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IL-12 Up-Regulates IL-18 Receptor Expression on T Cells, Th1 Cells, and B Cells: Synergism with IL-18 for IFN- γ Production¹

Tomohiro Yoshimoto,*[†] Kiyoshi Takeda,[‡] Takashi Tanaka,[‡] Kazunobu Ohkusu,* Shin-ichiro Kashiwamura,[†] Haruki Okamura,[†] Shizuo Akira,[‡] and Kenji Nakanishi^{2*†}

IL-18 is a product of macrophages and with IL-12 strikingly induces IFN- γ production from T, B, and NK cells. Furthermore, IL-18 and IL-12 synergize for IFN- γ production from Th1 cells, although this combination fails to affect Th2 cells. In this study, we show that IL-12 and IL-18 promptly and synergistically induce T and B cells to develop into IFN- γ -producing cells without engaging their Ag receptors. We also studied the mechanism underlying differences in IL-18 responsiveness between Th1 and Th2 cells. Pretreatment of T or B cells with IL-12 rendered them responsive to IL-18, which induces cell proliferation and IFN- γ production. These IL-12-stimulated cells had both high and low affinity IL-18R and an increased IL-18R mRNA expression. In particular, IL-12-stimulated T cells strongly and continuously expressed IL-18R mRNA. However, when T cells developed into Th1 cells after stimulation with anti-CD3 and IL-12, they lowered this IL-12-induced-IL-18R mRNA expression. Then, such T cells showed a dominant response to anti-CD3 by IFN- γ production when they were subsequently stimulated with anti-CD3 and IL-18. In contrast, Th2 cells did not express IL-18R mRNA and failed to produce IFN- γ in response to anti-CD3 and IL-18, although they produced a substantial amount of IFN- γ in response to anti-CD3 and IL-12. However, when Th1 and Th2 cells were stimulated with anti-CD3, IL-12, and IL-18, only the Th1 cells markedly augmented IFN- γ production in response to IL-18, suggesting that IL-18 responsiveness between Th1 and Th2 cells resulted from their differential expression of IL-18R. *The Journal of Immunology*, 1998, 161: 3400–3407.

Interleukin-18 is a product of activated macrophages or Kupffer cells (1–3). Like IL-1 β , this new cytokine is synthesized as a precursor protein that requires cleavage with the IL-1 β -converting enzyme (ICE) for activity (1–3). IL-18 induces IFN- γ production by Th1 cells, T cells, and B cells, in collaboration with IL-12 (1, 4–7), and by cloned NK cells without help from IL-12 (8). In addition, IL-18 augments Fas ligand expression on cloned Th1 cells and NK cells in vitro and on liver lymphocytes in vivo (8–10).

CD4⁺ T helper cells can be divided into Th1 and Th2 cells on the basis of their cytokine profile (11–13). Their development depends on the mode of priming: IL-12 and IL-4 induce differentiation of naive T cells toward Th1 or Th2 cells, respectively (14–21). IL-18 shares some of its biologic activities with IL-12, although the primary structures of the two cytokines show no homology (1). Importantly, these two factors show fundamental difference in their ability to induce Th1 cells: IL-12 induces naive CD4⁺ T cells to develop into Th1 cells in vivo and in vitro (18, 19, 22), whereas IL-18 cannot effect this development (5, 23, 24).

IL-18 by itself does not induce IFN- γ production by T cells and B cells. However, as we have reported, IL-12 and IL-18 promptly and synergistically induce anti-CD3-stimulated T cells or anti-CD40-stimulated B cells to develop into highly IFN- γ -producing cells (6), suggesting the possibility that IL-12 induces IL-18R on T cells or B cells. Indeed, using an IL-12-responsive cloned Th1 cells, 2D6, we were able to reveal that 2D6 cells maintained with IL-12 or IL-2 exhibited differential IL-18 responsiveness and that the former expressed IL-18R (25). Furthermore, IL-18 stimulates other cloned Th1 cells to proliferate and to produce IL-2, granulocyte/macrophage CSF, and IL-2R α , whereas IL-18 has no effect on Th2 cells (7). In this study, we investigated the mechanism whereby IL-18 and IL-12 synergize for IFN- γ production by T cells or B cells without engaging their Ag receptors. We also studied the mechanism underlying the differences in IL-18 responsiveness between Th1 cells and Th2 cells.

Recently, human IL-18R has been purified and characterized (26). Its internal amino acid sequence completely matched that of human IL-1R-related protein (IL-1Rrp),³ the ligand of which is unknown to date (27). IL-18R resembles the type 1 IL-1R and transduces a signal that activates IL-1R-associated kinase (IRAK) and induces nuclear translocation of NF- κ B (23, 26). Since the DNA sequence of murine IL-1Rrp is available, we have cloned murine IL-1Rrp (murine IL-18R) by PCR. Using this cDNA as a probe, we examined the regulation of IL-18R mRNA in T cells and B cells by IL-12. We also measured the number and affinity of IL-18R on both T and B cells stimulated with IL-12 in vitro. Furthermore, we examined the action of anti-CD3 on the expression of IL-18R. Finally, we examined whether differential responsiveness

*Department of Immunology and Medical Zoology, [†]Laboratory of Host Defenses, Institute for Advanced Medical Sciences, and [‡]Department of Biochemistry, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

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² Address correspondence and reprint requests to Dr. Kenji Nakanishi, Department of Immunology and Medical Zoology, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo, 663-8501 Japan. E-mail address: nakaken@hyo-med.ac.jp

³ Abbreviations used in this paper: IL-1Rrp, IL-1R-related protein; IRAK, IL-1R-associated kinase; CsA, cyclosporin A; K_d , dissociation constant; Acp, accessory protein.

of Th1 and Th2 cells to IL-18 results from preferential expression of IL-18R on Th1 cells.

Materials and Methods

Animals and reagents

Virus-free BALB/c mice, 8 to 12 wk of age, were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Recombinant mouse IFN- γ , IL-12, and IL-18 were kindly provided by Hayashibara Biochemical Laboratories (Okayama, Japan). Mouse rIL-4 was obtained and purified from a recombinant baculovirus (AcMNPV.IL-4) prepared in our laboratory. Anti-CD3 Ab (145-2C11, hamster IgG2a directed against the ϵ -chain) (28) and goat anti-mouse IgM Ab were prepared and used for cell stimulation and/or fluorocytometric analysis. FITC-rat anti-mouse B220 (RA3-6B2), FITC-rat anti-mouse Mac-1 (M1/70), FITC-goat anti-mouse IgM, and phycoerythrin-rat anti-mouse IL-2R β -chain (TM β 1) were purchased from PharMingen (San Diego, CA). Cyclosporin A (CsA) was a generous gift of Sandoz (Basel, Switzerland).

Culture medium

RPMI 1640 supplemented with 10% FBS (HyClone, Logan, UT), 2-ME (50 μ M), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and sodium pyruvate (1 mM) was used as the culture medium.

B and T cell preparation

Highly purified splenic B cells were prepared from BALB/c mice pretreated with anti-asialo GM1, which was used to eliminate NK cells *in vivo* (29), followed by passage of spleen cells over a Sephadex G10 column and two rounds of complement-mediated lysis of T cells with monoclonal anti-Lyt1.2 and anti-Thy1.2 Abs. This procedure routinely yields cells that are >99% surface IgM, B220, as well as Ia-positive and <1% CD3-positive. Highly purified splenic T cells were prepared from anti-asialo GM1-treated mice by passing their spleen cells through a nylon wool column, followed by treatment of resultant cells with 10 μ g/ml of FITC-anti-B220 and FITC-Mac-1 for 30 min at 4°C on a turning wheel. The cells were then washed twice and resuspended with magnetic beads coated with sheep anti-FITC Abs (Advanced Magnetics, Cambridge, MA). Cells that had bound magnetic beads were depleted by two rounds of exposure to a magnetic field. The residual cells were collected and washed twice, yielding 98% CD3-positive cells.

Cell cultures for IFN- γ production and cell proliferation

Splenic T cells ($2 \times 10^5/0.2$ ml/well) were cultured alone or stimulated with immobilized anti-CD3 (10 μ g/ml for coating), IL-12, and IL-18 (160 pg/ml to 100 ng/ml), either alone or in various combinations, in 96-well plates for 72 h. Splenic B cells ($2 \times 10^5/0.2$ ml/well) were cultured alone or stimulated with anti-IgM (5 μ g/ml), IL-12, and IL-18 (160 pg/ml to 100 ng/ml), either alone or in various combinations, in 96-well plates for 72 h. Supernatants in triplicate cultures were measured for their IFN- γ content by ELISA. In some experiments, T cells or B cells (4×10^7) were stimulated with 20 ng/ml of IL-12 in 24-cm² flask in a total 8-ml vol for 72 h, then collected, washed well, and subsequently stimulated with IL-18 (156 pg/ml to 40 ng/ml) for 72 h. After incubation, supernatants in triplicate cultures were measured for their IFN- γ content by ELISA. IL-18-induced DNA synthesis was measured by adding 1 μ Ci of [³H]thymidine during the final 16 h.

Induction of Th1 or Th2 cells

Th1 and Th2 cells were induced by stimulating naive splenic T cells (5×10^6) with 10 U/ml of IL-2 plus immobilized anti-CD3 (10 μ g/ml for coating) in the presence of 20 ng/ml of IL-12 or 1000 U/ml of IL-4, respectively, in a 6-well plate in a total 3-ml vol. After 72 h of priming, IL-12-induced Th1 cells or IL-4-induced Th2 cells ($2 \times 10^5/0.2$ ml/well) were recultured with immobilized anti-CD3 for 48 h. Their supernatants were measured for IFN- γ or IL-4 content by ELISA or CT.4S, an IL-4-dependent cell line (30). In some experiments, the priming period was shortened to 3 h.

Induction of IL-18R

T cells (4×10^7) or B cells (4×10^7) were stimulated with 20 ng/ml of IL-12 in 24-cm² flask in a total 8-ml vol for 3 to 72 h. After incubation, these variously stimulated cells were used for preparation of RNA and a ¹²⁵I-IL-18 binding study.

Radiolabeled IL-18 binding assay

IL-18 was radiolabeled with Enzymobeads (Bio-Rad Laboratories, Richmond, CA). The specific activity of ¹²⁵I-IL-18 was 3736 cpm/ng. A binding assay was performed as described by Robb et al. (31) and detailed in our previous report (32). The specific binding was calculated by subtracting the background binding observed in the presence of a 200-fold molar excess of unlabeled IL-18. The dissociation constant (K_d) and number of binding sites were calculated from Scatchard plots.

Analysis of expression of IL-18R mRNA

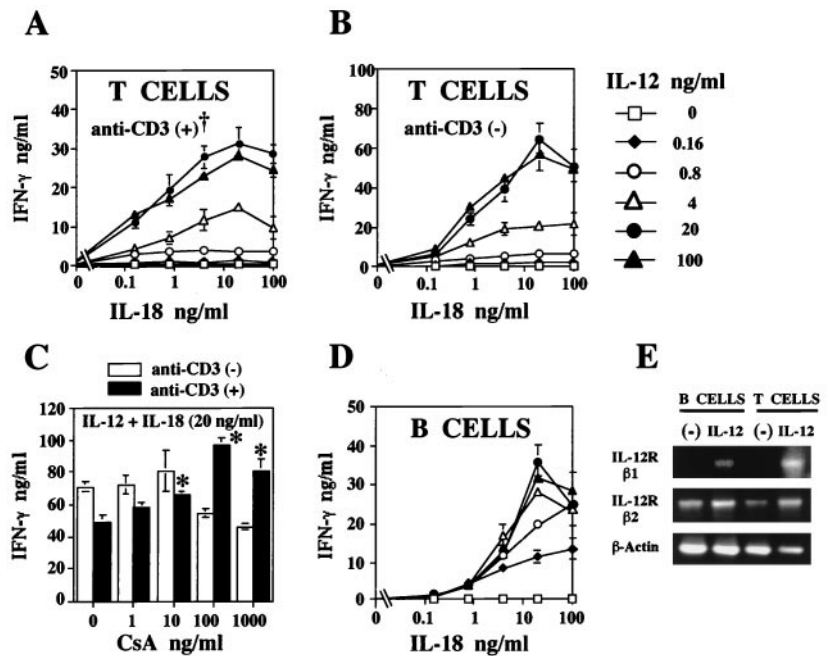
Cytoplasmic RNA was prepared using the guanidinium method as described previously (33). As positive controls for IL-18R, RNA extracted from cloned hepatic NK cells (5E3) (10) was used. For Northern blot analysis, RNA (20 μ g/lane) was electrophoresed through a 1% agarose/formaldehyde gel and blotted onto a nitrocellulose-Nytran (Schleicher and Schuel, Inc., Keene, NH) membrane. Since the internal amino acid sequence of human IL-18R is identical to that of IL-1R β (26) and the DNA sequence of murine IL-1R β has already been published (27), we cloned murine IL-1R β (IL-18R) by PCR. IL-1R β is a recently cloned protein bearing a strong resemblance to type I IL-1R, T1/ST2, and IL-1R accessory protein (Acp) (27). Using this cDNA as a probe, we measured the expression of IL-18R mRNA. We also measured expression of type I IL-1R, type II IL-1R mRNA, IL-1R Acp, IL-12R β 1, or IL-12R β 2 by RT-PCR. mRNAs were amplified by a modified standard RT-PCR amplification procedure as described in our previous paper (33). Primer sequences were as follows: IL-18R: sense, CGTGACAAGCAGAGATGTTG, antisense, ATGTTGTCTCGTCTCCTCTG; type I IL-1R: sense, TCITTTGTTTGTACTCTGCCA, antisense, TATTACTCGTGTGACCGGAT; type II IL-1R: sense, GATCAAATGTCTGTGGAAC, antisense, ATGATGCTGGTATTGTCTCC; IL-1R Acp: sense, GAAGTACAACACTACAGACTG, antisense, AAGTGACCCGATGGTTTGACA; IL-12R β 1: sense, GCAAACACATCACCTTCTCTCTGC, antisense, GTGTGTACCTTG GCAGGATC; IL-12R β 2: sense, GGCACAGACTGTTAGAGAATGC TC, antisense, TGCAGAAGCGCCTTTTGAGTTGGC; and β -actin: sense, GATGACGATATCGCTGCG CTG, antisense, GTACGACCAG AGGCATACAGG. cDNAs were amplified for 35 cycles, each composed of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (IL-18R, type I IL-1R, type II IL-1R, and IL-1R Acp) or 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min (IL-12R β 1, IL-12R β 2, and β -actin), with a further extension at 72°C for 7 min. At the end of 35 cycles, samples were stored at 4°C until analyzed. After amplification, PCR products were separated by electrophoresis in 1% agarose gels and visualized by UV light illumination.

Results

IL-18 and IL-12 synergistically induce IFN- γ production from T cells and B cells

We first compared the ability of IL-12 and IL-18 to induce IFN- γ production from T cells (\sim 98% CD3⁺) in the presence or absence of anti-CD3. A representative IL-12 and/or IL-18 stimulation experiment is illustrated in Figure 1, A and B. A very low level IFN- γ production was obtained as a result of stimulation with IL-12 or IL-12 plus immobilized anti-CD3 for 72 h. This induction was strongly enhanced by IL-18, although IL-18 or IL-18 plus anti-CD3 did not induce any IFN- γ production. Levels of IFN- γ in the culture supernatants of T cells stimulated with IL-12 plus IL-18 in the presence or absence of anti-CD3 revealed that anti-CD3 partially but significantly inhibited IFN- γ production ($p < 0.01$). Furthermore, addition of CsA (1 to 1000 ng/ml), capable of blocking anti-CD3-induced translocation of cytoplasmic nuclear factor of activated T cells (NFAT) (34), significantly ($p < 0.01$) enhanced IFN- γ production (Fig. 1C), substantiating further the capacity of anti-CD3 to inhibit IFN- γ production from T cells. We also stimulated B cells with IL-12 and/or IL-18 for 3 days and then measured IFN- γ production. As we have reported previously (6), B cells (\sim 99% IgM⁺) showed dose-dependent IFN- γ production in response to IL-12 and IL-18 (Fig. 1D). Since anti-CD3 down-regulates T cell IFN- γ production, we separately examined the effect of anti-IgM stimulation on B cells. We found that, in sharp contrast to the action of anti-CD3, anti-IgM stimulation did not

FIGURE 1. IL-12 and IL-18 synergize for IFN- γ production from T cells or B cells. *A–C*, Splenic T cells ($2 \times 10^5/0.2$ ml/well) were cultured alone or stimulated with immobilized anti-CD3 (10 μ g/ml for coating), IL-12, and IL-18 (160 pg/ml–100 ng/ml), either alone or in various combinations in the presence or absence of CsA (~ 1000 ng/ml), in 96-well plates for 72 h. \dagger , Anti-CD3 significantly inhibited IFN- γ production by T cells stimulated with 4, 20, or 100 ng/ml of IL-12 at 4, 20, or 100 ng/ml of IL-18 ($p < 0.01$). $*$, CsA significantly enhanced IFN- γ production by T cells stimulated with anti-CD3, IL-12 (20 ng/ml), and IL-18 (20 ng/ml) ($p < 0.01$). *D*, Splenic B cells ($2 \times 10^5/0.2$ ml/well) were cultured alone or stimulated with IL-12 and/or IL-18 (160 pg/ml–100 ng/ml) in 96-well plates for 72 h. Culture supernatants were harvested and tested for production of IFN- γ by ELISA. Results are mean \pm 1 SD. *E*, Demonstration of IL-12R mRNA in freshly prepared T cells and B cells. One microgram of total RNAs from T cells or B cells cultured by themselves or with IL-12 (20 ng/ml) for 72 h was amplified by RT-PCR.



affect IFN- γ production by B cells stimulated with IL-12 and IL-18 (data not shown).

Naive T cells develop into Th1 cells after stimulation with anti-CD3 and IL-12 for 3 or 4 days (18). However, these Th1 cells cannot produce IFN- γ during this priming period (Fig. 1*A*) and require anti-CD3 challenge to produce IFN- γ in the subsequent culture. Interestingly, as noted above, naive T cells stimulated with anti-CD3, IL-12, and IL-18 strikingly produced IFN- γ (Fig. 1*A*) but no IL-4 and IL-5 (data not shown) during this 72-h culture. Further kinetic study revealed that they produced a comparable level of IFN- γ within 48 h. Thus, stimulation of naive T cells with anti-CD3, IL-12, and IL-18 promptly induces them to develop into Th1 cells and to produce IFN- γ -producing cells in the same culture.

Because NK cells produce IFN- γ in response to IL-12 and/or IL-18 (8, 35), we sought to assess any possible contamination with NK cells by FACS analysis and found no B220⁺IL-2R β ⁺ cells (NK cells) in freshly purified T cells or B cells from anti-asialo GM1-treated mice (data not shown). Recently, IL-12R β 2, a second component of the IL-12R, was identified and cloned (36). IL-12R β 2 is not expressed by naive resting CD4⁺ T cells (Mel-14⁺ CD4⁺ T cells from TCR-transgenic mice) and requires Ag activation for its expression (37). However, freshly purified T cells and B cells expressed IL-12R β 2 when examined by RT-PCR and responded to IL-12 by up-regulation of IL-12R β 1 (Fig. 1*E*). This difference may be explained by the difference between Mel-14⁺ CD4⁺ T cells from TCR-transgenic mice (37) and freshly purified splenic T cells from nontransgenic mice.

T cells or B cells stimulated with IL-12 are competent to respond to IL-18 by IFN- γ production

The requirement for IL-12 and IL-18 for high level IFN- γ production suggests that IL-12 makes T cells or B cells sensitive to IL-18. Therefore, we stimulated T cells or B cells with IL-12 for 72 h, then collected, washed, and subsequently stimulated them with IL-18 for 48 h. Since 20 ng/ml of IL-12 and IL-18 maximally induced IFN- γ production from T cells or B cells (Fig. 1), we used this amount of IL-12 for the stimulation of T cells or B cells. As shown in Figure 2, these T cells or B cells showed dose-dependent

proliferation and IFN- γ production in response to IL-18. In contrast, IL-18-pretreated T cells or B cells did not increase IFN- γ production as a result of subsequent stimulation with IL-12 (data not shown). These results show that T cells or B cells stimulated with IL-12 for 72 h and then washed became competent to respond to IL-18 by IFN- γ production and cell proliferation. IL-18 has been shown to induce IL-2 production from Th1 clones (7). We tested whether IL-18 induced proliferation of IL-12-stimulated T cells by causing them to produce IL-2. We found that addition of Abs against IL-2R $\alpha\beta$ -chains did not inhibit their proliferation (data not shown).

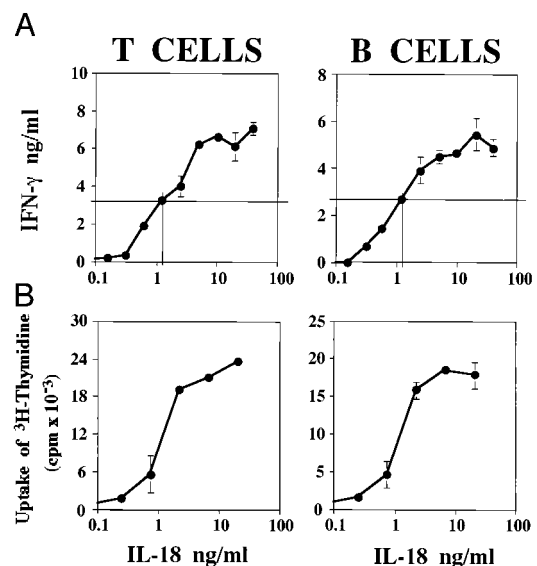


FIGURE 2. IL-18 induced IL-12-stimulated T cells or B cells to produce IFN- γ and to proliferate. T cells or B cells initially cultured at 5×10^6 /ml (8 ml/flask) with IL-12 (20 ng/ml) for 72 h were collected, washed, and recultured at $2 \times 10^5/0.2$ ml/well (*A*) or $5 \times 10^4/0.2$ ml/well (*B*) with various amounts of IL-18 (0.156–40 ng/ml) for 48 h for induction of IFN- γ production (*A*) or for induction of proliferation (*B*), respectively. Results are mean \pm 1 SD of triplicate cultures.

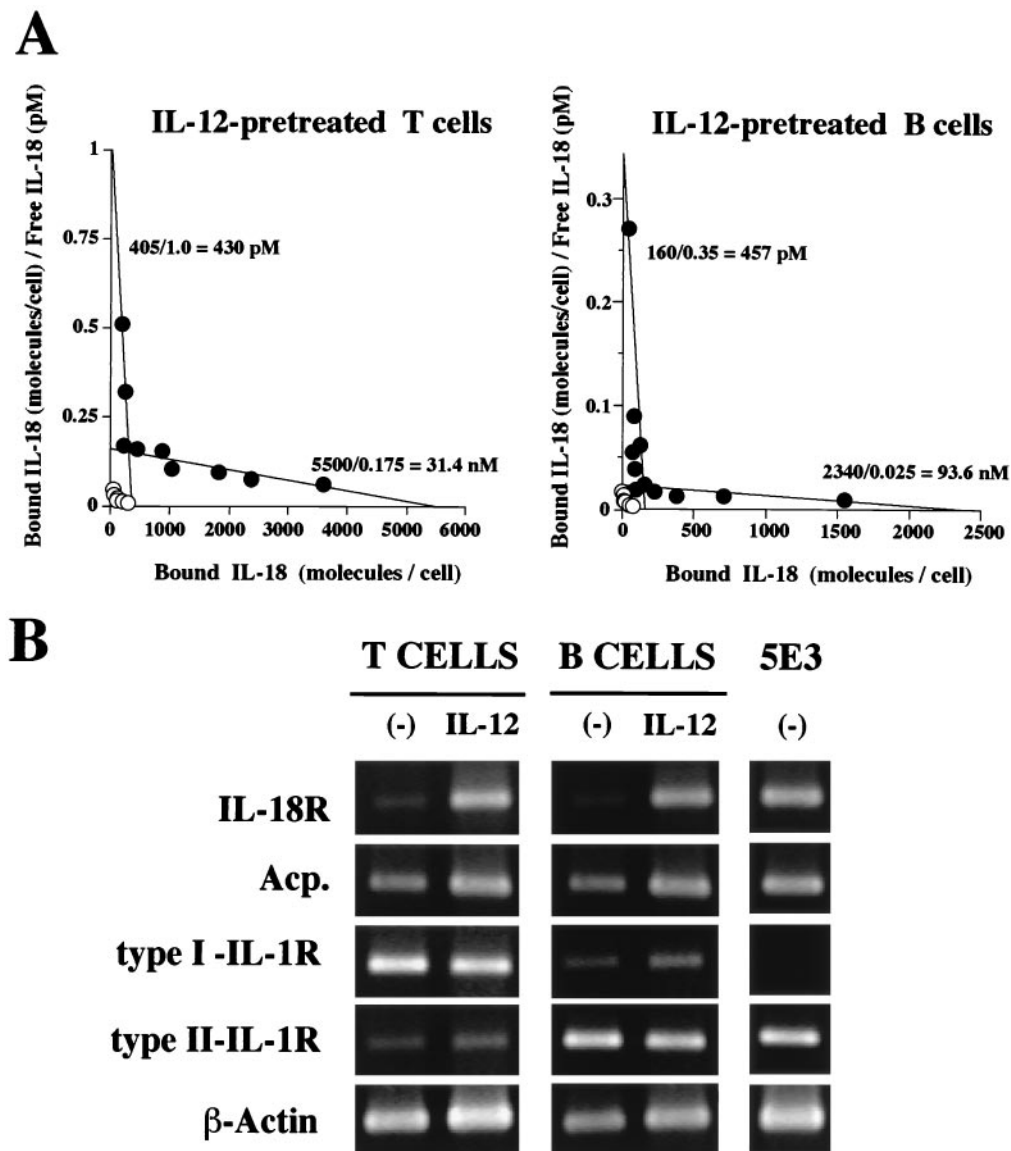


FIGURE 3. Induction of IL-18R on T cells and B cells by IL-12 stimulation. Forty million splenic T cells or B cells were cultured alone (○) or with 20 ng/ml of IL-12 (●) in 24-cm² flasks in a total 8-ml volume for 72 h. *A*, Binding assays were performed as described by Robb et al. (30) and detailed in our previous reports (31). One of three independent binding experiments, which provided identical results, was presented. Number of bound ¹²⁵I-IL-18 molecules per cell is plotted on the abscissa, and the ratio of bound ¹²⁵I-IL-18 molecules over free concentration of ¹²⁵I-IL-18 is plotted on the ordinate. *B*, Total RNA was extracted, and IL-18R mRNA was measured by RT-PCR. mRNA expression of IL-1R type I, IL-1R type II, and IL-1R Acp was also examined. As positive controls for IL-18R mRNA, mRNA extracted from cloned hepatic NK cells (5E3) (10) was used.

Since IL-12 activates STAT4 (38–41), we examined tyrosine phosphorylation of the STAT family in T cells that had been cultured with IL-12 (20 ng/ml) for 72 h, followed by 2 h of starvation and then stimulation with IL-12 or IL-18 (20 ng/ml) for 30 min. IL-12-stimulated T cells expressed tyrosine phosphorylation of STAT4 even with 2 h of starvation, and IL-12 stimulation enhanced this expression. However, IL-18 failed to enhance and activate any STAT (data not shown), although STAT proteins were detectable by Western blot analysis (data not shown), indicating that known members of the STAT family are not involved in IL-18 signaling and that activation of STAT4 might be important for rendering T cells or B cells to be responsive to IL-18.

Expression of IL-18R on IL-12-stimulated T cells and B cells

Because concentrations of 440 pM (8 ng/ml) or greater of IL-18 were required to obtain maximal IFN- γ production, we exam-

ined the number and affinity of IL-18R on T cells or B cells before and after stimulation with IL-12 for 72 h. T cells or B cells cultured by themselves did not specifically bind ¹²⁵I-IL-18, whereas T cells or B cells cultured with IL-12 alone had the capacity to specifically bind ¹²⁵I-IL-18. As shown in Figure 3A, the shape of the Scatchard plot obtained from the binding study is consistent with the presence of high affinity and low affinity IL-18R binding sites. Measurement of binding of ¹²⁵I-IL-18 on IL-12-stimulated T and B cells revealed that they expressed 405 high affinity IL-18R (K_d = 430 pM)/5500 low affinity IL-18R (K_d = 31.4 nM) and 160 high affinity IL-18R (K_d = 457 pM)/2340 low affinity IL-18R (K_d = 93.6 nM), respectively. These results taken together indicate that IL-12 induces high and low affinity IL-18R and that such IL-12-stimulated T cells or B cells exhibit the capacity to proliferate and to produce IFN- γ in response to IL-18.

We next examined the capacity of IL-12 to induce an increase in the expression of IL-18R mRNA in splenic T or B cells. As shown in Figure 3B, T cells cultured by themselves expressed very little IL-18R mRNA, whereas T cells cultured with IL-12 for 72 h clearly did. B cells cultured with IL-12 for 72 h also clearly expressed IL-18R mRNA. In this experiment, we used mRNA extracted from cloned hepatic NK cells (5E3) (10) as a positive control for IL-18R mRNA. We also examined mRNA expression of IL-1R type 1, IL-1R type 2, and IL-1R Acp. No mRNAs except IL-18R mRNA were up-regulated by IL-12 stimulation (Fig. 3B).

Anti-CD3 stimulation down-regulated IL-18R mRNA expression in IL-12-stimulated T cells

Development of naive CD4⁺ T cells into Th1 or Th2 cells depends on their mode of priming (14–21). Induction of Th1 cells in vitro requires stimulation of T cells with anti-CD3 and IL-12 for 72 to 96 h (18). However, as shown in Figure 1A, anti-CD3 stimulation partially but significantly ($p < 0.01$) inhibited the capacity of IL-12 and IL-18 to induce IFN- γ production from T cells. Furthermore, CsA significantly ($p < 0.01$) diminished this inhibitory action of anti-CD3 (Fig. 1C). To understand this inhibitory mechanism of anti-CD3, we compared the levels of expression of IL-18R mRNA in T cells that had been stimulated with IL-12 in the presence or absence of anti-CD3 for 3 to 72 h or with anti-CD3 and IL-12 in the presence or absence of CsA for 72 h. As shown in Figure 4A, IL-18R mRNA expression was detectable at 3 to 72 h after stimulation with IL-12. In contrast, the expression of IL-18R mRNA was only transiently detectable at 3 h after stimulation with anti-CD3 and IL-12, after which time this expression rapidly declined. However, CsA markedly abrogated this inhibitory action of anti-CD3 (Fig. 4A). Thus, IL-12 stimulation continuously up-regulated IL-18R mRNA in T cells and its protein product (Fig. 3), whereas anti-CD3 stimulation down-regulated this IL-12-induced IL-18R mRNA expression in T cells at 6 h and thereafter, leading to markedly diminished expression of IL-18R as measured by binding with ¹²⁵I-IL-18 (data not shown).

To show that this diminished IL-18R mRNA in T cells resulted in their diminished capacity to produce IFN- γ in response to IL-18, we restimulated these pretreated T cells with anti-CD3 and/or IL-18 for 48 h (Fig. 4B). T cells pretreated with anti-CD3 and IL-12 for 3 to 24 h produced a substantial amount of IFN- γ in response to IL-18, suggesting that these T cells expressed IL-18R. However, when they developed into Th1 cells after stimulation with anti-CD3 and IL-12 for 48 to 72 h, they showed a dominant response to anti-CD3 by production of IFN- γ in response to anti-CD3 and IL-18. In contrast, T cells pretreated with IL-12 in the absence of anti-CD3 for 24 h or 72 h produced similar level of IFN- γ in response to IL-18. However, as these T cells failed to produce IFN- γ in response to anti-CD3 (Fig. 4B), these IL-12-stimulated T cells were not Th1 cells. These results, taken together, indicated that signaling through CD3 plays an important role in the down-regulation of IL-18R mRNA and the induction of commitment of naive T cells into Th1 cells. However, CsA markedly abrogated this down-regulation of IL-18R mRNA by anti-CD3 and enhanced the IFN- γ production induced by IL-18 (Fig. 1C). Thus, when the T cells developed into Th1 cells, they diminished their IL-18R expression and gradually became less sensitive to IL-18 stimulation. However, since these T cells transiently but strongly expressed IL-18R mRNA and presumably sustained IL-18R expression in some period after stimulation with anti-CD3 and IL-12, they showed conspicuous IFN- γ production in response to IL-18.

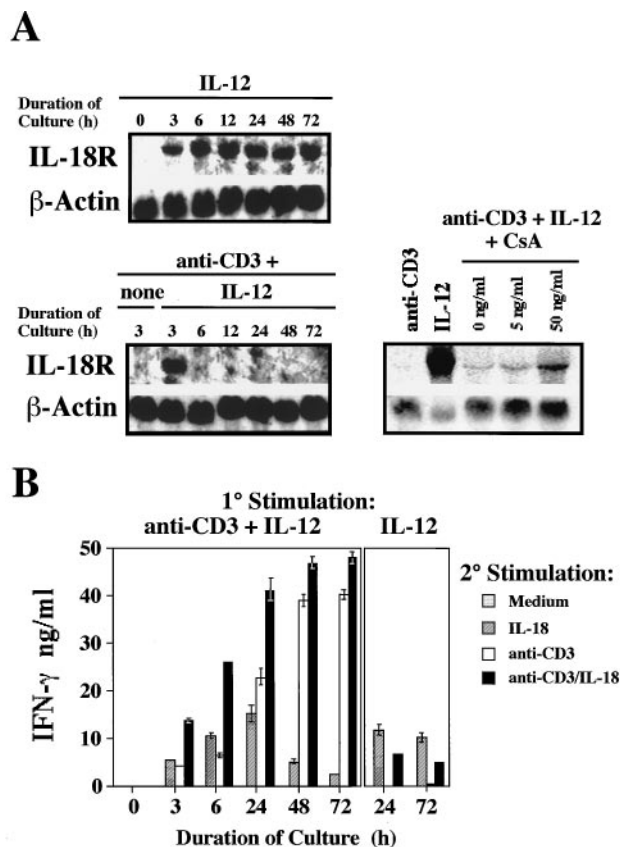


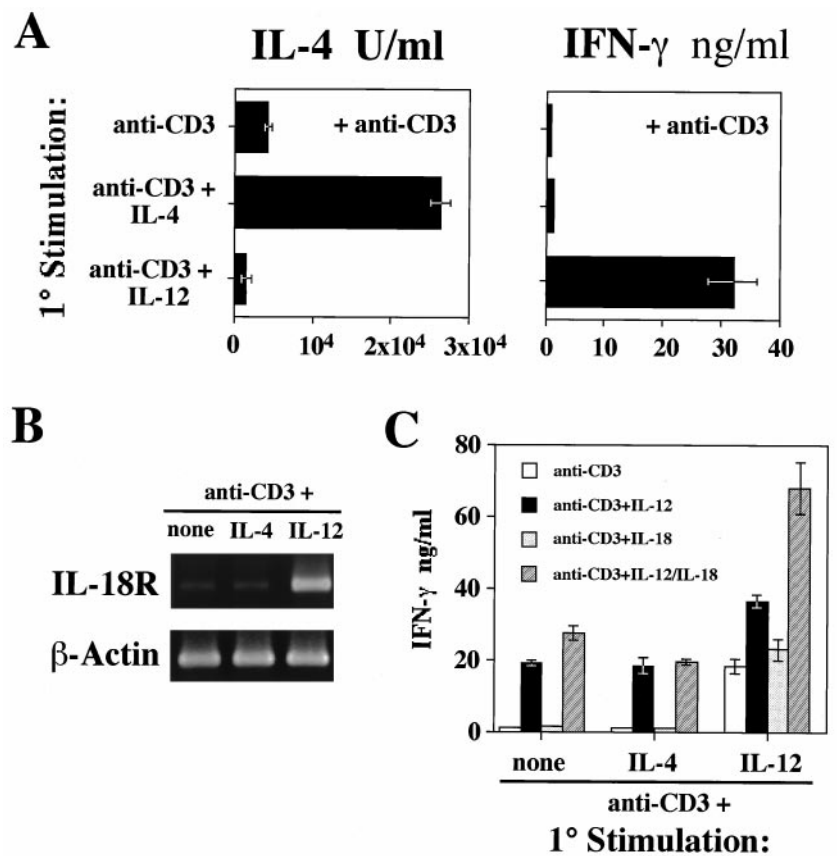
FIGURE 4. Kinetics of induction of IL-18R mRNA and IFN- γ production by IL-12. Splenic T cells (5×10^6) were cultured alone or with 20 ng/ml of IL-12 in 6-well plates in a total 3-ml vol, either with immobilized anti-CD3 (10 μ g/ml) or not, for 3 to 72 h or with anti-CD3 and IL-12 in the presence or absence of CsA (~ 50 ng/ml) for 72 h. After initial culture, cells were washed and A, mRNAs were extracted and examined for their expression of IL-18R mRNA by Northern blot analysis or B, recultured at $2 \times 10^5/0.2$ ml/well with medium alone or IL-18 (20 ng/ml) in 96-well plates either coated with anti-CD3 (10 μ g/ml) or not for 48 h. Culture supernatants were harvested and tested for production of IFN- γ by ELISA. Results are mean \pm 1 SD.

Preferential expression of IL-18R mRNA in IL-12-induced Th1 cells

IL-18 and IL-12 synergize for IFN- γ production from Th1 clones, whereas this combination fails to affect Th2 cells (7). To understand the mechanism underlying this difference in IL-18 responsiveness between Th1 and Th2 cells, we tested whether IL-18R mRNA is preferentially expressed in Th1 cells. For this purpose, we induced naive T cells to develop into Th1 cells or Th2 cells by stimulating them with anti-CD3 and IL-12 (20 ng/ml) or IL-4 (1000 U/ml) for 72 h, respectively (Fig. 5). Since we could not detect or detected meagerly expressed IL-18R mRNA in T cells stimulated with anti-CD3 and IL-12 for 72 h by Northern blot analysis (Fig. 4A), we performed RT-PCR analysis for this detection. As shown in Figure 5B, only the T cells stimulated with anti-CD3 and IL-12 for 72 h clearly expressed IL-18R mRNA.

We finally compared the ability of Th1 and Th2 cells to produce IFN- γ in response to anti-CD3 and IL-12 and/or IL-18. Again, after developing into Th1 cells, these Th1 cells diminished their responsiveness to IL-18 and predominantly responded to anti-CD3 by IFN- γ production when they were subsequently stimulated with anti-CD3 plus IL-18. As expected, Th2 cells did not produce IFN- γ in response to anti-CD3 plus IL-18. However, when Th1

FIGURE 5. Up-regulated IL-18R mRNA expression in IL-12-induced Th1 cells but not in IL-4-induced Th2 cells. Splenic T cells (5×10^6) were cultured with 10 U/ml of IL-2 plus immobilized anti-CD3 (10 μ g/ml) in the presence or absence of 20 ng/ml of IL-12 or 1000 U/ml of IL-4 in 6-well plates in a total 3-ml vol for 72 h. After initial priming, cells were washed and recultured at $2 \times 10^5/0.2$ ml/well with immobilized anti-CD3 in the presence or absence of IL-12 and/or IL-18 for 48 h for induction of cytokine production (A and C), or they were immediately examined by RT-PCR for expression of IL-18R mRNA (B).



cells and Th2 cells were stimulated with anti-CD3, IL-12, and IL-18, only the Th1 cells showed a further augmentation of IFN- γ production in response to IL-18 stimulation, suggesting that differences in IL-18 responsiveness between Th1 and Th2 cells resulted from their differential expression of IL-18R.

Discussion

We showed that resting T cells or B cells do not express IL-18R and fail to produce IFN- γ in response to IL-18. Pretreatment of T cells or B cells with IL-12 rendered them responsive to IL-18 by activation of STAT4 and induction of IL-18R. However, these IL-12-stimulated T cells did not produce IFN- γ in response to anti-CD3. Thus, T cells stimulated with IL-12 and IL-18 promptly and strikingly produce IFN- γ without their TCR engagement and Th1 development. We also showed that when T cells developed into Th1 cells after stimulation with anti-CD3 and IL-12, they lowered IL-12-induced IL-18R mRNA expression but gained the capacity to respond predominantly to anti-CD3 by IFN- γ production in response to anti-CD3 and IL-18. In contrast, Th2 cells did not express IL-18R mRNA and showed no IL-18 responsiveness. Moreover, we showed that when Th1 cells and Th2 cells were stimulated with anti-CD3, IL-12, and IL-18, only Th1 cells showed a further augmentation of IFN- γ production in response to IL-18, strongly indicating that the differences in IL-18 responsiveness between Th1 and Th2 cells resulted from their differential expression of IL-18R.

IL-12-stimulated T cells or B cells express both high and low affinity IL-18R (Fig. 3), whereas COS-1 cells transfected with IL-18R (IL-1Rrp) cDNA express only low affinity IL-18R (26). There are two possibilities that may account for this discrepancy. First, high affinity IL-18R consists of two chains: one is an IL-18-binding subunit (IL-1Rrp) found in Hodgkin's lymphoma cells that

were used for preparation of IL-18R (IL-1Rrp) and the other is a missing subunit that exists in IL-12-stimulated T or B cells in association with IL-1Rrp to form high affinity IL-18R. Second, high affinity IL-18R may be a homodimer of IL-1Rrp, although this possibility is very slight, because COS-1 cells transfected with IL-18R (IL-1Rrp) cDNA express only low affinity IL-18R (26). We need further study to elucidate the second chain of high affinity IL-18R.

As shown in Figure 3B, stimulation of T cells or B cells with IL-12 induces an increase in the expression of IL-18R mRNA without affecting the expression of IL-1R or IL-1R Acp. These IL-12-stimulated T cells rapidly and continuously expressed IL-18R mRNA (Fig. 4A) and produced IFN- γ in response to IL-18 (Figs. 2 and 4B), while they showed no response to IL-1 stimulation (our unpublished observation). Importantly, these T cells did not belong to Th1 cells because they could not produce IFN- γ in response to anti-CD3 (Fig. 4B). However, T cells stimulated with IL-12 and IL-18 without anti-CD3 produce IFN- γ more strikingly than T cells stimulated with anti-CD3, IL-12, and IL-18 (Fig. 1). Recently, we observed that IL-12 synergizes with IL-18 for IFN- γ production from spleen cells of SCID mice lacking T cells and B cells but having NK cells that constitutively express IL-18R and IL-12R mRNA (our unpublished observation). Like these NK cells, T cells produce IFN- γ in response to IL-12 and IL-18 without their TCR engagement or development into Th1 cells. Furthermore, B cells also produce IFN- γ in response to IL-12 and IL-18 (6). The physiologic relevance of these IFN- γ -producing T cells and B cells is uncertain. However, since they promptly and strikingly produce IFN- γ in response to IL-12 and IL-18 without developing into memory cells such as Th1 cells, they may play an important role as potent host defensive cells in the innate immune response. However, since these IL-12-stimulated T cells were

highly resistant to CsA treatment, it may be important to consider the presence of these IL-12 plus IL-18-stimulated cells in the treatment of some immunologic disorders with CsA.

The signal transduction pathways, after the activation of the receptors for IL-12 and IL-18, are quite complicated. IL-12 stimulation activates STAT4 (38–41). Indeed, IL-12-pretreated T cells showed tyrosine phosphorylation of STAT4 even after 2 h of starvation, whereas IL-18 stimulation did not phosphorylate any members of STAT family. Recently, Matsumoto et al. (42) reported that IL-18 activates NF- κ B in murine Th1 cells. More recently, Robinson et al. (23) proved that, like IL-1 α , IL-18 activates IRAK and NF- κ B. Thus, we assume that NF- κ B and STAT4 synergize to activate the IFN- γ promoter, resulting in a striking level of production of IFN- γ by T cells.

As shown in Figure 4, T cells gained the Th1 phenotype even at 48 h after stimulation with anti-CD3 and IL-12, because they produced IFN- γ in response to subsequent stimulation with anti-CD3 for 48 h. However, T cells stimulated with anti-CD3 and IL-12 for 72 h did not produce IFN- γ during this period (Fig. 1A), although they produced IFN- γ in response to stimulation with anti-CD3 or anti-CD3 and IL-12 in the subsequent culture (Figs. 4 and 5). To our surprise, such a two-step culture was not required for induction of IFN- γ production by T cells stimulated with anti-CD3, IL-12, and IL-18. As shown in Figure 1, IL-12 and IL-18 synergistically induced IFN- γ production by T cells in the presence and absence of anti-CD3 within 72 h. Thus, stimulation with anti-CD3, IL-12, and IL-18 induces Th1 cell development and IFN- γ production in the same culture.

In contrast to the IL-12-stimulated T cells that continuously expressed high level of IL-18R mRNA, T cells stimulated with anti-CD3, IL-12, and IL-18 only transiently expressed high level of IL-18R mRNA at 3 h (Fig. 4A). Furthermore, we demonstrated that when T cells develop into Th1 cells, they diminished their IL-18R expression and gradually became less sensitive to IL-18 but showed a dominant response to anti-CD3 by production of IFN- γ in response to anti-CD3 and IL-18 (Fig. 4B). Moreover, treatment with CsA enhanced IFN- γ production by T cells in response to anti-CD3, IL-12, and IL-18 presumably by inhibiting the action of anti-CD3 to down-regulate IL-18R mRNA (Fig. 4A). Thus, CD3-transduced signaling plays an important role in the down-regulation of IL-18R and the induction of naive T cells to develop into Th1 cells. Nevertheless, when naive T cells were stimulated with anti-CD3, IL-12, and IL-18, they were strikingly able to produce IFN- γ in response to IL-18 (Fig. 1A). It is intriguing to assume that anti-CD3 and IL-12 increased the number of cells entering into the G₁ phase of the cell cycle and augmented IL-18R mRNA at 3 h. Therefore, IL-18 in collaboration with IL-12 strikingly enhanced IFN- γ production by these T cells. Furthermore, IL-18 enhanced the number of cells entering into the S phase of the cell cycle. Thus, anti-CD3, IL-12, and IL-18 may strikingly induce T cell IFN- γ production, first by induction of IL-18R-positive cells and then by subsequent IL-18-dependent cell cycle progression, leading to striking IFN- γ production.

We examined the expression of IL-18R mRNA in Th1 cells and Th2 cells that had been induced by stimulation of naive T cells for 72 h with anti-CD3 and IL-12 or IL-4, respectively. We found that only Th1 cells express IL-18R mRNA (Fig. 5B). When Th1 cells and Th2 cells were restimulated with anti-CD3, IL-12, and IL-18, only Th1 cells augmented IFN- γ production in response to additional IL-18 stimulation (Fig. 5C). Stimulation of Th1 cells with anti-CD3, IL-12, and IL-18 may induce them to recycle and to produce more IFN- γ ; IL-12-dependent induction of IL-18R was followed by binding of IL-18 to this up-regulated IL-18R, whereas Th2 cells may be insensitive to such action, although they pro-

duced IFN- γ in response to anti-CD3 and IL-12 (37, 43–45). In this study, we have shown two distinct IFN- γ induction pathways: one is an IL-12- and IL-18-dependent pathway, without induction of Th1 cells; and the other is an anti-CD3-, IL-12-, and IL-18-dependent pathway, with induction of Th1 cells. This first pathway is especially important, because T cells produce IFN- γ without TCR engagement by Ag or anti-CD3.

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