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Immunology

Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist

(natural immunity/cytokines/macrophages/microbial infection)

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ABSTRACT **Listeriosis in mice with the severe combined immunodeficiency (SCID) mutation is an established model *in vivo* and *in vitro* of interferon γ (IFN-γ)-dependent macrophage activation by natural killer (NK) cells during the development of natural immunity. We demonstrate that IFN-γ production from SCID splenocytes is stimulated by interleukin (IL) 12, tumor necrosis factor α (TNF-α), and IL-2 but is inhibited by IL-10. IL-10, IL-12, and TNF are induced by heat-killed *Listeria monocytogenes* (hk-LM) from SCID splenocytes and peritoneal macrophages. IL-12 production is necessary for hk-LM to stimulate IFN-γ production by SCID splenocytes since neutralization of IL-12 totally blocks IFN-γ production in this system. TNF-α and IL-2 act synergistically with IL-12 to augment IFN-γ production. Also, exogenous IL-2 increases the response of NK cells to hk-LM or to IL-12 and TNF-α. In contrast, IL-10 inhibits hk-LM-induced IFN-γ production at two levels: (*i*) by inhibiting TNF and IL-12 production from these cultures (presumably from the macrophage) and (*ii*) by inhibiting the stimulatory effects of IL-12 and TNF-α on NK-cell IFN-γ production. Thus, these data indicate that macrophage production of TNF-α and IL-12 stimulates the release of IFN-γ by NK cells and that IL-10 produced in response to hk-LM inhibits this response at the level of the macrophage and the NK cell.**

Interferon γ (IFN-γ) production by natural killer (NK) cells is necessary in combating infection with *Listeria monocytogenes* in severe combined immunodeficiency (SCID) mice, which lack T and B lymphocytes (for review, see ref. 1), and in immunocompetent mice (2). In the SCID model of listeriosis, tumor necrosis factor α (TNF-α) is an important cofactor for NK-cell IFN-γ production *in vivo* (3) and *in vitro* (3, 4). Neutralization of TNF with monoclonal antibodies (mAbs) inhibits NK-cell IFN-γ production *in vitro* and inhibits macrophage activation *in vivo*, resulting in increased systemic bacterial spread (3). Exogenous TNF-α alone, however, is an ineffective stimulus of NK-cell IFN-γ production *in vitro* but acts synergistically with a factor in conditioned medium (CM) generated from peritoneal macrophages stimulated with several different organisms, including *Listeria* (4). So TNF-α is necessary but not sufficient in stimulating NK-cell production of IFN-γ. Recently, interleukin (IL) 12 has been cloned (5, 6) and described by several laboratories (7–9) as a potent inducer of IFN-γ production by NK cells and various T cells. Also, IL-2 acts synergistically with IL-12 in the stimulation of IFN-γ. Thus, IL-12 is an important positive modulator of NK- and T-cell responses. In contrast, IL-10 is a potent inhibitor of NK-cell and Th_1 helper T cell IFN-γ production and an inhibitor of macrophage/monocyte activation and cytokine synthesis (for review, see refs. 10 and 11).

In this report, we examine the effects of TNF-α, IL-12, IL-2, and IL-10 on the production of IFN-γ in response to heat-killed *L. monocytogenes* (hk-LM) by SCID spleen cells *in vitro*. We establish that IL-12 and TNF-α are costimulators for IFN-γ production, that IL-2 maximizes the IFN-γ response, and that IL-10 inhibits production of IL-12, TNF, and IFN-γ.

MATERIALS AND METHODS

Mice. CB-17/ICR SCID (SCID) mice (12) were bred in specific-pathogen-free conditions at Washington University. Within each experiment, all mice were age (6–10 weeks)- and sex-matched.

Cell Culture. All cells were cultured in supplemented RPMI medium 1640 containing 10% (vol/vol) fetal calf serum (HyClone) as described (3, 4). All reagents were free of lipopolysaccharide, as determined by the *Limulus* assay (Whittaker Bioproducts).

Single-cell suspensions from spleens of uninfected SCID mice were cultured at 2.5×10^6 cells per ml in a total volume of 200 μl in a 96-well flat-bottom plate. Reagents were added to the cultures simultaneously, as detailed in each figure. The supernatants were harvested at 48 h and IFN-γ or TNF was measured by ELISA (13, 14). Resident peritoneal macrophages were isolated by peritoneal lavage from uninfected SCID mice and 2×10^6 cells per ml were allowed to adhere to plastic tissue culture dishes for 4 h. Nonadherent cells were removed with three washings. LM-elicited peritoneal macrophages were isolated from SCID mice infected i.p. with 3×10^3 live LM, 3–5 days prior to isolation, purified, and cultured as described above. CM was generated by stimulating macrophage cultures with 2×10^7 hk-LM per ml (15) for 16 h. The CM was centrifuged at 3000 rpm in a Sorvall H100B rotor for 15 min, filtered through 0.22-μm (pore size) sterile filters, and stored at −80°C.

mAbs, Cytokines, and Reagents. The anti-IL-4 mAb 11B11 (10 μg/ml) (16) and anti-IL-10 mAb 2A5 (10 μg/ml) (17) were as described. Anti-IL-1α mAb Alf-161 (10 μg/ml) (18), anti-IL-1β mAb B122 (10 μg/ml) (19), anti-TNF mAb TN3-19.12 (150 μg/ml) (4), and anti-IFN-γ mAb (20) were generous gifts from R. D. Schreiber (Washington University). Mouse anti-transforming growth factor β 1, 2, 3 mAb (10 μg/ml) was obtained from Genzyme. Recombinant mouse IL-12 (specific

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Abbreviations: IL, interleukin; IFN-γ, interferon γ; hk-LM, heat-killed *Listeria monocytogenes*; NK, natural killer; mAb, monoclonal antibody; SCID, severe combined immunodeficiency; TNF-α, tumor necrosis factor α; CM, conditioned medium.

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activity, 4.75 × 10^6 units/ml), rabbit preimmune serum (1:1000 dilution), and rabbit anti-mouse IL-12 polyclonal serum (1:1000 dilution) were produced at Genetics Institute (21). Recombinant mouse IL-10 (38,000 units/ml) was obtained from PharMingen (San Diego) and recombinant human IL-2 (2 × 10^5 units/ml, used at 200 units/ml) was a gift from Hoffmann–La Roche. Each experiment is represented as the mean ± SEM of triplicate determinations within a given experiment. The experiments were repeated 2–4 times.

RESULTS

hk-LM induces IFN-γ production by SCID splenocytes. This IFN-γ production is mediated by NK cells stimulated by cytokines released by macrophages (for review, see ref. 1). In this report, we used three approaches to identify the macrophage-derived cytokines induced by hk-LM stimulation that regulate IFN-γ production by NK cells: (*i*) the use of specific neutralizing mAbs directed against a number of cytokines added to SCID splenocyte cultures stimulated with hk-LM, (*ii*) addition of exogenous recombinant cytokines to reconstitute IFN-γ production from SCID splenocytes, and (*iii*) the use of CM from purified SCID macrophages stimulated with hk-LM to induce IFN-γ by SCID spleen cells.

IFN-γ production from SCID splenocytes requires addition of hk-LM (1). There was 5–10 times greater IFN-γ production under all conditions with added IL-2 (Fig. 1). Neutralization of IL-10, IL-12, and/or TNF dramatically affected IFN-γ production. If IL-10 was blocked with specific mAb, IFN-γ production was markedly augmented by hk-LM with or without the addition of IL-2. Neutralization of TNF partially inhibited IFN-γ production, whereas anti-IL-12 antibody completely abolished it. Additionally, mAb to IL-10 reversed the inhibition caused by anti-TNF but not that caused by anti-IL-12.

These data imply that IL-12 is produced by these cultures in response to hk-LM and that its production is necessary for IFN-γ stimulation by NK cells. Although TNF and IL-2 augmented IFN-γ production mediated by IL-12, IL-12 alone induced low levels of IFN-γ. IL-12 has been shown to act synergistically with IL-2 to produce IFN-γ in other systems as well (7–9). Likewise, the effects of anti-IL-10 indicated that this cytokine was being stimulated by hk-LM to inhibit IFN-γ production. When IL-10 was measured by ELISA, we found that these cultures did indeed produce this cytokine in response to hk-LM (5–15 units/ml).

The effects of endogenously produced IL-10 were confirmed and extended in the experiment of Fig. 2. The production of IFN-γ and TNF in response to hk-LM was augmented when IL-10 was neutralized with mAb. Furthermore, IL-2 could overcome the inhibition by IL-10, but maximal levels of IFN-γ were only produced by blocking IL-10 in the presence of IL-2.

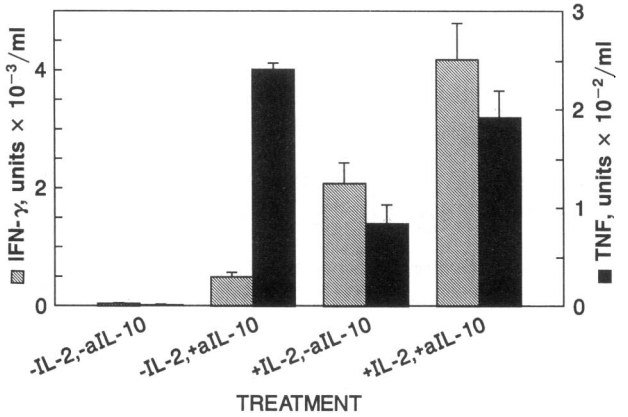

FIG. 2. Effect of IL-2 and anti-IL-10 mAb on IFN-γ and TNF production by SCID splenocytes. Spleen cells were cultured and stimulated with hk-LM in the presence and absence of IL-2 and/or anti-IL-10 mAb (aIL-10) as indicated. IFN-γ and TNF were measured in the supernatants by ELISA.

To further characterize the stimulation of IFN-γ by IL-12 and TNF in this system, we examined the effects of exogenous IL-12 added to SCID splenocytes (Figs. 3 and 4). IL-12 alone minimally stimulated IFN-γ production (Fig. 3). Addition of IL-12 and TNF-α increased IFN-γ production with an EC_{50} value for IL-12 of 1 unit/ml. IFN-γ production was augmented by addition of IL-12 plus IL-2, but TNF production was unaffected (Fig. 4). IL-12 and TNF-α acted synergistically with IL-2 to maximally increase IFN-γ production. The EC_{50} value for IL-12 remained 1 unit/ml in the presence of IL-2, but more IFN-γ was produced. Neither TNF-α nor IL-2, alone or together, could stimulate IFN-γ in the absence of IL-12. Addition of hk-LM increased IL-12-dependent production of IFN-γ (Figs. 3 and 4). The maximum amount of IFN-γ produced in the presence of hk-LM and IL-12 was always slightly higher than with exogenous TNF-α and IL-12. This effect of hk-LM was seen both in the presence and absence of IL-2. Whether other factors from the cells themselves or from the hk-LM are also augmenting IFN-γ production remains to be elucidated. Also noted is that hk-LM alone stimulated limited TNF production unless IL-12 (Fig. 3) or IL-2 (Fig. 4) was present.

We further analyzed the inhibition produced by IL-10 by addition of exogenous IL-10 to SCID splenocytes (Figs. 5 and 6). IL-10 inhibited IFN-γ production stimulated by exogenous IL-12 and TNF-α with an IC_{50} value of 50 units/ml (Fig. 5). In contrast, IL-10 had no effect on the small production of IFN-γ caused by IL-12 alone. In the presence of IL-2 (Fig. 6), exogenous IL-12, alone or with exogenous TNF-α, maximally stimulated IFN-γ production and these stimulations were not inhibited by exogenous IL-10. However, in the

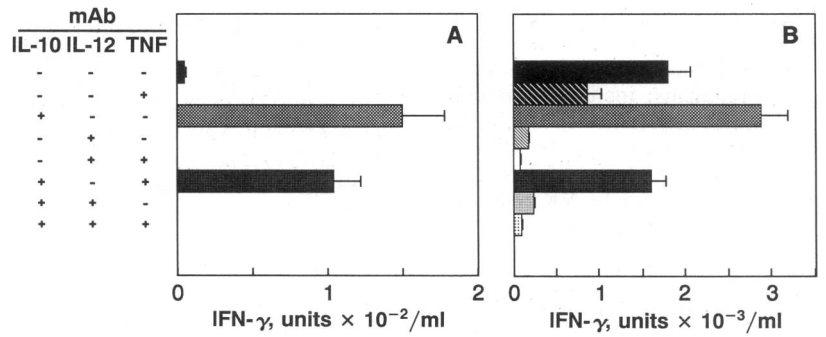

FIG. 1. Effect of mAbs to TNF, IL-12, and IL-10 on IFN-γ production by SCID spleen cells. Spleen cells were cultured and stimulated with hk-LM in the absence (*A*) or presence (*B*) of IL-2. mAbs to TNF, IL-12, and/or IL-10 were added simultaneously with the hk-LM as shown. No IFN-γ production was seen without hk-LM. IFN-γ was measured in the supernatant by ELISA.

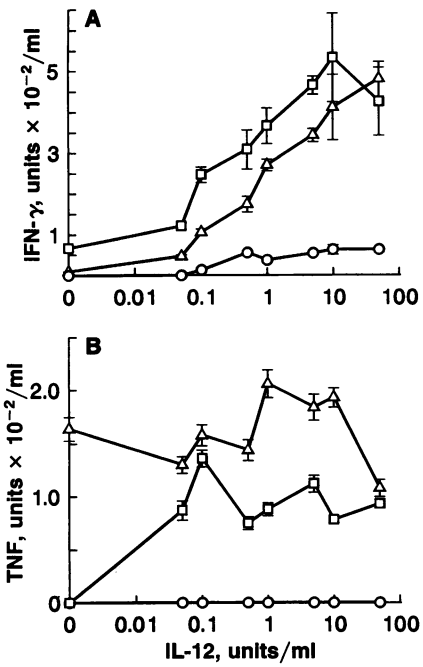

FIG. 3. Effects of IL-12 on IFN-γ and TNF production by SCID splenocytes in the absence of IL-2. Spleen cells were cultured with increasing doses of IL-12 alone (○) and in the presence of TNF-α (300 units/ml; △) or hk-LM (5 × 10^6 bacteria per ml; □). IFN-γ and TNF were measured in the supernatants by ELISA.

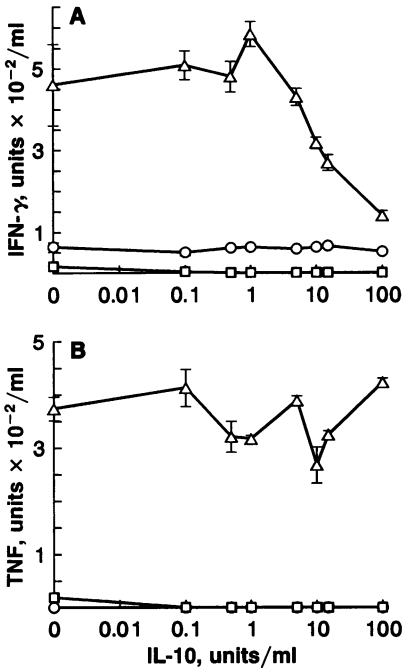

FIG. 5. Effects of IL-10 on IFN-γ and TNF production by SCID splenocytes in the absence of IL-2. Spleen cells were cultured with increasing doses of IL-10 in the presence of the following material. □, hk-LM (5 × 10^6 bacteria per ml); ○, IL-12 (10 units/ml); △, IL-12 (10 units/ml) plus TNF-α (300 units/ml). IFN-γ and TNF were measured in the supernatants by ELISA.

presence of IL-2, hk-LM stimulated both IFN-γ and TNF production, which were now inhibited by exogenous IL-10 with an IC_{50} value of 0.1 unit/ml [these data are in agreement with results to be published by G. Bancroft (personal communication)]. The IC_{50} value for IL-10 inhibition of TNF-α/IL-12 induction of IFN-γ in the absence of IL-2 was 100 times higher than its inhibition of hk-LM-stimulated IFN-γ production in the presence of IL-2. These data imply two mechanisms of IL-10 inhibition. These differential effects of IL-10 need to be further evaluated on purified macrophages and NK cells from SCID spleen cells.

We next examined the effects of CM from purified macrophages stimulated with hk-LM on splenocyte IFN-γ production in the presence of anti-cytokine antibodies (Fig. 7).

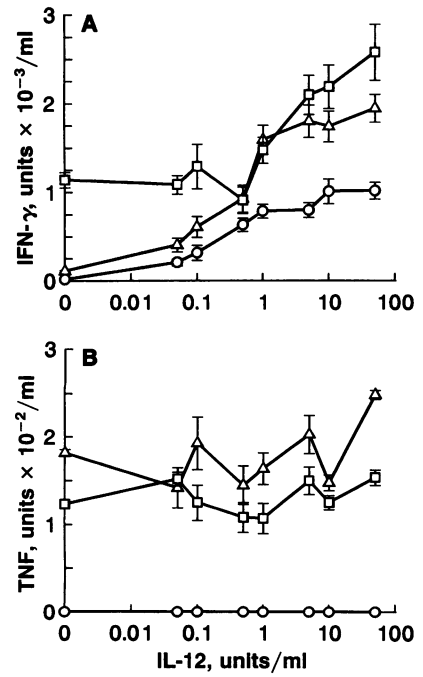

FIG. 4. Effects of IL-12 on IFN-γ and TNF production by SCID spleen cells in the presence of IL-2. Spleen cells were cultured with IL-2 and increasing doses of IL-12 alone (○) and in the presence of TNF-α (△) or hk-LM (□) as described in Fig. 3. IFN-γ and TNF were measured in the supernatants by ELISA.

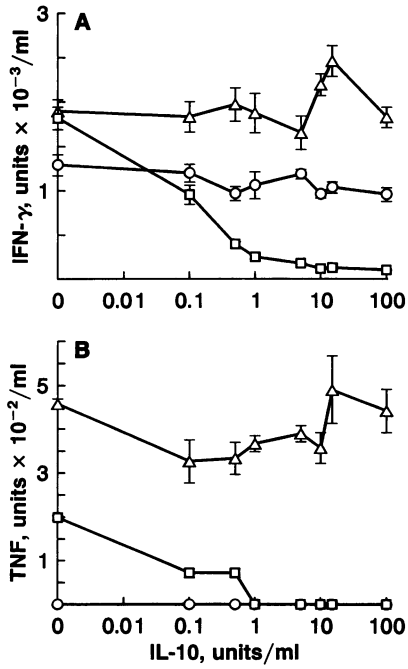

FIG. 6. Effects of IL-10 on IFN-γ and TNF production by SCID splenocytes in the presence of IL-2. Spleen cells were cultured with IL-2 and increasing doses of IL-10 in the presence of hk-LM (□), IL-12 (○), and IL-12 plus TNF-α (△) as described in Fig. 5. IFN-γ and TNF were measured in the supernatants by ELISA.

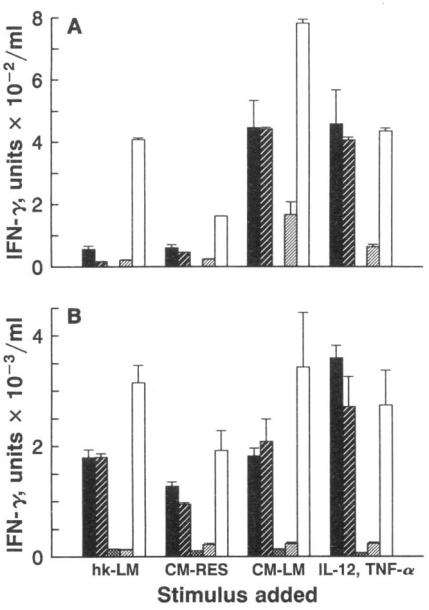

FIG. 7. CM from various peritoneal macrophage populations stimulates IFN-γ production by SCID spleen cells. The medium was prepared from resident (CM-RES) and *Listeria*-elicited (CM-LM) peritoneal macrophages. SCID spleen cells were stimulated in the absence (*A*) or presence (*B*) of IL-2 with hk-LM (5×10^6 bacteria per ml), CM [50% (vol/vol)], or IL-12 (10 units/ml) and TNF-α (300 units/ml) as indicated without (solid bars) or with (cross-hatched bars) mAbs to IL-12, TNF (lightly hatched bars), or IL-10 (open bars). Preimmune serum is shown as a control antibody (darkly hatched bars). IFN-γ was measured in the supernatants by ELISA.

Addition of this CM from resident peritoneal macrophages (CM-RES) stimulated IFN-γ production by purified NK cells (4) or by SCID spleen cells. CM generated in the same fashion from *Listeria*-elicited peritoneal macrophages (CM-LM) was a more potent stimulant. There was five times more TNF in CM-LM than in CM-RES (216 ± 78 units/ml vs. 45 ± 26 units/ml, respectively). These stimulations with CM are inhibited by antibodies to IL-12 and TNF but potentiated by anti-IL-10. In the presence of IL-2 (Fig. 7*B*), the production of IFN-γ in response to each stimulus was augmented as shown before, and the differences between the two CMs were less pronounced, with the synergy provided by IL-2. mAbs to IL-1α, IL-1β, IL-4, and transforming growth factor β had no effect on the production of IFN-γ in response to any of these stimuli (C.S.T., unpublished data).

DISCUSSION

The SCID mouse is an established model for examining the mechanisms of innate immunity. In this model, IFN-γ is produced during listeriosis by NK cells *in vivo* and *in vitro* (1). In this paper we demonstrate that IL-12 is a necessary cytokine required for the production of IFN-γ by SCID spleen cells in response to hk-LM *in vitro*. TNF-α is also required as a costimulator for hk-LM-induced IFN-γ production, as shown here and described previously (3, 4). Thus, both cytokines must be present for production of IFN-γ by NK cells within the SCID spleen. However, in the presence of IL-2, IFN-γ production occurs in response to IL-12 alone and is further augmented by TNF-α. IL-2 induces maximum production of IFN-γ, in response to either hk-LM or IL-12 and TNF-α. hk-LM-induced IL-10 appears to be a very potent inhibitor of macrophage cytokine synthesis as shown here and described previously (22, 23). Furthermore, IL-10 possibly inhibits the IFN-γ production by NK cells directly.

We propose the following model for the interaction of these cytokines in this SCID system. LM is phagocytosed by macrophages, which then release TNF-α, IL-12, and IL-10 and digest and process LM. Production of these three cytokines by monocyte/macrophages has been demonstrated (22–24). TNF-α and IL-12 stimulate the NK cells to produce IFN-γ. Furthermore, processed bacterial products may augment the cytokine stimulation of IFN-γ production, as suggested here (Figs. 2–4) and previously (4). IFN-γ then stimulates macrophage activation, which among numerous responses, includes increased cytokine synthesis, ultimately leading to even more NK-cell IFN-γ production. However, IL-10, also produced by macrophages, inhibits the effects of IL-12 and TNF-α on IFN-γ production by (*i*) inhibiting macrophage cytokine synthesis shown here (Fig. 6) and by others (22, 23) and (*ii*) by inhibiting the ability of NK cells to respond to IL-12 and TNF-α (Fig. 5). Since IL-2 is absent in the SCID model, the inhibition by IL-10 is more pronounced so that maximal IFN-γ production cannot be achieved. It is conceivable that certain conditions or stimuli may favor either IL-12/TNF-α or IL-10 production differentially, resulting in either enhanced or diminished IFN-γ production. Stimuli like hk-LM appear to produce all three cytokines, resulting in an intermediate response. When the T-cell product IL-2 is exogenously added to this system, both TNF and IFN-γ productions are maximally enhanced.

In listeriosis, IFN-γ has been shown to be necessary *in vivo* for the development of resistance in immunocompetent and SCID animals (1, 2). Thus, the regulation of IFN-γ production is of critical importance in surviving an infection. One difference between SCID and normal mice in response to *Listeria* is that SCID mice develop a chronic infection with persistent immune-cell activation (for review, see ref. 1), whereas the immunocompetent control mice clear the organism and return to a quiescent state. Of course, T cells are necessary and important to clear the infection. However, one possible role of the T cell is to maximize the early IFN-γ production in response to *Listeria* by producing IL-2. Without IL-2, production of IFN-γ is more profoundly inhibited by IL-10 at the level of macrophage cytokine synthesis and at the response of the NK cell to TNF-α and IL-12. Furthermore, maximum IFN-γ production does not occur in the absence of IL-2. Similarly, in a transgenic system of CD4 development, hk-LM-stimulated IL-12 enhances IFN-γ production during a primary stimulation of $CD4^+$ cells and directly promotes the rapid acquisition of the Th_1 phenotype by naive T cells (25). Thus, regulation of IFN-γ production by NK cells and Th_1 cells appears to be finely balanced by the release of both inhibitory and stimulatory cytokines.

In the SCID model, where IL-2 is absent, T-cell production of IFN-γ is absent and the inhibition of IFN-γ production by NK cells caused by IL-10 is exaggerated. This inhibition may be particularly profound in the absence of IL-2, possibly resulting in a weakened immune response with ensuing chronic infection. Whether IL-2 alone or in the presence of Th_1 cells is necessary for the resolution of the infection remains to be elucidated.

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