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Natural killer cells determine the outcome of B cell–mediated autoimmunity

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Natural killer (NK) cells can affect the outcome of adaptive immune responses. NK cells, but not NK1.1⁺ T cells, were found to participate in the development of myasthenia gravis (a T cell–dependent, B cell– and antibody-mediated autoimmune disease) in C57BL/6 mice. The requirement for NK cells was reflected by the lack of a type 1 helper T cell response and antibodies to the acetylcholine receptor in both NK1.1⁺ cell–depleted and NK cell–deficient IL-18$^{-/-}$ mice. These findings establish a previously unrecognized link between NK cells and autoreactive T and B cells.

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Autoimmune diseases are inflammatory disorders, many of which have a suspected infectious etiology. Natural killer (NK) cells, as a first line of defense in combating infections, may be involved in the initiation of autoimmunity and accumulate in the target organs of certain autoimmune diseases[1–3]. It has been suggested that NK or NK1.1⁺ T (NKT) cells serve as regulatory cells in some T cell–mediated experimental autoimmune disease, including murine models of encephalomyelitis[4,5], colitis[6] and diabetes[7]. However, several issues regarding the role of NK cells in the development of autoimmune diseases remain unresolved. Few studies have been able to distinguish between effects of NK cells and effects of NKT cells, and the mechanism for the regulatory effect of NK cells in murine models remains unclear. Also still unknown is the point at which NK cells impact on the development of autoimmune disease, and the contribution of NK cells to the development of those autoimmune diseases that are primarily mediated by pathogenic antibodies.

Autoantibodies produced by B cells are the primary cause of disease in a variety of autoimmune conditions, including hemolytic anemia, thyroiditis, stiff man syndrome, pemphigus vulgaris and systemic lupus erythematosus[8]. Myasthenia gravis (MG) is one of the best characterized antibody-mediated autoimmune diseases because the target antigen, the nicotinic acetylcholine receptor (AChR) of neuromuscular junctions, has been well defined[9]. Experimental autoimmune myasthenia gravis (EAMG) in C57BL/6 (B6) mice, induced by repeated immunizations with *Torpedo* AChR emulsified in complete Freund's adjuvant (CFA), is a particularly useful model for identification of the pathogenic mechanisms that cause MG in humans[10]. In both MG and EAMG, autoreactive CD4⁺ T cells provide help for B cells to produce antibodies to AChR[9]. Although the T_H1 cytokines interferon γ (IFN-γ) and interleukin 12 (IL-12) are critically important for the generation of EAMG in B6 mice[11,12], the production of both T helper cell subsets 1 and 2 (T_H1 and T_H2) cytokines may be required for the development of full-blown EAMG[13].

To obtain a more comprehensive view of the potential role of NK and NKT cells in B cell–mediated autoimmune diseases, we examined the development of antibody-mediated EAMG in mice depleted of NK1.1⁺ cells, in NK cell–deficient IL-18$^{-/-}$ mice, and in NKT cell–deficient mice. Our results demonstrate that NK cells determine the outcome of autoantibody responses to AChR via control of autoreactive T

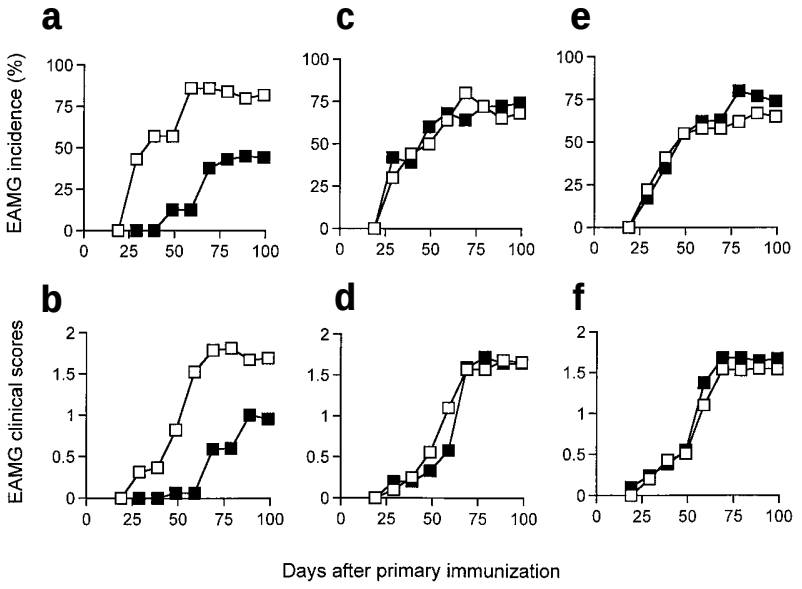

Figure 1. Incidence and severity of EAMG in NK1.1⁺ cell–depleted and NKT cell–deficient mice. Mice were immunized with AChR and CFA, boosted on day 30 and 60 after immunization, and monitored for development of EAMG. (**a,b**) B6 mice (□, *n*=35) treated with isotype control antibody and B6 mice depleted of NK1.1⁺ cells (■, *n*=36). (**c,d**) Wild-type (□, *n*=15) and Jα281$^{-/-}$ mice (■, *n*=17). (**e,f**) Wild-type (□, *n*=8) and CD1d1$^{-/-}$ mice (■, *n*=8).

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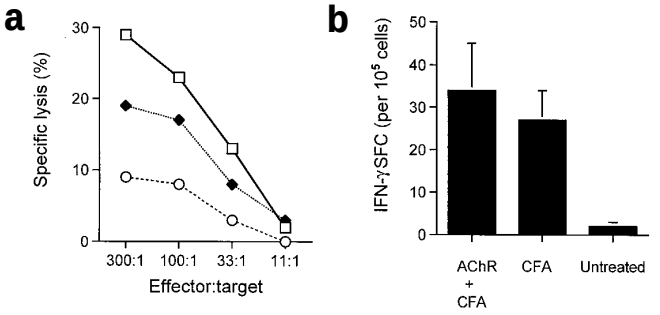

Figure 2. Functional properties of NK cells in EAMG. (**a**) NK cell–mediated cytotoxicity of splenocytes from naïve B6 mice (○), B6 mice immunized with CFA alone (◆) or B6 mice immunized with AChR in CFA (□) 7 days after immunization. All effectors were tested against YAC-1 target cells. (**b**) Mean numbers (± s.d.) of IFN-γ spot–forming NK cells (SFC) measured by a solid-phase enzyme-linked immunospot (ELISPOT). NK cells were sorted from spleen cells of naïve B6 mice, B6 mice immunized with CFA alone or B6 mice immunized with AChR in CFA by flow cytometry on day 7 after immunization and cultured for 48 h without antigen stimulation. (**a**) and (**b**) represents one of two independent experiments. (*n*=4 mice per group).

cells.

Results

Onset and severity of murine EAMG

To determine whether NK1.1$^+$ cells (NK cells and NKT cells) are important for the development of EAMG, B6 mice or B6 mice depleted of NK1.1$^+$ cells were immunized with AChR and CFA, boosted on days 30 and 60 after immunization, and monitored for 100 days after immunization for signs of MG. In B6 mice, the median EAMG onset was day 37, whereas in NK1.1$^+$–depleted mice, the median onset was day 65. Compared to control mice, the incidence (P<0.001 on day 49 and P<0.01 on day 59 after immunization) and severity (P<0.001 on day 49 and P<0.01 on day 59 after immunization) of EAMG were significantly lower in mice depleted of NK1.1$^+$ cells (**Fig. 1a,b**). Mice depleted of NK1.1$^+$ cells had also higher AChR levels in their muscle tissue than control mice (35±5% *versus* 68±12%, P<0.05).

To determine whether NKT cells are important for the development of EAMG in this model, NKT cell–deficient $J_\alpha281^{-/-}$ (ref. 14) and CD1d1$^{-/-}$ (ref. 15) mice were immunized with AChR and CFA as above. Both $J_\alpha281^{-/-}$ mice and CD1d1$^{-/-}$ mice developed EAMG at a similar rate to their corresponding wild-type controls (**Fig. 1c–f**). Thus NK cells, but not NKT cells, affect the development of AChR-induced EAMG.

NK cell activation during primary immunization

To examine the mechanisms by which NK cells influence the development of EAMG in mice, we first examined the status of NK cells in mice actively immunized with AChR + CFA. NK cell activity in mice immunized with AChR + CFA, or CFA alone, was higher than in nonimmunized mice (**Fig. 2a**). The frequency of NK cells producing IFN-γ was also higher in mice immunized with AChR + CFA, or CFA alone, than in nonimmunized mice (P<0.01) (**Fig. 2b**). Therefore, NK cells are activated after primary immunization with AChR + CFA.

NK1.1$^+$ cell depletion: proliferation and cytokine responses

To determine whether NK1.1$^+$ cells influence autoreactive T cell responses, lymph node mononuclear cells (MNCs) from NK1.1$^+$ cell–depleted or control mice were isolated at days 7 and 100 after immunization, and examined for proliferative and cytokine responses. Depletion of NK1.1$^+$ cells did not significantly alter T cell proliferative responses to AChR or its dominant epitope the peptide AChRα(146–162)[13] (**Fig. 3a,b**). When lymph node MNCs were examined for cytokine responses, it was found that IFN-γ production by AChR-specific T cells was reduced on day 7 and 100 after immu-

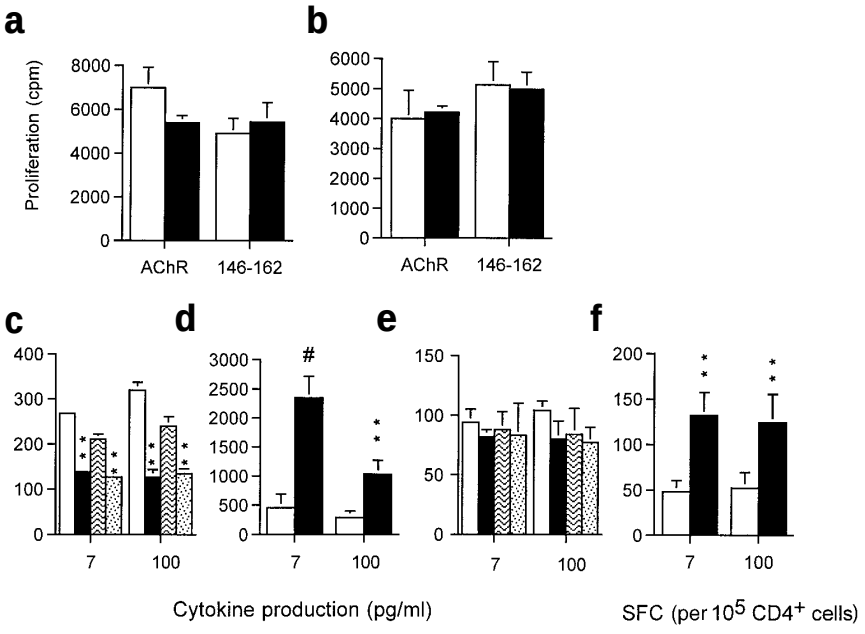

Figure 3. NK1.1$^+$ cell depletion before immunization with AChR + CFA leads to enhanced TGF-β1 production by T cells that down-regulate T_H1 type immune responses. B6 mice were immunized with AChR + CFA on days 0, 30 and 60. NK1.1$^+$ cells were depleted as described in the Methods. Mice were killed at day 7 or day 100 after immunization and MNCs isolated from draining lymph nodes were cultured. Proliferative responses to AChR and AChRα(146–162) on (**a**) day 7 and (**b**) day 100 after immunization (background cpm 1306±110 for day 7 after immunization, 1130±50 for day 100 after immunization; no difference was found between control and NK1.1$^+$–depleted mice). (**c**) AChR-specific IFN-γ production in NK1.1$^+$ cell–depleted mice or in mice treated with isotype control antibody (spontaneous release: 67±21 pg/ml). Neutralizing transforming growth factor β1 monoclonal antibody (TGF-β1 mAb) injected, as described in the Methods, on the day of immunization. (**d**) AChR-specific TGF-β1 production in mice treated with anti-NK1.1$^+$ or in mice treated with isotype control antibody (spontaneous release: 273±69 pg/ml). (**e**) AChR-specific IL-4 production in NK1.1$^+$ cell–depleted mice or mice treated with control antibody. Neutralizing anti–TGF-β was injected, as described in the Methods (spontaneous release: undetectable). (**f**) Mean numbers of AChR-specific TGF-β SFC per 10^5 CD4$^+$ cells measured by ELISPOT in control and NK1.1$^+$ cell–depleted mice (background spots: 3–5). All results are expressed as mean values ± s.d. **a** and **b** represent one of eight independent experiments; **c–f** represent one of three independent experiments. Cytokine release in response to myelin basic protein (MBP) was similar to spontaneous cytokine release. Comparative statistical analysis of control and experimental groups was done: **, P<0.01; #, P<0.001. (open bars, control antibody; filled bars, anti-NK1.1; wavey-lined bars, anti-NK1.1 + anti–TGF-β, dotted bars anti-NK1.1 + control antibody).

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nization in mice depleted of NK1.1$^+$ cells compared to control mice (**Fig. 3c**; $P<0.01$ at both time points). In contrast, TGF-β1 production by AChR-specific T cells was markedly elevated in NK1.1$^+$ cell–depleted mice on days 7 and 100 after immunization (**Fig. 3d**; $P<0.001$ and 0.01, respectively). No significant difference in IL-4 production was observed in NK1.1-depleted mice *versus* control mice (**Fig. 3e**).

Table 1. EAMG development in NK1.1$^+$ cell–depleted B6 mice inoculated with neutralizing antibodies to TGF-β1

Number of B6 mice	Treatment[a]	Muscle weakness[b] 0	1	2	3	Disease incidence
35	Control mAbs	5	6	15	9	30/35 (86%)
36	Anti-NK1.1$^+$[c]	23	6	4	3	13/36 (36%)
6	Anti-NK1.1$^+$+ anti–TGF-β1[c]	1	3	1	1	5/6 (83%)

[a]NK1.1 mAb treatment was initiated 2 days before immunization. Anti–TGF-β treatment was initiated at the time of immunization. Data on mice injected with control mAb or NK1.1 mAb only are from **Fig. 1**. [b]Mice were immunized with AChR and CFA, and monitored for EAMG (muscle weakness) graded 0–3 as described in Methods. [c]$P<0.01$ between groups with anti-NK1.1–treated mice *versus* anti-NK1.1–treated mice given neutralizing anti–TGF-β1.

The number of CD4$^+$ TGF-β1–producing cells, measured by ELISPOT was also higher in NK1.1$^+$ cell–depleted mice (**Fig. 3f**). Similar results were obtained for mice depleted of NK1.1$^+$ cells 7 days before immunization and onwards, as described in Methods (data not shown). Taken together, these results suggest that NK1.1$^+$ cell depletion differentially regulates cytokine production by AChR-specific T_H subsets.

Neutralization of TGF-β in NK1.1$^+$ cell–depleted mice

TGF-β is a potent inhibitor of T cells[16,17]. To examine whether the suppression of IFN-γ–producing T_H1 cells observed in NK1.1$^+$ cell–depleted mice was associated with increased production of TGF-β, we inoculated NK1.1$^+$ cell–depleted mice with neutralizing antibodies to TGF-β. The suppression of IFN-γ production in NK1.1$^+$ cell–depleted mice could be reversed, at least in part, by neutralizing antibodies to TGF-β. Because concentrations of the T_H2 cytokine IL-4 were unaltered after NK1.1$^+$ cell depletion, it is less likely that the suppression of T_H1 cells was attributed to effects mediated by T_H2 cells (**Fig. 3c**). In line with the cytokine data, EAMG development in NK1.1$^+$ cell–depleted mice inoculated with neutralizing antibodies to TGF-β was significantly enhanced compared to NK1.1$^+$ cell–depleted mice receiving no antibodies to TGF-β (**Table 1**).

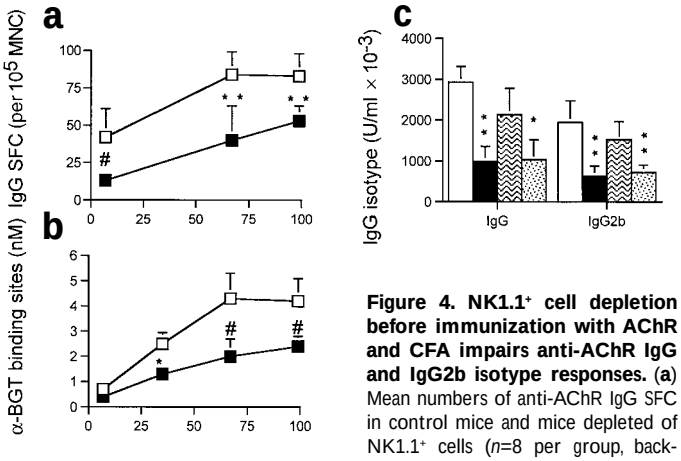

Figure 4. NK1.1$^+$ cell depletion before immunization with AChR and CFA impairs anti-AChR IgG and IgG2b isotype responses. (**a**) Mean numbers of anti-AChR IgG SFC in control mice and mice depleted of NK1.1$^+$ cells (n=8 per group, background spots 0–3). (**b**) Serum anti-AChR measured by radioimmunoassay using ^{125}I–α-bungarotoxin (α-BGT). (**c**) Anti-AChR IgG isotypes measured by ELISA on day 100 after immunization, and effects of neutralizing TGF-β (see Methods). All results are expressed as mean values ± s.d. n=20 mice per group for **b** and **c**. Comparative statistical analysis of control and experimental groups was done: *, $P<0.05$, **, $P<0.01$, #, $P<0.001$. (Open squares, control antibody; filled squares, anti-NK1.1; wavey-lined bars, anti-NK1.1 + anti-TGF-β, dotted bars anti-NK1.1 + control antibody.)

Antibody responses to AChR

In MG and EAMG, the pathogenic antibodies to AChR consist predominantly of all IgG subtypes[9]. These antibodies are responsible for the functional loss of AChR in the neuromuscular junctions, resulting in muscle weakness[9]. The number of anti-AChR IgG–secreting cells was reduced in mice depleted of NK1.1$^+$ cells (**Fig. 4a**). Accordingly, the concentration of AChR-specific IgG in serum was reduced in these mice (**Fig. 4b**). Compared to control mice, AChR-specific IgG2a and IgG1 concentrations were not significantly altered in mice depleted of NK1.1$^+$ cells, but IgG2b was significantly reduced ($P<0.05$, **Fig. 4c**). In NK1.1$^+$ cell–depleted mice receiving anti–TGF-β, the concentrations of anti–AChR IgG and IgG2b were similar to those in control mice (**Fig. 4c**). These effects were not specific for AChR, as NK1.1$^+$ cell–depleted mice immunized with keyhole limpet haemocyanin also had a reduced concentration of specific antibody (data not shown).

NK1.1$^+$ cells influence EAMG during priming

To determine the time point at which NK1.1$^+$ cells affect development of EAMG, mice were treated with anti-NK1.1 every 5–7 days starting on day 7 or day 37 after immunization. Mice depleted of NK1.1$^+$ cells from days 7 and 37 after immunization developed EAMG at a similar rate to control mice (MG incidence 83.3%, maximum MG severity 1.9; compare with data in **Fig. 1** and **Table 1**). AChR-specific T cell proliferation (4,220±604 cpm *versus* 5,170±567 cpm) and cytokine production (IFN-γ 267±45 pg/ml *versus* 300±60 pg/ml; IL-4 77±11 pg/ml *versus* 70±7 pg/ml; TGF-β 455±123 pg/ml *versus* 423±109 pg/ml) were similar in control mice and in mice depleted of NK1.1$^+$ cells at day 7 after immunization These groups of mice had similar anti-AChR IgG (1.23±0.05 *versus* 0.89±0.24), detected by enzyme-linked immunosorbent assay (ELISA) at A_{405} and of IgG isotypes (IgG1, 0.14±0.03 *versus* 0.1±0.04; IgG2a, 0.15±0.08 *versus* 0.12±0.05; IgG2b, 0.84±0.60 *versus* 0.61±0.13 detected by ELISA at A_{405}). Likewise, depletion of NK1.1$^+$ cells from day 37 after immunization had no impact on the development of MG or T and B cell responses to AChR (data not shown).

AChR responses in mice lacking NKT cells

To find whether the effects observed after removal of NK1.1$^+$ cells were due to NK cells or NKT cells, we immunized NKT cell–deficient $J_\alpha281^{-/-}$ and CD1d1$^{-/-}$ mice, and corresponding wild-type controls, with AChR in CFA. Seven days after immunization lymph node MNCs were prepared and analyzed for proliferative and cytokine responses. AChR- and AChRα(146–162)–specific proliferation did not differ significantly between $J_\alpha281^{-/-}$ and wild-type mice (**Fig. 5a,b**). MNC from $J_\alpha281^{-/-}$ mice released slightly more AChR-

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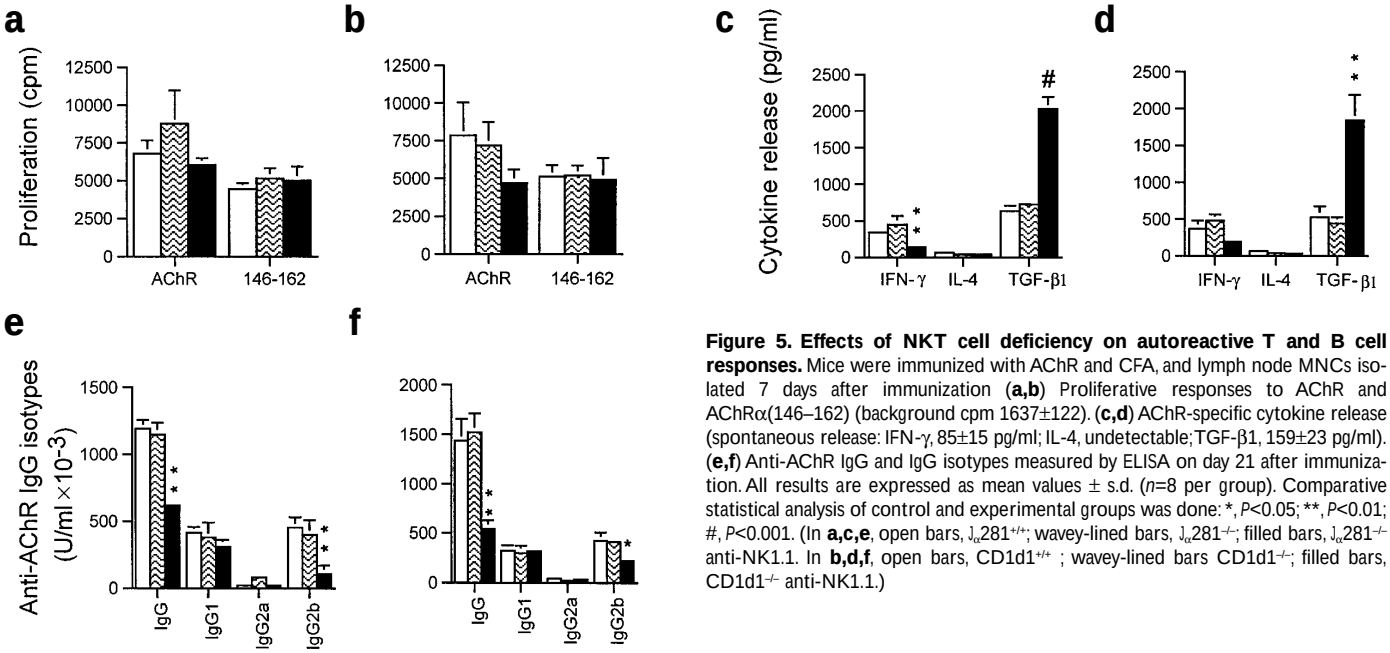

Figure 5. Effects of NKT cell deficiency on autoreactive T and B cell responses. Mice were immunized with AChR and CFA, and lymph node MNCs isolated 7 days after immunization (**a,b**) Proliferative responses to AChR and AChRα(146–162) (background cpm 1637±122). (**c,d**) AChR-specific cytokine release (spontaneous release: IFN-γ, 85±15 pg/ml; IL-4, undetectable; TGF-β1, 159±23 pg/ml). (**e,f**) Anti-AChR IgG and IgG isotypes measured by ELISA on day 21 after immunization. All results are expressed as mean values ± s.d. (n=8 per group). Comparative statistical analysis of control and experimental groups was done: *, P<0.05; **, P<0.01; #, P<0.001. (In **a,c,e**, open bars, $J_\alpha281^{+/+}$; wavey-lined bars, $J_\alpha281^{-/-}$; filled bars, $J_\alpha281^{-/-}$ anti-NK1.1. In **b,d,f**, open bars, CD1d1$^{+/+}$; wavey-lined bars CD1d1$^{-/-}$; filled bars, CD1d1$^{-/-}$ anti-NK1.1.)

specific IFN-γ and slightly less IL-4 than wild-type mice (not statistically significant). No differences in TGF-β1 production were observed (**Fig. 5c,d**). Total IgG, as well as IgG1, concentrations were not statistically different in $J_\alpha281^{-/-}$ mice as compared to wild-type mice (**Fig. 5e,f**). IgG2a was undetectable at the same dilution of the sera. CD1d1$^{-/-}$ mice exhibited immune responses to AChR similar to $J_\alpha281^{-/-}$ mice (**Fig. 5 a–f**). $J_\alpha281^{-/-}$ and CD1d1$^{-/-}$ mice treated with antibodies against NK1.1 on day 2 before immunization with AChR in CFA behaved in a similar way as corresponding wild-type mice treated with the same antibodies (**Fig. 5**; data not shown).

Immune responses to AChR in IL-18$^{-/-}$ mice

IL-18, originally identified as IFN-γ–inducing factor, promotes the production of IFN-γ by NK cells and T_H1 cells and enhances NK cell activity[18]. To further clarify the mechanisms by which NK cells affect EAMG development, we characterized the immune responses to AChR in IL-18$^{-/-}$ mice[19] immunized with AChR and CFA. Compared to wild-type mice, IL-18$^{-/-}$ mice were resistant to the induction of MG (**Fig. 6a,b**; incidence P<0.01 on day 55 after immunization, severity P<0.01 on day 78 after immunization). This was associated with defective T_H1 responses and lower levels of antibodies to AChR IgG and IgG2b, and increased TGF-β1 production (**Fig. 6c,d**), whereas AChR-reactive T cell proliferative responses were not impaired in the IL-18$^{-/-}$ mice (data not shown). Therefore, the clinical and immunological parameters in the induction of MG appear similar in B6 mice receiving anti-NK1.1 and in IL-18$^{-/-}$ mice.

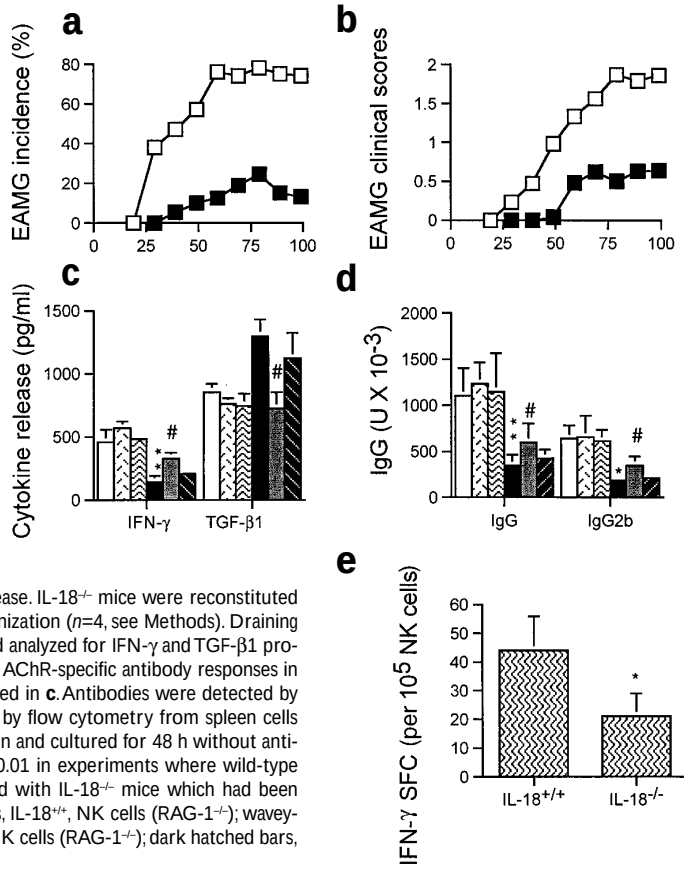

Figure 6. Resistance to EAMG induction in IL-18$^{-/-}$ mice is associated with defective NK cell function. Mice were immunized with AChR and CFA, boosted on day 30 and 60 after immunization, and monitored for development of EAMG. (**a**) EAMG incidence and (**b**) severity in wild-type (n=19) and IL-18$^{-/-}$ mice (n=20). (**c**) Effects of reconstitution of NK cells in IL-18$^{-/-}$ mice on AChR-specific cytokine release. IL-18$^{-/-}$ mice were reconstituted with 10^7 NK cells from either RAG-1$^{-/-}$ or RAG-1$^{-/-}$ IFN-γ$^{-/-}$ mice one day before immunization (n=4, see Methods). Draining lymph node MNC culture supernatants were collected on day 7 after immunization and analyzed for IFN-γ and TGF-β1 production by ELISA (spontaneous release: IFN-γ, 58±23 pg/ml; TGF-β1, 122±15 pg/ml). (**d**) AChR-specific antibody responses in IL-18$^{-/-}$ mice. Serum samples were collected on day 7 after immunization from mice used in **c**. Antibodies were detected by ELISA (n=4). (**e**) Numbers of IFN-γ SFC measured by ELISPOT. NK cells were sorted by flow cytometry from spleen cells of wild-type and IL-18$^{-/-}$ mice immunized with AChR in CFA on day 7 after immunization and cultured for 48 h without antigen stimulation (n=4). All results are expressed as mean values ± s.d. *, P<0.05; **, P<0.01 in experiments where wild-type mice were compared with IL-18$^{-/-}$ mice. #, P<0.05 when IL-18$^{-/-}$ mice were compared with IL-18$^{-/-}$ mice which had been reconstituted with NK cells (RAG-1$^{-/-}$–derived). (Open bars, IL-18$^{+/+}$; light hatched bars, IL-18$^{+/+}$, NK cells (RAG-1$^{-/-}$); wavey-lined bars, IL-18$^{+/+}$, NK cells (RAG-1$^{-/-}$ IFN-γ$^{-/-}$); filled bars, IL-18$^{-/-}$; shaded bars, IL-18$^{-/-}$, NK cells (RAG-1$^{-/-}$); dark hatched bars, IL-18$^{-/-}$, NK cells (RAG-1$^{-/-}$ IFN-γ$^{-/-}$)).

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Table 2. EAMG development in IL-18$^{-/-}$ mice reconstituted with NK cells

Number of IL-18$^{-/-}$ mice	Treatment[a]	Muscle weakness[b] 0	1	2	3	Disease incidence
7	PBS buffer[c]	5	1	1	0	2/7 (29%)
5	NK cells (RAG-1$^{-/-}$)[c,d]	1	2	2	0	4/5 (80%)
5	NK cells (RAG-1$^{-/-}$ IFNγ$^{-/-}$)[d]	4	1	0	0	1/5 (20%)

[a]Mice were intravenously inoculated with 10^7 NK cells at the time of immunization. [b]Mice were immunized with AChR and CFA, and monitored for EAMG (muscle weakness) graded 0–3 as described in Methods. [c]$P < 0.01$ for PBS buffer–inoculated mice *versus* NK cell (RAG-1$^{-/-}$–derived) inoculated mice. [d]$P < 0.05$ for NK cell (RAG-1$^{-/-}$ IFN-γ$^{-/-}$ –derived) inoculated mice *versus* NK cell (RAG-1$^{-/-}$–derived) inoculated mice.

Reconstitution of NK cells in IL-18$^{-/-}$ mice

Because early production of IFN-γ by NK cells may promote subsequent T_H1 responses[20,21], the defective AChR reactive T_H1 responses could be due to either the absence of direct IL-18 action on T_H1 cells, reduced IFN-γ production by NK cells, 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[19] (**Fig. 6e**). RAG-1$^{-/-}$ mice[22] have normal NK cells but no NKT cells. To address directly whether IFN-γ derived from NK cells can influence autoreactive T_H1 cell responses and disease development in the EAMG model, we sorted NK cells from RAG-1$^{-/-}$ or RAG-1$^{-/-}$ IFN-γ$^{-/-}$ (double mutant)[23] mice. Although NK cells from RAG-1$^{-/-}$ mice readily produced IFN-γ as determined by ELISPOT analysis, only background levels of IFN-γ production were detected in NK cells from RAG-1$^{-/-}$ IFNγ$^{-/-}$ mice (data not shown).

We then reconstituted IL-18$^{-/-}$ mice with these two types of NK cells 1 day before immunization with AChR and CFA. Transfer of 5×10^6 or 1×10^7 NK cells from RAG-1$^{-/-}$ IFNγ$^{-/-}$ mice to IL-18$^{-/-}$ mice had no detectable effects on cytokine production by T cells (data not shown). However, transfer of 5×10^6 or 1×10^7 NK cells from RAG-1$^{-/-}$ mice lead to increased levels of IFN-γ production by MNCs from IL-18$^{-/-}$ mice and the production of anti-AChR IgG2b (**Fig. 6c,d**; data not shown). TGF-β1 production was suppressed, whereas IL-4 production was unaltered by reconstitution of NK cells (**Fig. 6c**; data not shown). IFN-γ producing CD4$^+$ T cells from IL-18$^{-/-}$ mice, as measured by ELISPOT, were also rescued (data not shown). AChR-immunized IL-18$^{-/-}$ mice reconstituted with NK cells derived from RAG-1$^{-/-}$ mice exhibited clinical MG similar to that of wild-type mice (**Table 2**). Taken together, these results suggest that IFN-γ production by activated NK cells can promote T_H1 responses and enhance B cell–mediated autoimmunity.

Discussion

In this study we provide evidence that NK cells, a major arm of innate immunity, participate in the development of MG, a primary B cell– and antibody–mediated autoimmune disease, in mice. The requirement of NK cells was reflected by the failure to mount T_H1 type and anti-AChR responses after NK1.1$^+$ cell depletion in wild-type mice and in NK cell–deficient IL-18$^{-/-}$ mice. NK cells were involved in the initiation of disease, and absence of NK cells resulted in enhanced TGF-β production by AChR-specific T cells. These effects were not observed in mice lacking NKT cells. Collectively, these data reveal a critical link between NK cells and autoreactive T and B cells.

The mechanisms by which NK cells modulate adaptive immune responses are not fully understood. Factors that determine the differentiation of naïve T cells into either T_H1 (IL-2, IFN-γ, TNF-α), or T_H2 (IL-4, IL-10) phenotypes would be expected to have an impact on the development of autoimmune responses. It has been proposed that intracellular bacteria and viruses stimulate NK cells to produce IFN-γ and by this means promote the differentiation of antigen-specific T cells into a T_H1 phenotype[20,21]. Our findings support this notion. However, the production of IFN-γ by NK cells, although important, may not be sufficient for the induction of a full-blown T_H1 response[20] (and as this study shows).

TGF-β is a potent inhibitor of T cell–mediated responses[16,17]. Antigen-specific triggering of T_H3 cells to produce TGF-β confers protection against EAMG induction[24]. TGF-β appears to contribute to the suppression of disease development in the current model. Our data suggest an interplay between NK cells and antigen-specific T and B cells: NK cells promote T_H1 responses in part by early production of IFN-γ, and further by suppression of TGF-β producing T cells. These effects of NK cells influence the antigen-specific antibody response. These observations do not exclude the possibility that other signals from NK cells, or other inhibitory molecules involved in NK cell–T cell interactions, may contribute to down-regulation of T_H1 responses in this model. It has been proposed that an "inhibitory circuit" in which TGF-β produced by NK cells serves as a critical costimulatory signal to induce CD8$^+$ T suppressor cells[25,26]. It is also possible that cytokines produced by NK cells collaborate with cytokines produced by T cells to regulate autoreactive B cell responses. NK cells may also directly interact with B cells[27].

We observed profound effects of NK1.1$^+$ cell depletion on antibody responses. However, in another study it was shown that depletion of NK cells by anti-NK1.1 reduced host defense against malignancy without loss of cellular or humoral immunity[28]. These different results are most likely due to the different status of the NK cells in the experimental systems used. In an earlier study, NK cells did not receive an infectious signal and thus may not have been activated. The frequent association between infections and autoimmune diseases in humans and the use of bacterial adjuvant to induce experimental autoimmune diseases indicates that initiation of autoimmunity often involves NK cell activation. The findings presented here further demonstrate that NK cell activation can result in altered antibody responses, including those directed against autoantigens.

In relation to the effects observed on IgG2b concentrations in NK cell–depleted mice, it has been proposed that both IFN-γ and TGF-β regulate production of IgG2b to T cell dependent antigens[29]. However, TGF-β does not affect IgG2b production in all experimental systems[29,30]. In situations when levels of both IFN-γ and TGF-β are altered, it is unclear which one of these cytokines is most critical for control of IgG2b production. In EAMG, results from several laboratories indicate that anti-AChR IgG2b is preferentially subject to regulation by IFN-γ, in particular in situations where multiple cytokines are altered[11–13]. This pattern is in line with the observations presented here.

Recent studies suggest that susceptibility to T_H1-mediated autoimmune diseases in mice can be attributed, at least in part, to the available numbers of NKT cells[7,31]. It has been suggested that NKT cells confer protection due to their ability to promote T_H2 development[31].

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We also observed that the NKT cell–deficient mice exhibited a tendency toward reduced IL-4 production and a concomitant increase in IFN-γ production. However, the two mouse strains deficient in NKT cells developed MG to a similar extent as wild-type mice, suggesting that this defect is not sufficient to enhance disease. Nevertheless, these findings do not exclude a possible role for NKT cells in the current model.

The current data provide insights into the possible mechanisms of autoimmune pathology. In a tentative scenario, one may envisage an intracellular bacterial or virus infection that activates NK cells. Next, NK cells may promote autoaggresssion via control of autoreactive T and B cells during the initial activation phase of these cells. Decreased NK cell function has been observed in fulminant MG[32], as well as in some other autoimmune diseases[33]. These studies of human subjects may represent only part of the history of autoimmune diseases, essentially events in the established phase. Our study also suggests that an orchestra of NK cells and autoreactive T and B cells contribute to the generation of destructive autoimmunity. Thus a therapy taking into account all these cellular components, and targeting the critical link between them, could be successful in preventing autoimmune diseases.

Methods

Mice. B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The NKT cell–deficient strains $J_\alpha281^{-/-}$ (ref. 14), $CD1d1^{-/-}$ (ref. 15), $IL\text{-}18^{-/-}$ (ref. 19), $RAG\text{-}1^{-/-}$ (ref. 22) and $IFN\text{-}\gamma^{-/-}$ (ref. 23) were all backcrossed to a B6 background. All mice were housed in a specific pathogen-free condition at the animal facilities of the Microbiology and Tumor Biology Center, Karolinska Institutet. Female mice, aged 8–10 weeks at the initiation of the experiments, were used.

Antigens. AChR was purified from the electric organs of *Torpedo californica* (Pacific Biomarine, Venice, CA) by affinity chromatography on a α-cobrotoxin–agarose resin (Sigma)[34]. The isolated product was pure as judged by SDS-PAGE. The purified *Torpedo* AChR was used to induce EAMG and for stimulation *in vitro*. Muscle AChR extract from B6 mice was prepared[34] and used as antigen for detection of antibodies to mouse AChR. MBP used as control antigen was purified from normal mouse brains[35]. AChRα(146–162): LGIWTYDGTKVSISPES[13] was synthesized at the Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

Antibodies. The clone PK136 (anti-NK1.1)[36] was obtained from American Type Culture Collection (Manassas, VA). Mouse IgG2a (Sigma) was used as isotype control antibody. For *in vivo* depletion of $NK1.1^+$ cells, 300 μg monoclonal antibody to NK1.1 was administered by intraperitoneal (i.p.) injection to each mouse 2 days before immunization unless otherwise stated. Every 5–7 days thereafter, 150 μg anti-NK1.1, or control antibodies were injected via the i.p. route until the termination of experiments. Depletion efficacy was checked by flow cytometry with anti-NK1.1 or anti-DX5 (both from PharMingen, La Jolla, CA). In some experiments, the anti–TGF-β 1D11.16 (mouse IgG1) specific for TGF-β1, TGF-β2, and TGF-β3 and 1410KG7 (mouse IgG1) isotype control mAb[37] were injected via the i.p route to neutralize TGF-β. In these experiments, mice were treated with 1 mg anti–TGF-β or control mAb at the time of immunization, followed by 500 μg weekly administration until the termination of the experiments.

Induction of EAMG and measurement of muscle AChR content. Mice were immunized subcutaneously with 20 μg AChR in CFA in a total volume of 100 μl, and at days 30 and 60 after immunization boosted twice with 20 μg of AChR in CFA with subcutaneous injection. The mice were scored every other day after the second immunization for signs of muscle weakness characteristic of EAMG. The disease symptoms were graded between 0 and 3[10]: 0, no definite muscle weakness; 1, normal strength at rest, but weak with chin on the floor and inability to raise the head after exercise consisting of 20 consecutive paw grips; 2, as grade 1, and weakness at rest; 3, moribund, dehydrated and paralyzed. Clinical EAMG was confirmed by injection of neostigmine bromide and atropine sulphate[10]. For measurement of muscle AChR content, two pM aliquots of ^{125}I–α-bungarotoxin– (Amersham) labeled Triton X-100–solubilized mouse muscle extract was mixed with standard pooled mouse AChR antiserum in triplicate. After incubation, rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) was added. The precipitates were counted in a γ-counter (Packard Instrument Co., Meriden, CT)[34].

Culture medium. Cells were suspended in Dulbecco's modification of Eagle medium (Gibco, Paisley, UK) supplemented with 1% (v/v) minimum essential medium (Gibco), 2 mM glutamine (Flow Lab., Irvine, UK), 50 IU/ml penicillin and 50 mg/ml streptomycin and 10% (v/v) fetal calf serum (both from Gibco). Supernatants to be assayed for TGF-β1 content were generated in Aim V serum-free medium (Life Technologies, Grand Island, NY).

Cytotoxicity assay. NK cell–mediated cytotoxicity was measured using a standard ^{51}Cr-release assay[38]. Spleen cells were incubated with ^{51}Cr-labeled YAC-1 target cells at the indicated effector/target ratios. After 4 h of culture, supernatants were counted for ^{51}Cr release in a γ-counter (Packard Instrument Co.).

Cell isolation, sorting and transfer. MNC were obtained by mincing the popliteal and inguinal lymph nodes through a wire mesh. Spleen NK cells were sorted using a FACStar^{plus} (Becton Dickinson, Mountain View, CA). The sorted cells were >99% pure upon reanalysis by flow cytometry. After overnight culture, the sorted NK cells were injected via the intravenous route into recipient mice.

T cell proliferation and cytokine induction. 4×10^5 MNC were incubated in 200 μl culture medium in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). 10 μl aliquots of either AChR, AChRα(146–162), MBP or Con A (both from Sigma) were added into wells at final concentrations of 10 μg/ml (AChR, peptide or MBP) or 5 μg/ml (LPS and Con A). After 4 days of incubation, the cells were pulsed for 18 h with 10 μl aliquots containing 1 μCi of [^{3}H]methylthymidine (specific activity 42 Ci/mmol; Amersham, Arlington Heights, IL). Cells were collected onto glass fiber filters and thymidine incorporation was measured. For cytokine induction, supernatants were collected at 48 h after *in vitro* boosting. IFN-γ and IL-4 were measured by optEIA kits (PharMingen). TGF-β1 was measured with an ELISA kit (Promega). The sensitivities of these ELISA assays were 31.3 pg/ml for IFN-γ, 7.8 pg/ml for IL-4 and 30 pg/ml for TGF-β1.

Cytokine ELISPOT. An ELISPOT assay was used to detect cytokine secretion at the single cell level[33]. Plastic plates (Dynatech, Chantilly, VA) were coated with 100 μl IFN-γ capture antibody (Innogenetics, Genth, Belgium) at 15 μg/ml. 10^5 sorted $NK1.1^+$ cells were cultured for 48 h. Secreted and bound IFN-γ were visualized by application of biotinylated detector antibody (Innogenetics), and ABC (Dakopatts). Similarly, IFN-γ and TGF-β1 cytokine–producing $CD4^+$ lymph node cells were detected as described previously[39].

Assays of antibodies to AChR IgG. An ELISPOT assay was used for enumerating anti-AChR IgG–secreting cells[13,24]. Anti-AChR were measured by radioimmunoassay[34]. Isotypes of anti-AChR IgG were detected by ELISA using rabbit anti-mouse IgG1, IgG2a, or IgG2b (Dakopatts) as described[24].

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

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