A possible difference between the results presented here and those of others is that in our 4-d culture system with anti-CD3 and cytokines, T cells were cultured with immobilized anti-CD3 for only the first 2 d to reduce cell death as the result of repeated stimulation via the TCR. Culture of $CD4^+$ T cells with anti-CD3 and IL-18 failed to induce upregulation of the IL-18Rα. However, IL-18 could synergize with IL-12 to induce IL-18Rα upregulation in the presence of anti-CD3. Collectively, these results are most consistent with a model in which IL-12 dictates IL-18 responsiveness in $CD4^+$ T cells. Further evidence of this is supported by the observation that the ability of IL-12 to upregulate IL-18Rα expression is abrogated in Stat4$^{-/-}$ mice (data not shown).

The capacity of IL-12 to induce upregulation of the IL-18Rα was completely IFN-γ dependent, as T cells from IFN-$\gamma^{-/-}$ mice did not upregulate their IL-18Rα when stimulated with anti-CD3 and IL-12. Although reconstitution of these cultures with IFN-γ restored IL-18Rα expression, IFN-γ itself did not enhance IL-18Rα expression in the absence of IL-12. Thus, IFN-γ increases T cell responsiveness to IL-12 mostly likely via its effects on induction/maintenance of expression of the IL-12Rβ2 chain.

The detection of IL-18Rα on resting $CD4^+$ T cells prompted us to examine the factors responsible for the absence of the IL-18Rα on Th2 clones. We have demonstrated that IL-4 rapidly downregulates the expression of the IL-18Rα during short-term stimulation of naive $CD4^+$ T cells with anti-CD3. When $CD4^+$ T cells were primed under Th2 conditions, marked downregulation was seen after 1 wk of culture and after 3 wk of culture under Th2 conditions, there was no detectable IL-18Rα expression (data not shown). There was no observable downregulation of IL-18Rα when resting T cells were cultured with IL-4 (data not shown) in the absence of TCR stimulation, suggesting that TCR-mediated signals are also required. The effect of IL-4 on IL-18Rα expression was even more dra-

matic when T cells from IL-4$^{-/-}$ mice were used. IL-4$^{-/-}$ T cells expressed higher levels of IL-18Rα after the 4-d culture when compared with wild-type cells; however, the addition of exogenous IL-4 resulted in downregulation of IL-18Rα to levels similar to wild-type. This is consistent with the notion that endogenous IL-4 contributes to IL-18Rα downregulation during anti-CD3 stimulation. Further support for the role of IL-4 in negative regulation of IL-18Rα is that Stat6$^{-/-}$ T cells, which are impaired in their ability to respond to IL-4, also express higher levels of IL-18Rα after T cell activation. In addition, the ability of IL-12 to upregulate IL-18Rα is enhanced in Stat6$^{-/-}$ T cells. Surprisingly, the addition of TGF-β to either anti-CD3 or antigen-primed cultures led to an increase in IL-18Rα expression. TGF-β has been shown by others to inhibit IL-4 production (31), and we hypothesize that the inhibition of IL-4 production by TGF-β is responsible for the observed increase in IL-18Rα expression.

In addition to examining the cytokine requirements for expression of the IL-18Rα during Th1/Th2 differentiation, we have also examined their effects on the induction of both IL-12 and IL-18 responsiveness. Restimulation of CD4$^+$ T cells that had been cultured for 4 d with anti-CD3, IL-2, and the combination of IL-12 and IL-18, but not either cytokine alone, resulted in production of large amounts of IFN-γ. More importantly, when CD4$^+$ T cells were cultured for 4 d with anti-CD3 and IL-12, they produced significant amounts of IFN-γ when restimulated with either IL-12 or IL-18 alone. Identical results were observed with 1-wk Th1 cells. The capacity to respond to IL-18 alone correlated with enhanced level of cell surface IL-18Rα expression. Although CD4$^+$ T cells cultured with anti-CD3 and IL-4 for 4 d retained their capacity to respond to the combination of IL-12 and IL-18, 1-wk Th2 cells were completely nonresponsive. This is probably due to the absence of endogenous IL-12 and IFN-γ in Th2-priming experiments. Similar results have been reported by others (7, 32). The ability of IL-4 to regulate IL-18–induced IFN-γ production was also evident in T cells from Stat6$^{-/-}$ mice, which secreted significantly more IFN-γ in response to IL-18/IL-12 plus IL-18 when compared with controls. This enhanced production of IFN-γ correlated with increased levels of IL-18Rα expression. Although IFN-γ production in response to IL-18 appeared to correlate with expression of the IL-18Rα, we cannot exclude the possibility that increased expression/induction of IL-18Rβ accounted, in part, for enhanced IFN-γ production in response to IL-18. Very little information is available concerning the regulation of IL-18Rβ expression (20).

Intriguingly, 1-wk Th null cells still responded vigorously to the combination of IL-12 and IL-18 by producing significant amounts of IFN-γ. These in vitro findings raise a number of questions about the possible role of IL-12 and IL-18 in inducing IFN-γ production during the course of an inflammatory response in vivo. As IL-12 and IL-18 may be produced by different cell types under different conditions, one possibility is that T cells primed in an IL-12 rich environment will subsequently be able to respond by producing IFN-γ in response to either cytokine alone in the absence of reactivation by their TCR. Although the in vivo equivalent of our Th null cell population has not been directly demonstrated, immunization of mice with autoantigens under certain conditions results in a population of primed T cells that only produce IL-2 and not Th1/Th2 cytokines (33–34). It is possible that when these cells are exposed to IL-12 and IL-18 in a paracrine fashion during the course of an infectious insult they would complete their differentiation into pathogenic Th1 cells.

Apart from the known effects of IL-18 in synergizing with IL-12 to drive Th1 responses, recent reports have shown that IL-18 can induce Th2-type responses (i.e., IgE, IL-4, IL-13) (12–15). One interpretation of these studies is that IL-18 is a potent inducer of IL-4 and IL-13 production by basophils and mast cells, or possibly CD4$^+$ T cells. Indeed, induction of IL-4 production by IL-18 in NK T cells has recently been reported (35), which is consistent with our observation that unstimulated CD4$^+$ NKT cells express high levels of IL-18Rα similar to NK cells (data not shown). Such a systemic production of IL-4 may facilitate the priming of antigen-specific CD4$^+$ Th2 cells. Our observation that IL-4 leads to downregulation of the IL-18Rα on CD4$^+$ T cells is very compatible with such a model, as the absence of the IL-18Rα on the antigen-responsive T cells would further prevent the potential costimulatory effects of IL-18 on IL-12–mediated Th1 differentiation. Since this IL-4–mediated downregulation of IL-18Rα was only observed when T cells were triggered through the TCR, bystander effects of IL-4 on resting cells that express the IL-18Rα would not occur. Ultimately, the major factor that determines whether IL-18 potentiates Th1- or Th2-type immune responses is likely to be the balance between IL-12 and IL-4 in the microenvironment during T cell priming. The presence of IL-12, as well as the expression of IL-12 receptor components, would allow IL-18 to synergize with IL-12 in the development of Th1 development. Conversely, the presence of specialized cell types capable of making IL-4 in response to IL-18 (i.e. mast cells) would favor IL-18 as a potentiator of Th2 responses.

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