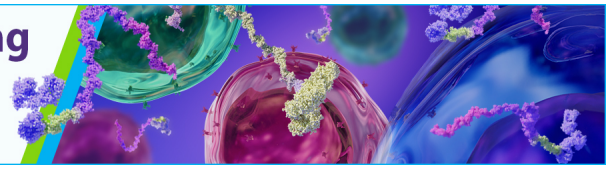

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IL-18 Directs Autoreactive T Cells and Promotes Autodestruction in the Central Nervous System Via Induction of IFN-γ by NK Cells[1]

Fu-Dong Shi,[2]*† Kiyoshi Takeda,‡ Shizuo Akira,‡ Nora Sarvetnick,† and Hans-Gustaf Ljunggren*

IL-18 promotes NK cell and Th1 cell activity and may bridge innate and adaptive immune responses. Myelin oligodendrocyte glycoprotein (MOG) is a myelin component of the CNS and is a candidate autoantigen in multiple sclerosis. In the present study we show that IL-18-deficient (IL-18$^{-/-}$) mice are defective in mounting autoreactive Th1 and autoantibody responses and are resistant to $MOG_{35–55}$ peptide-induced autoimmune encephalomyelitis. IL-18 administration enhances the disease severity in wild-type mice and restores the ability to generate Th1 response in the IL-18$^{-/-}$ mice. This restoration was abrogated in NK cell-depleted mice, indicating that the action of IL-18 in promoting the generation of MOG-specific Th cells was dependent on NK cells. Furthermore, transfer of NK cells from recombinase-activating gene 1$^{-/-}$ mice, but not from recombinase-activating gene 1/IFN-$\gamma^{-/-}$ mice, rescued the defective Th1 responses in IL-18$^{-/-}$ mice and rendered IL-18$^{-/-}$ mice susceptible to the induction of autoimmune encephalomyelitis. Thus, IL-18 can direct autoreactive T cells and promote autodestruction in the CNS at least in part via induction of IFN-γ by NK cells. *The Journal of Immunology,* **2000, 165: 3099–3104.**

The cause of multiple sclerosis (MS)[3] remains a mystery. Epidemiological data suggest that MS is related to an infectious agent encountered early in life. An infection during the course of MS often exacerbates the demyelination within the CNS. Molecular mimicry with viral Ags, bacterial LPSs, or superantigens may trigger activation of self-reactive T cells (1, 2). However, the exact pathogenesis of the disease is not clear. A favored hypothesis is that when these T cells encounter putative MS Ags, e.g., myelin basic protein, myelin-associated glycoprotein, myelin oligodendrocyte glycoprotein (MOG), or proteolipid protein in the context of complex genetic components associated with susceptibility to disease in a given individual, they become autodestructive and cause demyelination.

Experimental autoimmune encephalomyelitis (EAE) in C57BL/6 (B6) mice is one of the animal models of MS (3). EAE in B6 mice can be induced by immunization with $MOG_{35–55}$ peptide in adjuvant (3). Myelin basic protein-specific $CD4^+$ T cells have been shown to transfer EAE in SJL mice (4). Similarly, $MOG_{35–55}$-specific $CD4^+$ T cells can also transfer EAE in B6 mice (5). EAE is a prototype Th1 cell-mediated disease, although the exact sequence of events as well as molecular mediators of this CNS inflammatory response have not been clearly defined. The induction of EAE, like the induction of many other experimental autoimmune diseases, requires the use of complete adjuvants. Adjuvant contains bacterial Ags that activate innate immunity (6). Therefore, immunization of experimental animals with concoctions of autoantigen and adjuvant may mimic the initial events involved in the development of autoimmune diseases in humans. We hypothesize that infection might participate in driving autoreactivity to autodestruction. The signals from innate immune responses combating infectious agents, e.g., the early production of key cytokines, may also have an instructive role in the development of autoreactive T cells in both MS and EAE. Thus, identification of cells and molecules of innate immunity and the pathways involved in the subsequent development of EAE may shed light on the etiology of MS.

IL-18, originally designated IFN-γ-inducing factor, is a cytokine produced by activated macrophages and dendritic cells during the innate immune response (7). IL-18 shares structural features with the IL-1 family of proteins (8) and shares some of the biological activities with IL-12 (7, 9). IL-18 is capable of promoting the production of IFN-γ by NK and Th1 cells and enhances NK cell activity (7, 9). Recent studies suggest that not only is IL-18 essential for the host defense against intracellular infection, but it also plays a critical role in regulating the synthesis of inflammatory cytokines (10). This evidence suggests the possible participation of IL-18 in the development of autoimmune diseases. Indeed, expression of IL-18 transcripts occurred with the onset of insulitis in the nonobese diabetic mouse (11). Neutralizing Abs to IL-18 prevented EAE in rat (12). However, the nature of IL-18 in the initiation and maintenance of autoimmunity remains largely elusive.

To identify the contribution of IL-18 in autoimmune disease, we compared the development of EAE in wild-type (WT) and IL-18-deficient (IL-18$^{-/-}$) mice. We show that IL-18$^{-/-}$ mice are defective in mounting autoreactive Th1 and autoantibody responses and are resistant to $MOG_{35–55}$ peptide-induced encephalomyelitis. Furthermore, we demonstrate that IL-18 promotes autoreactive Th1 cell development at least in part via induction of IFN-γ by NK cells.

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[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MNC, mononuclear cells; WT, wild type; RAG, recombinase-activating gene; p.i., postimmunization.

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Materials and Methods

Mutant mice

IL-18$^{-/-}$ (9), recombinase-activating gene 1 (RAG1)$^{-/-}$ (13), and IFN-$\gamma^{-/-}$ (14) mice were backcrossed at least 12 times to a B6 background. Mice were housed in specific pathogen-free conditions at the animal facilities of the Microbiology and Tumor Biology Center, Karolinska Institute (Stockholm, Sweden), and The Scripps Rodent Colony (La Jolla, CA). Female mice, 8–10 wk of age at the initiation of the experiments, were used.

Ags, Abs, and recombinant cytokines

The murine $MOG_{35–55}$ peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) was synthesized at the Swedish Institute for Infectious Disease Control (Stockholm, Sweden). PK136 clone (anti-NK1.1) (15) was obtained from American Type Culture Collection (Manassas, VA). Mouse IgG (Sigma, St. Louis, MO) was used as the isotype control Ab for anti-NK1.1 Ab. For in vivo depletion of NK1.1$^+$ cells, 100 μg of anti-NK1.1 mAb was injected i.p. into each mouse on day −2 postimmunization (p.i.). Every 5–7 days thereafter, 50 μg of anti-NK1.1 mAb was injected i.p. until the termination of experiments. Depletion efficacy was confirmed by flow cytometry with PE-NK1.1 Ab (PharMingen, San Diego, CA). Recombinant IL-18 was purchased from PeproTech (Rocky Hill, NJ). Each mouse received 1000 ng of IL-18 i.p. daily for 5 consecutive days as previously described (9).

Induction of EAE

EAE was induced by s.c. flank and tail base injections of 200 μg of MOG peptide in CFA (Difco, Detroit, MI) containing 500 μg of heat-inactivated *Mycobacterium tuberculosis* on days 0 and 7, supplemented by i.v. injections of 200 ng of pertussis toxin on day 2 (List Biologic, Campbell, CA). The mice were observed daily for clinical signs of disease and were scored on a arbitrary scale of 0–5, with graduations of 0.5 for intermediate scores (16): 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness or abnormal gait; 3, complete hind limb paralysis; 4, complete hind limb paralysis with forelimb weakness or paralysis; and 5, moribund or deceased. For passively transferred EAE, mice were immunized with $MOG_{35–55}$ as described above. Fourteen days later, spleen cell suspensions from the immunized mice were cultured for 4 days with 10 μg/ml $MOG_{35–55}$ (15 U/ml IL-2; PeproTech). After washing them with HBSS, 5×10^7 cells were injected i.v. into recipient mice.

Culture medium

Cells were suspended in DMEM (Life Technologies, Paisley, U.K.) supplemented with 1% (v/v) MEM (Life Technologies), 2 mM glutamine (Flow Laboratories, Irvine, U.K.), 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% (v/v) FCS (all three from Life Technologies).

Cytotoxicity assay

NK cell-mediated cytotoxicity was assayed using a standard ^{51}Cr release assay (17). Spleen cells were incubated with ^{51}Cr-labeled YAC-1 target cells at the indicated E:T cell ratios. After 4 h of culture, supernatants were counted for ^{51}Cr release in a gamma counter (Packard, Meriden, CT).

Cell isolation, sorting, and transfer

Mononuclear cells (MNC) were obtained by mincing the popliteal and inguinal lymph nodes through a wire mesh. Spleen DX5$^+$ cells were sorted using a FACStar^{Plus} (Becton Dickinson, Mountain View, CA). DX5$^+$ spleen cells were >99% pure upon reanalysis by flow cytometry. Alternatively, NK cells were purified using anti-NK cell (DX5) MicroBeads (Miltenyi Biotech, Auburn, CA) from spleen cells immunized with CFA. After overnight culture, the purified NK1.1$^+$ cells were injected i.v. into recipient mice.

T cell proliferation

MNC (4×10^5) were incubated in 200 μl of culture medium in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). Ten-microliter aliquots of $MOG_{35–55}$ peptide or Con A (Sigma) were added to wells at final concentrations of 5, 10, 15, and 20 μg/ml ($MOG_{35–55}$) and 5 μg/ml (Con A). After 4 days of incubation, the cells were pulsed for 18 h with 10-μl aliquots containing 1 μCi of [*methyl*-^{3}H]thymidine (sp. act., 42 Ci/mmol; Amersham, Arlington Heights, IL). Cells were harvested onto glass-fiber filters, and thymidine incorporation was measured.

Cytokine induction

CD4$^+$ T cells were purified from spleen cells of $MOG_{35–55}$/CFA-immunized mice by magnetic separation using anti-CD4 Ab conjugated to magnetic beads (Miltenyi Biotech). T cells were eluted by flushing the magnetic column with PBS containing 0.5% FCS as recommended by the manufacturer's protocol. The CD4$^+$ T cells were cultured in duplicate with irradiated (3000 rad) B6 spleen cells in 1.5 ml of culture medium containing 10 μg/ml $MOG_{35–55}$ peptide. For cytokine induction, supernatants were collected at 48 h after in vitro boosting. IFN-γ and IL-4 were measured by optEIA kits (PharMingen). The sensitivities of these ELISAs were 31.3 pg/ml for IFN-γ and 7.8 pg/ml for IL-4. TNF-α was measured by Quantikine M Mouse TNF-α Immunoassay (sensitivity, 15 pg/ml; R&D Systems, Minneapolis, MN).

Anti-$MOG_{35–55}$ IgG Abs

Microtiter plates (Nunc) were coated with 100 μl/well of $MOG_{35–55}$ peptide at a concentration of 5 μg/ml. $MOG_{35–55}$-specific IgG and IgG isotypes were detected by ELISA using rabbit anti-mouse IgG1, IgG2a, or IgG2b (Dakopatts, Glostrup, Denmark) as previously described (18).

Statistical analysis

Differences between groups were evaluated by ANOVA. Disease incidence and severity were analyzed by Fisher's exact test and Mann-Whitney *U* test, respectively.

Results

IL-18$^{-/-}$ mice are resistant to MOG-induced EAE

Given the prominent role of IL-18 in promoting Th1 and NK cell activities, we determined the involvement of this cytokine in the development of EAE in WT and IL-18$^{-/-}$ mice. Subcutaneous immunization of WT mice with 200 μg of $MOG_{35–55}$ peptide in CFA on days 0 and 7 and with pertussis toxin i.v. on day 2 resulted in moderate to severe acute encephalomyelitis, with clinical signs appearing on or around day 13 and rapid progression thereafter. On day 19 p.i., disease incidence reached 89% (17 of 19 animals), and the mean severity was 3.8 ± 0.9. Clinical motor defect was present for 10 days and then spontaneously remitted to leave a mild, chronic, nonrelapsing motor deficit (approximate disease score, 1.5; Fig. 1*A*). In contrast, only 2 of 22 IL-18$^{-/-}$ (11%) mice exhibited clinical signs of EAE. The signs were mild and started from day 16. The mice were completely recovered by day 22 ($p < 0.01$ for both comparisons of disease incidence and severity with WT mice on day 19 p.i.). This observation is in agreement with a recent study showing that EAE in rats could be prevented by neutralizing Abs to IL-18 (12).

The conversion of susceptible B6 mice to a state of high resistance by disruption of the IL-18 gene suggests that IL-18 is critical in the pathogenesis of $MOG_{35–55}$-induced EAE. To further examine this, we performed adoptive transfer experiments. Intravenous injection of 5×10^7 splenocytes from $MOG_{35–55}$-sensitized WT mice to recipient WT or IL-18$^{-/-}$ mice, respectively, resulted in similar severe EAE starting on day 8. The disease lasted for 10 days and spontaneously remitted to a mild or moderate deficiency. These results indicate that IL-18 is not required for disease progression once Th1 cells have been generated. Transfer of the same number of splenocytes from $MOG_{35–55}$-sensitized IL-18$^{-/-}$ mice to recipient WT mice or IL-18$^{-/-}$ mice, respectively, caused very mild EAE (Fig. 1*B*; $p < 0.01$ for both comparisons of disease incidence and severity with transfer cells from WT mice on day 11). Collectively, IL-18 plays a critical role in the inductive stage of EAE, but may not be required for the progression of the disease in the CNS.

Generation of encephlitogenic Th1 cells and autoantibodies to $MOG_{35–55}$ are impaired in IL-18$^{-/-}$ mice

The CNS demyelination is considered an outcome of coordinated immune attaches initiated from both encephalomyelitis T cells and

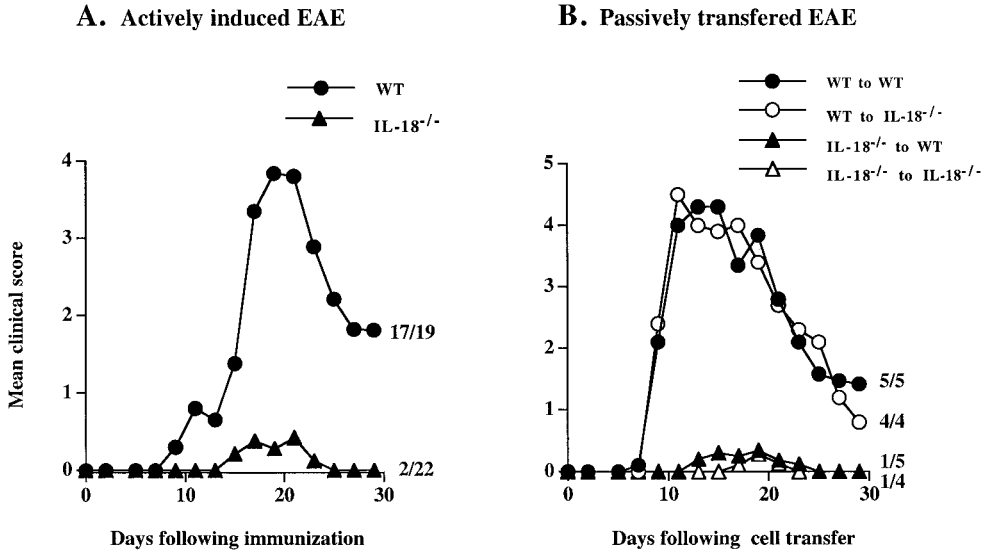

FIGURE 1. IL-18$^{-/-}$ mice are resistant to $MOG_{35–55}$ peptide-induced EAE. *A*, Mice were primed with $MOG_{35–55}$/CFA and monitored for development of EAE. *B*, Passive transfer of spleen cells from $MOG_{35–55}$/CFA-sensitized WT and IL-18$^{-/-}$ mice to WT and IL-18$^{-/-}$ mice, respectively.

pathogenic autoantibodies in MOG-induced EAE (19). We first assessed Ag-specific spleen cell proliferation and cytokine induction in $MOG_{35–55}$-sensitized WT and IL-18$^{-/-}$ mice. Spleen cells from WT and IL-18$^{-/-}$ mice proliferated equally well to $MOG_{35–55}$ peptide (Fig. 2*A*), indicating that the absence of IL-18 did not alter Ag-specific T cell proliferation in this model. However, compared with WT mice, CD4$^+$ T cells from IL-18$^{-/-}$ mice produced severely reduced levels of IFN-γ and TNF-α in response to the $MOG_{35–55}$ peptide (Fig. 2*B*). In contrast, levels of IL-4 production by CD4$^+$ T cells were comparable in IL-18$^{-/-}$ and WT mice. These results indicated that IL-18 is required for autoreactive Th1, but not for Th2 cell development.

We then examined anti-$MOG_{35–55}$ Ab responses in $MOG_{35–55}$-immunized mice. Compared with WT mice, IL-18$^{-/-}$ mice had lower circulating anti-$MOG_{35–55}$ IgG and IgG2b levels by day 20 p.i., while levels of anti-$MOG_{35–55}$ IgG2a and IgG1 were not significantly different from those in WT and IL-18$^{-/-}$ mice (Fig. 2*C*).

Because IFN-γ was shown not to be required for the generation of MOG induced-EAE (20), the resistance to $MOG_{35–55}$-induced EAE could be a result of a more general malformation of Th1 cells (e.g., production of TNF-α and/or other inflammatory cytokines) and autoantibodies to MOG rather than only depend on the IFN-γ producing Th1 cells.

NK cell functions are impaired in $MOG_{35–55}$-sensitized IL-18$^{-/-}$ mice

Recent studies have suggested that NK cells may regulate the adaptive immune response, including the development of autoimmune and hypersensitivity reactions (21, 22). IL-18 can promote NK cell functions (9). Therefore, we asked whether the resistance to EAE induction in IL-18$^{-/-}$ mice could be associated with an altered NK cell function. To explore this possibility, the killing of YAC-1 cells by NK cells and the production of IFN-γ by NK cells were examined. Spleen cells from $MOG_{35–55}$-sensitized WT mice could readily kill YAC-1 target cells. Sorted NK cells from these mice were capable of producing IFN-γ (Fig. 3). In contrast, levels of YAC-1 killing and production of IFN-γ were significantly lower in nonimmunized mice (Fig. 3). Therefore, NK cells are activated quickly after primary immunization with $MOG_{35–55}$ plus CFA. In contrast, both killing of YAC-1 cells and the production of IFN-γ by NK cells were lower in IL-18$^{-/-}$ mice immunized with $MOG_{35–55}$ and CFA than in WT mice ($p < 0.05$; Fig. 3*B*). Collectively, these data suggest that NK cell functions were reduced in the autoantigen-sensitized IL-18$^{-/-}$ mice.

The pathogenic role of IL-18 in EAE is dependent on NK cell function

To explore whether the resistance to EAE induction in IL-18$^{-/-}$ mice could be attributed to the defective NK functions observed above, we first injected IL-18 to WT and IL-18$^{-/-}$ mice. IL-18 injection enhanced the severity of EAE as well as the production of IFN-γ and TNF-α in WT mice (Table I, groups 1 and 2). IL-18 injection could break the resistance to EAE induction and restore the defective Th1 response in IL-18$^{-/-}$ mice (Table I, groups 4 and 5). However, when host NK1.1$^+$ cells were depleted by administration of anti-NK1.1 mAb, the effects of IL-18 injection were abrogated in both WT and IL-18$^{-/-}$ mice (Table I, groups 3 and 6). This suggested that the effects of IL-18 are dependent on the presence of NK cells. Notably, the role of NK cells appeared critical at the time of primary immunization, because depletion of NK1.1$^+$ cells after primary immunization had no detectable effect (data not shown). When WT mice were treated with anti-NK1.1 mAb, EAE development was suppressed. This was associated with a reduced production of IFN-γ and TNF-α (Table I, group 9).

NK cell-derived IFN-γ directs autoreactive Th1 cell development

Early production of IFN-γ by NK cells has been suggested to promote subsequent Th1 responses during host defense against infections (23, 24). It is not known whether this mechanism operates an autoimmune process. In the current model the defective $MOG_{35–55}$-reactive Th1 responses in the IL-18$^{-/-}$ mice could be attributed to the direct absence of IL-18, the reduced IFN-γ production by NK cells as a consequence of the absence of IL-18, or both. Despite normal development of NK cells, NK cell activity with respect to cytotoxicity and production of IFN-γ is severely impaired in IL-18$^{-/-}$ mice (6) (Fig. 3). Thus, IL-18$^{-/-}$ mice provide a very useful model for functional NK cell deficiency. RAG1$^{-/-}$ mice have normal NK cells, but no NK T cells (13). To address whether NK cell-derived IFN-γ can influence autoreactive Th1 cell responses, we purified NK cells (activated by $MOG_{35–55}$ and CFA) from RAG1$^{-/-}$ or RAG1/IFN-$\gamma^{-/-}$ (double-mutant) mice and transferred the NK cells to IL-18$^{-/-}$ mice at the time of

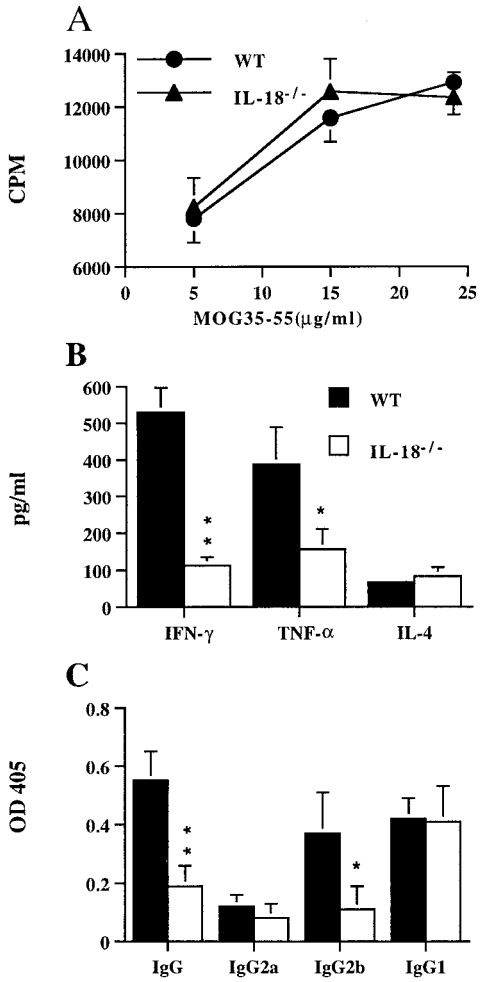

FIGURE 2. Defective $MOG_{35–55}$-specific Th1 and anti-$MOG_{35–55}$ IgG Ab responses in IL-$18^{-/-}$ mice. WT and IL-$18^{-/-}$ mice were primed with $MOG_{35–55}$ peptide in CFA. *A*, Mice were killed on day 10 p.i., and MNC were isolated from draining lymph nodes. Proliferative responses to different concentrations of $MOG_{35–55}$ peptide were assessed. Background proliferation was 1,306 ± 110 cpm; Con A-induced proliferation was 15,420 ± 4,300 cpm. No significant difference was found between control and IL-$18^{-/-}$ mice. *B*, $MOG_{35–55}$ peptide-specific cytokine production of $CD4^+$ T cells from $MOG_{35–55}$/CFA-immunized mice. Spontaneous cytokine release: IFN-γ, 58 ± 23 pg/ml; TNF-α, 67 ± 21 pg/ml; IL-4, undetectable. No difference was revealed in spontaneous cytokine release between WT and IL-$18^{-/-}$ mice. All results are expressed as the mean ± SD. *A* and *B* represent one of two independent experiments with similar results ($n = 4$). *C*, Anti-$MOG_{35–55}$ IgG and IgG isotypes measured by ELISA on day 14 p.i. ($n = 15$). All results are expressed as the mean ± SD. Statistical evaluation was performed between the different experimental groups and control groups, respectively. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

immunization with $MOG_{35–55}$ and CFA. IL-$18^{-/-}$ mice that received 10^7 NK cells from RAG1$^{-/-}$ mice before immunization with $MOG_{35–55}$/CFA developed EAE at a similar magnitude as control WT mice (Table I, group 7). In contrast, no development of EAE was observed after transfer of NK cells from RAG1/IFN-$\gamma^{-/-}$ mice (Table I, group 8). The mice were killed on 25 days p.i., and $CD4^+$ T cells were purified from local lymph nodes. Transfer of 10^7 NK cells from RAG1$^{-/-}$ mice largely rescued the impaired IFN-γ and TNF-α production by $CD4^+$ T cells from IL-$18^{-/-}$ mice (Table I, groups 4 and 7). In contrast, transfer of 10^7 NK cells from RAG1/IFN-$\gamma^{-/-}$ mice to IL-$18^{-/-}$ mice had no detectable effect on cytokine production by T cells. IL-4 production was not

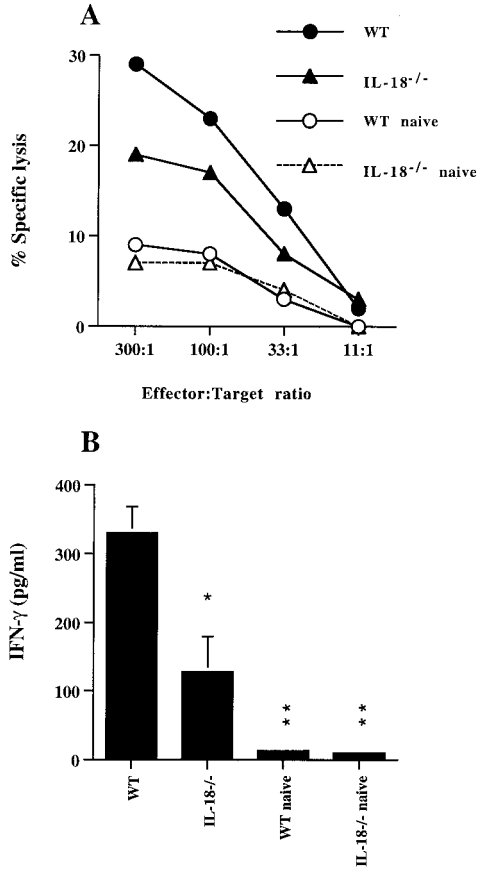

FIGURE 3. Functional properties of NK cells in $MOG_{35–55}$/CFA immunized mice. *A*, NK cell-mediated cytotoxicity of splenocytes from WT (●) or IL-$18^{-/-}$ (▲) mice immunized with $MOG_{35–55}$ in CFA on day 7 p.i. as well as from native WT (○) and IL-$18^{-/-}$ mice (△). NK cell effectors were tested against YAC-1 target cells. *B*, IFN-γ production by NK cells. NK cells were sorted with anti-DX5 Abs by flow cytometry from spleen cells WT or IL-$18^{-/-}$ mice immunized with $MOG_{35–55}$ in CFA on day 7 p.i. as well as from naive WT mice and cultured for 48 h without Ag stimulation. *A* and *B* represent one of two independent experiments. $n = 4$ mice/group. Statistical evaluation was performed between the different experimental groups and control groups, respectively. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

significantly altered by reconstitution of NK cells (data not shown). Transfer of NK cells after induction of disease had no detectable effect (data not shown). Taken together, these results provide in vivo evidence that IFN-γ production by activated NK cells can promote autoreactive Th1 responses during the initiation stage of autoimmune responses.

Discussion

IL-18 is produced by macrophages and dendritic cells early during host defense against infectious pathogens. IL-18 synergizes with IL-12 in induction of IFN-γ by NK cells (7, 9). In this study we have focused attention on IL-18 in the pathogenesis of MOG-induced EAE in B6 mice, a murine model for MS. IL-18 gene disruption converts susceptible B6 mice to a state of high resistance to disease induction, while IL-18 administration enhances the severity of the disease. These results suggest that IL-18 is critical for the development of MOG-induced EAE. An impaired capacity of NK cells to release IFN-γ during the point of primary immunization, due to the absence of IL-18, appears to be a major mechanism underlying the resistance to EAE induction. Thus, our study suggests that IL-18 links the innate immune response involving NK cells to the generation of destructive autoimmunity.

Table I. *Defective NK cell function underlies the resistance to MOG_{35-55} peptide-induced EAE in IL-$18^{-/-}$ mice*[a]

Groups of Mice	No. of Mice	Treatment	Clinical Score ± SD (incidence)	IFN-γ (pg/ml)	TNF-α (pg/ml)
1. IL-$18^{+/+}$	4	PBS	3.3 ± 0.8 (4/4)	367.4 ± 23	302.2 ± 28
2. IL-$18^{+/+}$	6	Control Ab + IL-18	4.5 ± 1.1 (4/4)*	482.0 ± 47*	499.8 ± 47*
3. IL-$18^{+/+}$	6	Anti-NK1.1 + IL-18	2.2 ± 0.6 (4/4)	282.0 ± 22	279.4 ± 35
4. IL-$18^{-/-}$	4	PBS	0.0 ± 0.0 (0/4)	83.8 ± 33	147.4 ± 55
5. IL-$18^{-/-}$	5	Control Ab + IL-18	2.8 ± 0.6** (4/5)	310.4 ± 55*	347.8 ± 29*
6. IL-$18^{-/-}$	6	Anti-NK1.1 Ab + IL-18	1.6 ± 0.7 (2/5)	112.5 ± 14	88.9 ± 43
7. IL-$18^{-/-}$	5	NK cells (RAG1$^{-/-}$)	2.1 ± 0.9* (4/5)	322.1 ± 55*	332.2 ± 14*
8. IL-$18^{-/-}$	5	NK cells (RAG1/IFN$^{-/-}$)	0.5 ± 0.2* (1/5)	107.6 ± 72*	158.4 ± 33*
9. IL-$18^{+/+}$	8	Anti-NK1.1	0.9 ± 0.7 (1/8)**	145.8 ± 27*	77.9 ± 34**

[a] Mice were treated with control (anti-mouse IgG) or anti-NK1.1 mAb (see *Materials and Methods*) 2 days before primary immunization with MOG_{35-55} peptide in CFA. All other manipulations were initiated at the day of primary immunization. EAE development was monitored until 25 days postimmunization (p.i.). Mice were subsequently sacrificed and $CD4^+$ T cells from spleen were cultured. Cytokine production in response to the MOG_{35-55} peptide in culture supernatants were measured by ELISA. The range of cytokine spontaneous release: IFN-γ, 66 ± 47 pg/ml; TNF-α, 45 ± 30 pg/ml; IL-4, undetectable. No significant difference was found between WT and IL-$18^{-/-}$ mice. All results are expressed as mean values ± SD. Statistical evaluation was performed between experimental groups and corresponding control groups, respectively. *, $p < 0.05$; **, $p < 0.01$.

Myelin sheath destruction in MS and MOG-induced EAE appears to be a coordinated immunological attack initiated by both Th cells and autoantibodies (19). Factors that determine the differentiation of naive T cells into either Th1 (IL-2, IFN-γ, TNF-α), or Th2 (IL-4, IL-10) phenotypes would be expected to have an impact on the development of autoimmune responses. The most clearly defined factors determining Th subset differentiation from naive $CD4^+$ T cell precursors are cytokines present at the initiation of the immune response at the stage of ligation of the TCR (25). A number of inflammatory mediators are released promptly during the innate immune responses. For example, virus/bacterial stimuli activate macrophages and subsequently NK cells of the innate immune response to produce IL-12 and IFN-γ, respectively, which may drive the development of Th1 cells from naive Ag-specific T cells (23, 24). Beside its ability to produce IFN-γ, NK cells may produce a variety of other immunoregulatory mediators, including TGF-β, TNF-α, TNF-β, GM-CSF, macrophage inflammatory protein-1α, IL-1, IL-3, IL-5, IL-8, and IL-10 (21, 26). These soluble factors may also affect in different ways the development of autoimmune diseases.

Although NK cell numbers are normal in IL-$18^{-/-}$ mice (9), NK cell functions with regard to cytotoxicity and production of IFN-γ are reduced in MOG_{35-55}/CFA-immunized mice (Fig. 3). A critical question arises as to whether the failure to mount Th1 responses to MOG in IL-$18^{-/-}$ mice is due to the defective NK cell functions, the deficiency in IL-18, or both. IL-18 could not directly drive Th1 development in in vitro experiments (27), indicating that additional factors in vivo are required for the development of Th cells. In the present study, IL-18 injection restored the MOG_{35-55}-specific Th1 and autoantibody responses (data not shown). This restoration was abrogated when NK cells were depleted by administration of anti-NK1.1 mAb in IL-$18^{-/-}$ mice, indicating that the action of IL-18 in promoting the generation of strong MOG-specific Th cells was dependent on NK cells in this model. Furthermore, transfer of NK cells from RAG1$^{-/-}$ mice, but not from RAG1/IFN-$\gamma^{-/-}$(double-mutant) mice, rescued the defective Th1 responses in IL-$18^{-/-}$ mice and converted IL-$18^{-/-}$ mice so that they were susceptible to EAE induction. Collectively, these data demonstrate that IL-18 favors the differentiation of naive T cells into autoreactive Th1 cells. This effect is largely dependent on IFN-γ production by NK cells and cannot be compensated by other soluble factors, such as IL-12, in this system. Therefore, the present study provides in vivo supportive evidence for a role of NK cells in directing Th1 responses not only in the host defense against infections, but also in the generation of an autoimmune disease.

It has been demonstrated that NK cells could critically influence the initial differentiation of naive T cells into effector cells in response to foreign Ag, but not the secondary activation of Ag-specific T cells (21). This salient feature of NK cells is also well reflected in the current model, in which no detectable effects were seen upon depletion of NK cells or transfer of NK cells to IL-$18^{-/-}$ mice after induction of disease. Because most cytokines produced by NK cells can also be produced by other cell types, our study suggests that the timing of IFN-γ released by NK cells in regulating the autoreactive T cell response is critical and is indispensable by cytokines produced by other cells.

Our results suggests that defective NK cell function in IL-$18^{-/-}$ mice underlies the failure of the development of autoreactive Th1 cells and the resistance to EAE induction. These results seem at odds with a recent study showing that anti-NK1.1 mAb treatment enhanced the MOG_{35-55}-reactive Th1 cell response and the development of EAE (22). Currently, there is no clear explanation for this apparent discrepancy. It is noticeable that in that study induction of disease followed shortly after the administration of anti-NK1.1 mAb. Anti-NK1.1 mAb cross-link with NKR-P1 and trigger IFN-γ release by the NK cells before the actual depletion of the cells (28). In our hands, depletion of $NK1.1^+$ cells 2 days prior to immunization resulted in reduced disease development as well as Th1 responses (Table I, group 9). Determination of whether the timing and dose of Ab administration are responsible for these contrasting results requires further investigation.

Previous studies have demonstrated that EAE can be induced by IFN-γ-independent pathways as well (20). However, those results do not exclude a disease-promoting role for IFN-γ, including IFN-γ produced by NK cells. The present results support the idea of an important role for IFN-γ produced by NK cells in the course of disease development in the present model. It might be that Th1 differentiation is crucial, but that the relevant effector cytokine is T1 cytokines other than (in additional to) IFN-γ.

The current study provides evidence that IL-18 can interplay with NK cells and direct autoreactive T cells to a strong Th1 phenotype, and links innate immune responses to the autoimmune responses. In a more physiological context, one may envisage an intracellular bacterial or virus infection that induces monocytes/macrophages to promptly produce IL-18, which, in turn, independently or in synergy with IL-12 activates NK cells. These cytokine-activated NK cells may provide a unique endogenous milieu promoting downstream adaptive responses. Subsequently, NK cells may promote autoaggression via control of autoreactive T and B cells. This may be achieved by the production of IFN-γ and/or other inflammatory mediators during the initial activation

phase of these cells. Because progression of MS may be associated with successive rounds of activation of myelin-reactive Th1 cells (such as in epitope spreading) (4), selective targeting of IL-18 may prevent the development of such neo-generated autoimmune responses and may therefore provide a therapeutic alternative for MS.

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