Mice Lacking IL-12 Develop Polarized Th1 Cells During Viral Infection

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Studies in IL-12-deficient mice established the necessity for IL-12 to generate a Th1 cytokine response that is often required for elimination of intracellular pathogens. In this study, we demonstrate that mice with a targeted disruption of the IL-12p40 and/or p35 gene effectively control liver damage induced by mouse hepatitis virus (MHV) infection, similar to wild-type animals. In contrast, MHV-infected IFN-γ receptor-deficient (IFN-γR^{−/−}) mice showed an increased susceptibility to coronaviral hepatitis. Surprisingly, MHV-infected mice lacking IL-12 produced a polarized Th1-type cytokine response, as evidenced by high IFN-γ and nondetectable IL-4 production by CD4^{+} splenocytes and normal virus-specific serum IgG2a/IgG1 ratios. The virus-induced type 1 cytokine secretion pattern was not reversed in IL-12-deficient mice by in vivo neutralization of IFN-γ nor in IFN-γR^{−/−} mice receiving IL-12-neutralizing Abs. In IL-12-deficient mice, Th1-type responses were also generated upon immunization with inactivated MHV. In contrast, following immunization with keyhole limpet hemocyanin, mice lacking IL-12 mounted strongly reduced specific IgG2a and increased IgE responses, indicative of a type 2-dominated cytokine pattern. These findings demonstrate that following a virus infection, IL-12 is not essential for the generation of polarized T cell type 1 cytokine expression and associated immune responses, which is in marked contrast to nonviral systems. Our data suggest that viruses may selectively induce IFN-γ production and Th1-type immune reactions even in the absence of IL-12. *The Journal of Immunology,* **1998, 160: 3958–3964.**

Resistance or susceptibility to infectious diseases is strongly determined by the pattern of cytokines produced in response to the infection (1). The proinflammatory cytokine IL-12 is defined as an obligatory inducer of IFN-γ production (2), an important lymphokine in antiviral defenses (for review, see Ref. 3). In a model of acute progressive viral infection, we have demonstrated recently that mice with a defective IFN-γ receptor (IFN-γR^{−/−}) gene, generated by targeted disruption of the IFN-γR α-chain, develop an exacerbated hepatitis after infection with mouse hepatitis coronavirus (MHV)[2] (4). In comparison with wild-type 129/Sv/Ev mice that resolved the infection, their livers had an increased viral load, and most animals succumbed to the infection within 5 to 10 days. Notably, infected IFN-γR^{−/−} mice produced normal IFN-γ levels, but less IL-12 than MHV-infected wild-type mice, as evidenced from marginal levels of IL-12 gene expression (4, 5). This also has been observed after *Mycobacterium* infection (6) and demonstrates that in vivo IL-12 expression can be facilitated by endogenous IFN-γ. Reciprocally, endogenous IL-12 is required for IFN-γ production and for the establishment of a Th1-type response after infection with *Listeria*, *Leishmania*,

Schistosoma, and *Toxoplasma gondii*, and immunization with keyhole limpet hemocyanin (KLH) or collagen (7–12).

In a variety of viral infections, IL-12 gene expression has been observed within 24 to 48 h (5). Recently, Cousens and coworkers (13) have demonstrated that IFN-α/β produced during viral infection profoundly inhibits IL-12 and associated IFN-γ production. However, Ab-mediated in vivo neutralization of IL-12 before murine cytomegalovirus (MCMV) infection reduced IFN-γ synthesis by NK cells, but not their cytolytic activity (14); late CTL activation and IFN-γ production following lymphocytic choriomeningitis virus infection remained unaffected (14). Interestingly, neutralizing anti-IL-12 Abs increased MCMV burden in NK cell- and in T cell-deficient E26 mice (15), suggesting an IFN-γ-independent antiviral function for endogenous IL-12. To examine the in vivo role of IL-12 in viral hepatitis and especially its significance in the regulation of antiviral cytokine expression, we studied MHV-infected IL-12-deficient 129/Sv/Ev mice, generated by gene targeting of either the p35 gene (IL-12p35^{−/−}) or both the p40 and the p35 gene (IL-12p40/p35^{−/−}). For comparison, wild-type 129/Sv/Ev mice and IFN-γR^{−/−} mice were examined. Our observations indicate that endogenous IL-12 is dispensable for the control of coronavirus-induced acute hepatitis. In addition, IL-12 is not required for the differentiation of naive T cells toward a polarized Th1 phenotype during this infection.

Materials and Methods

Mice

The mutant (129/Sv/Ev) mouse strain deficient in expression of the intact IFN-γ receptor α-chain (IFN-γR^{−/−}), generated by gene targeting in murine embryonic stem cells (16), was kindly provided by Dr. M. Aguet (University of Zurich, Switzerland) and bred at the Central Animal Laboratory Utrecht. The IL-12p35^{−/−} and IL-12p40/35[−/−] mice on a 129/Sv/Ev background were generated by Dr. J. Magram and coworkers (9) and obtained from BRL Fullingdorf (Fullingsdorf, Switzerland). All animals were housed in filter top cages. The animal experiments had been approved by Institutional Animal Welfare Committee.

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[2] Abbreviations used in this paper: MHV, mouse hepatitis coronavirus; β-PL, β-propiolactone; KLH, keyhole limpet hemocyanin; MCMV, murine cytomegalovirus; PFU, plaque-forming unit.

Virus

Stocks of the virulent hepatotropic MHV strain A-59 had been generated originally on Sac$^{(-)}$ cells, and the virus was titrated on L cells. Inactivated MHV was prepared by β-propiolactone (β-PL) treatment in PBS. The inactivated MHV stock contained an antigenic mass equivalent to 10^8 plaque-forming units (PFU)/ml and contained no detectable IFN-α/β activity when examined in a virus inhibition assay against vesicular stomatitis virus on murine L cells (not shown).

In vivo treatment of mice

Six- to twelve-week-old mice were i.p. infected with 10^7 PFU of MHV. At designated time points after infection, the livers and spleens were removed, snap frozen in liquid nitrogen or embedded in paraffin, and analyzed immunohistochemically for viral Ag expression using polyclonal rabbit anti-MHV (K134), as described (4). In survival experiments, groups of MHV-infected mice were monitored daily for symptoms and mortality. Separate groups of mice were immunized either with 250 μl of a 1:1 suspension of KLH (100 μg) in aluminum hydroxide adjuvant, as described (17), or with 1 ml of β-PL-inactivated MHV (equivalent to 10^8 PFU/ml). Both KLH-immunized and virus-infected animals were bled from the retro-orbital plexus at the indicated time points for determination of Ag-specific Ab levels in sera. From other groups of mice, either infected or immunized, spleens were removed at indicated time points, and splenocyte single cell suspensions were tested for cytokine production. For in vivo neutralization of IL-12, mice were i.p. injected with 1 mg of highly purified IgG2a mAb C17.8, kindly provided by Dr. G. Trinchieri (18), at 5 h before and 24 h after infection. Endogenous IFN-γ was neutralized by injection of 5000 neutralizing units DB-1 mAb, provided by Dr. P. Van der Meide (Biomedical Primate Research Center, Rijswijk, The Netherlands) (19), at 2 h before and 24 h after infection. Control mice received isotype-matched monoclonals.

Analysis of lymphokine production

For the analysis of cytokine production, erythrocyte-depleted splenocytes (3×10^6 cells/ml) were cultured in 24-well plates (Nunc, Breda, The Netherlands) in conditioned medium or stimulated with β-PL-inactivated MHV (antigenic mass corresponding to 5×10^6 to 5×10^7 PFU MHV/ml) or Con A (2.5 μg/ml). In selected experiments, splenocytes were separated into CD4$^+$ and CD4$^-$ fractions, or into CD8$^+$ and CD8$^-$ fractions before cytokine production analysis. Briefly, splenocytes were incubated with MACS microbead-conjugated mAbs GK1.5 (anti-CD4) or 53-6.7 (anti-CD8) and then sorted on MiniMACS biomagnetic columns (Miltenyi Biotec, Bergisch Gladbach, Germany), exactly as recommended by the manufacturer. Subsequent FACScan analysis (Becton Dickinson, San Jose, CA) revealed that the purity of the selected lymphocyte subsets exceeded 93 to 96%. No CD4$^+$ or CD8$^+$ cells could be detected in the respective depleted splenocyte fractions. Supernatants of stimulated cultures were harvested at 48 h and stored at -20°C until use. IFN-γ and IL-4 were measured by two-site ELISA (Holland Biotechnology, Leiden, The Netherlands, and PharMingen, San Diego, CA) using standard curves established with known amounts of murine rIFN-γ (kindly provided by Dr. H. Heremans, Leuven, Belgium) and rIL-4 (Genzyme, Cambridge, MA). IL-2 levels were determined by an IL-2-dependent CTLL cell proliferation assay. The proliferative responses were measured by [³H]thymidine incorporation for 16 h after 3 days of culture (20).

Proliferative responses

The proliferative splenocyte responses were determined by [³H]thymidine incorporation. Splenocytes were isolated 4 days after infection with MHV or 6 days after immunization with inactivated MHV, and seeded at a concentration of 2 to 5×10^5 cells in 96-well round-bottom plates in 100 μl volumes per well. After 48-h incubation, 0.4 μCi [³H]thymidine was added per well. The cellular DNA was harvested on glass filters and measured by liquid scintillation counting (LKB-Wallac, Gaithersburg, MD).

Assay for serum Abs

Serum levels of KLH-specific Abs were determined by ELISA, as described (17). MHV-specific Ab isotype levels were determined by an ELISA; 96-well flat-bottom plates were incubated overnight at 4°C with MHV (equivalent to 10^7 PFU/well in NaHCO$_3$ 0.05 M, pH 9.6), washed with tap water, and saturated with 1% BSA (Sigma Chemical, St. Louis, MO) in PBS. Twofold serum dilutions were added and incubated for 1 h at 37°C. After washing with tap water, a 1/3200 dilution of isotype-specific peroxidase-coupled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was added. After incubation for 1 h at 37°C and another wash, the substrate was developed with tetramethylbenzidine for

10 min at room temperature. The reaction was stopped with 2 M sulfuric acid and read at an OD of 450 nm in a Titertek Multiskan MC. The titer was defined as the reciprocal of the highest dilution at which the absorbance was equal to two times the background value.

Statistical analysis

Group means of cytokine responses and Ig isotypes of wild-type and mutant mice were compared by using the independent sample Student's *t* test. To take into account the greater probability of a type I error due to multiple comparisons of the Ab isotypes, the level of significance was preset according to Bonferroni's adaptation at $p < 0.01$ instead of a fixed $p < 0.05$. Statistical significance of differences between data from wild-type mice vs those from mutant animals is indicated by an asterisk in the figures.

Results

Pathogenesis of MHV infection in IL-12-deficient mice

To assess the importance of in vivo IL-12 function for resistance to acute MHV infection, wild-type, IL-12$^{-/-}$, and for comparison IFN-γR$^{-/-}$ mice were inoculated with 10^7 PFU. Since the first MHV-infected IFN-γR$^{-/-}$ mice usually succumb after 5 days (4), livers were removed after 4 days and examined histologically. At this time point, the IFN-γR$^{-/-}$ animals already exhibited advanced clinical symptoms. Immunohistologic examination revealed increased numbers of viral Ag-positive cells and larger histopathologic lesions in the livers of the IFN-γR$^{-/-}$ mice (Fig. 1, *E* and *F*), as compared with wild-type animals (Fig. 1, *A* and *B*) (4). In contrast, no marked difference was noted in the number, size, and distribution per section of histopathologic foci and virus Ag-positive cells in the livers of IL-12p35$^{-/-}$ mice, when compared with wild-type mice (Fig. 1, *C* and *D*). Depending on the experiment, IFN-γR$^{-/-}$ mice suffered 56 to 95% mortality (Fig. 2) (4), while wild-type mice had mortality rates of 16 to 44%. Virus titers in the liver were not significantly increased in IL-12p35$^{-/-}$ mice, when compared with wild-type animals (not shown). Moreover, no difference was noted in survival rates between IL-12-deficient (p35$^{-/-}$ and p40/p35$^{-/-}$) and wild-type mice after infection with 10^7 PFU MHV (Fig. 2). These data establish the contribution of IFN-γ and the redundancy of IL-12 to install in vivo defenses against MHV infection.

MHV-infected IL-12-deficient mice produce normal levels of IFN-γ and fail to produce increased levels of IL-4

The undiminished antiviral resistance of mice lacking IL-12 is rather surprising in view of the exacerbated coronaviral hepatitis in IFN-γR-deficient mice, since the monokine is considered critical for induction of IFN-γ. Experiments using exogenous IL-12 (21), IL-12-neutralizing Abs (7), or mice with disrupted genes coding for IL-12 (9, 10) or STAT4, the IL-12-activated signaling molecule (23), have demonstrated a critical role for IL-12 in IFN-γ induction and Th1 development. Indeed, TCR stimulation of naive spleen cells from IL-12p35$^{-/-}$ mice with mitogen Con A yielded significantly reduced ($p = 0.018$), minimal to nondetectable amounts of IFN-γ (about 5.2 ± 1.2 U/ml), and increased levels of IL-4 ($p = 0.001$) in 48-h culture supernatants, when compared with responses from wild-type splenocytes that produced readily detectable amounts of IFN-γ (21 ± 7 U/ml; mean $\pm$ SEM) and minimal IL-4 (1.5 U/ml or less; mean $\pm$ SEM; Fig. 3*A*).

In other systems studied to date, endogenous IL-12 favored the expansion of IFN-γ-producing Th1 cells and concomitantly inhibited IL-4 production (24). Lack of IL-12 function led to increased IL-4 production. Gene-targeted IL-12-deficient mice, when infected with *Leishmania major* or injected with KLH or collagen, produce a Th2 characteristic cytokine profile (9, 10). To determine whether IL-12 deficiency leads to reduced IFN-γ and increased IL-4 production in virus-infected mice, we isolated spleen cells from IL-12p35$^{-/-}$ and wild-type mice 4 days after MHV infection
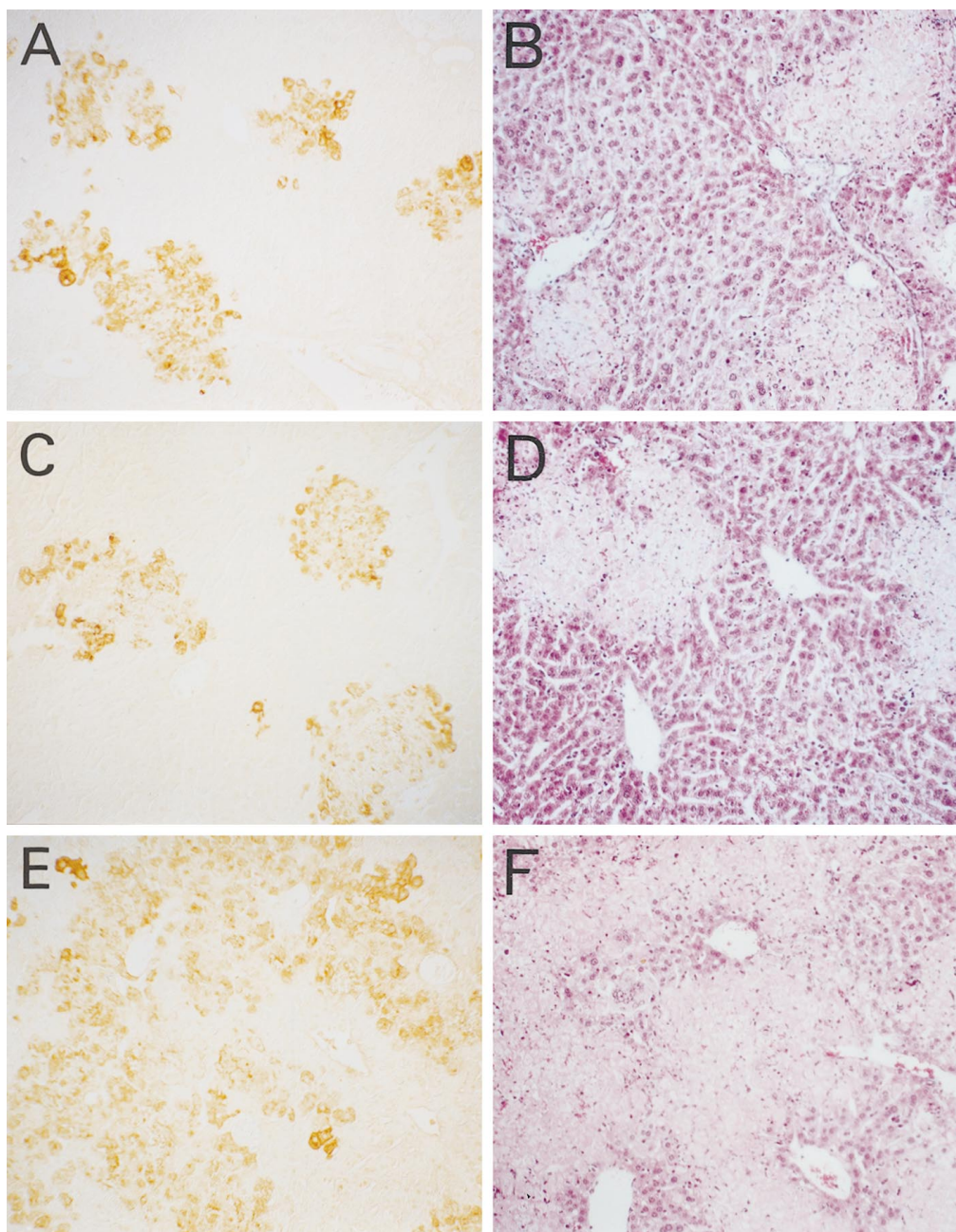
FIGURE 1. Histologic evidence for unaltered viral replication and histopathology in IL-12-deficient mice. Shown are representative sections of livers isolated from 129/Sv/Ev wild-type (*A, B*), IL-12p35$^{-/-}$ (*C, D*), and IFN-γR$^{-/-}$ (*E, F*) mice on day 4 after MHV infection (10^7 PFU/animal), stained with virus-specific Ab k134 (*A, C, E*; cryostat sections) or hematoxylin and eosin (*B, D, F*; paraffin sections). The groups were age and sex matched. Sections from naive animals showed no staining (not shown). Note similar histopathologic features and viral Ag expression in IL-12-deficient and normal mice (*A–D*), but increased replication and pathology in IFN-$\gamma^{-/-}$ mice (*E, F*). Original magnification, $\times$105.

and cultured them in conditioned medium or restimulated them with either inactivated virus (antigenic mass equivalent to 5×10^6 or 5×10^6 PFU/ml) or Con A mitogen (2.5 μg/ml) for 48 h before harvesting their supernatants. Strikingly, IFN-γ was readily produced by splenocytes derived from MHV-infected IL-12p35$^{-/-}$-deficient mice when stimulated with Con A or cultured in conditioned medium. Restimulation with viral Ag did not further increase IFN-γ production above levels observed in cultures maintained in conditioned medium only (not shown). The minimal, or absence of, increases in cytokine production by addition of viral Ag or Con A most likely result from saturating amounts of viral Ag in the spleens of infected mice. IL-4 production was not detectable in splenocyte cultures derived from MHV-infected IL-

12p35$^{-/-}$-deficient mice when stimulated with Con A or viral Ag nor when maintained in conditioned medium (less than 1.5 U/ml). Similarly, IL-4 levels were very low in cultures from virus-infected wild-type mice ($<$1.5 U/ml). Thus, similar levels of IFN-γ and IL-4 were noted in cultures from virus-infected wild-type and IL-12-deficient mice (Fig. 3*A*). Cytokine levels in supernatants from naive nonstimulated splenocyte cultures were below detection limit. These observations indicate that endogenous IL-12 is not involved in inhibition of the expansion and/or differentiation of Th2 cells during viral hepatitis.

In viral infections, generally a massive Ag nonspecific T cell proliferation ($>$90%) occurs, which has been ascribed to cytokine-mediated (IFN-α/β and IFN-γ) bystander stimulation (22). Since
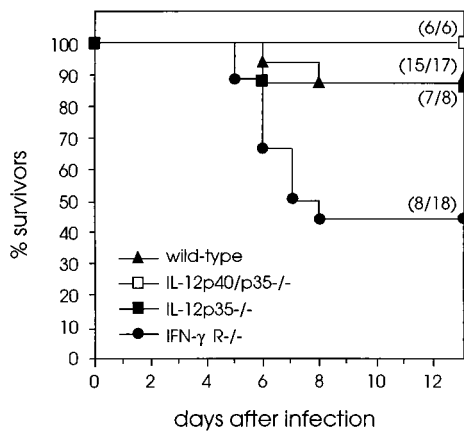
FIGURE 2. Survival of IL-12p35$^{-/-}$, IL-12p40/35$^{-/-}$, IFN-γR$^{-/-}$, and wild-type mice after infection with 10^7 PFU MHV/mouse. Note that the mortality rates in IL-12-deficient mice were similar to those in wild-type controls, while IFN-γR$^{-/-}$ mice typically showed impaired resistance. Between parentheses is indicated the number of surviving/infected animals.

IL-12 augments proliferation of preactivated T cells (2), we looked for alterations in proliferative responses in virus-infected IL-12$^{-/-}$ mice. As a measure of T cell function, we determined proliferative responses of IL-12$^{-/-}$ and normal splenocytes. Coronaviral infec-

tion-driven proliferative responses of splenocytes isolated 4 days after infection were normal in IL-12-deficient mice (not shown). This may be explained by the normal levels of IL-2 synthesis observed for splenic cells from both genotypes (not shown), an observation in line with other antigenic systems (9).

IL-12-deficient mice primed with replicating or inactivated MHV generate CD4$^+$ Th1 cells

To test whether viral replication is necessary for the induction of IFN-γ production, we immunized normal and mutant mice with β-PL-inactivated MHV. To our surprise, splenocytes derived from IL-12-deficient mice injected with noninfectious MHV particles also gave a type 1 characteristic cytokine secretion pattern, with high IFN-γ and low IL-4 secretion (Fig. 3A). This suggests that IL-12-independent IFN-γ production with low IL-4 synthesis is based on an intrinsic biochemical property of the virus particles, independent from their replication.

To investigate whether the virus-induced polarization toward a type 1 cytokine profile in IL-12-deficient mice is only transient and possibly reversible at later time points, we analyzed the cytokine expression pattern of IL-12p40/p35$^{-/-}$ splenocytes at 14 days after infection; again a sustained polarized type 1 pattern, with substantial IFN-γ and nonincreased levels of IL-4, was observed in IL-12-deficient mice, similar to the profile in the wild-type controls (Fig. 3B).
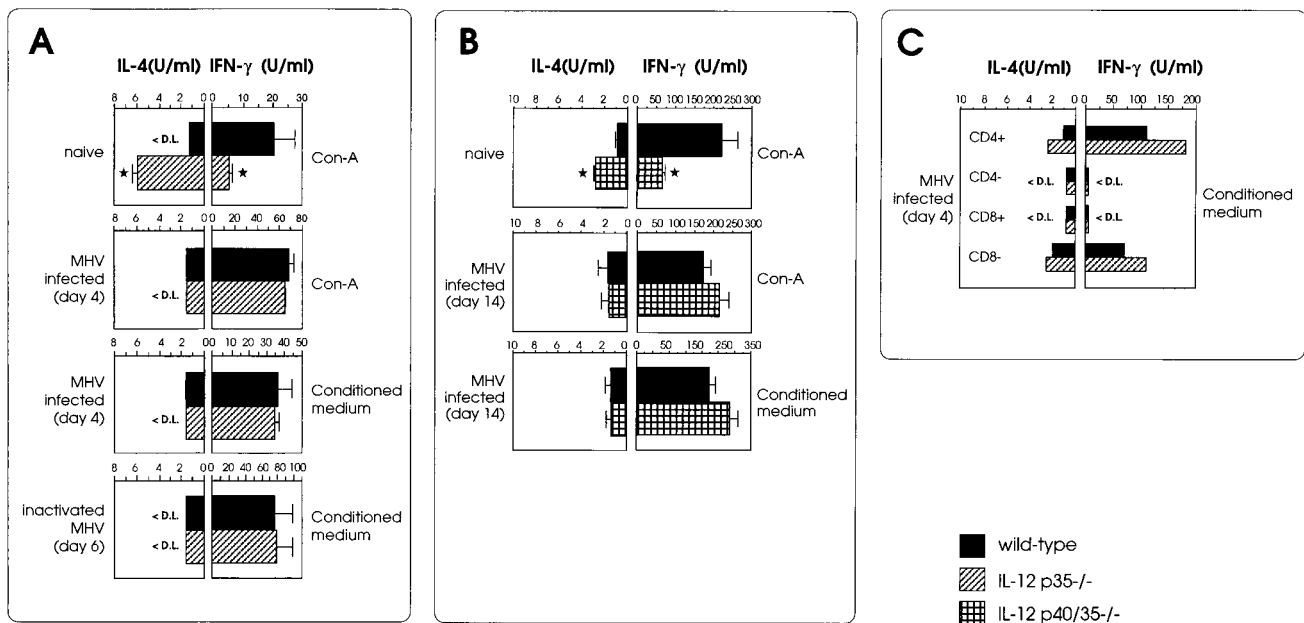

FIGURE 3. Unimpaired type 1 cytokine responses in IL-12-deficient mice following viral infection or priming with inactivated noninfectious virus. A and B, Splenocytes were isolated from uninfected (naive) mice, 4- or 14-day MHV-infected (10^7 PFU/animal) wild-type animals (dark bars), or IL-12-deficient mice (hatched and crossed bars), and after erythrocyte depletion (NH$_4$ Cl buffer) cultured in 24-well plates (Nunc) at a concentration of 3 $\times$ 10^6 cells/ml. Other splenocyte cultures (5 $\times$ 10^6 cells/ml) were generated from normal and IL-12p35-deficient mice 6 days after i.p. priming with 1 ml of β-PL-inactivated MHV in saline (equals 10^8 inactivated infectious particles/ml). The cells were either stimulated with the mitogen Con A or cultured in conditioned medium without stimulation or stimulated with inactivated MHV, which gave no higher levels of cytokines than the conditioned medium cultures (not shown). The levels of cytokines in 48-h supernatants were evaluated by ELISA (4) and standardized against recombinant IL-4 and IFN-γ. The limits of detection for IL-4 and IFN-γ were 0.5 to 1.5 U/ml and 4.5 U/ml, respectively. Statistical significance of differences between data from wild-type and mutant mice is indicated by an asterisk. Data are expressed as means $\pm$ SEM. C, Cytokine profiles of CD4$^+$ and CD8$^+$ splenic T cells from MHV-infected wild-type and IL-12-deficient mice. Splenocytes were isolated 4 days after infection and cultured as described in the legend of A and B. Positive selection of CD4$^+$ and CD8$^+$ cells was performed by immunomagnetic selection using microbead-conjugated mAbs GK1.5 (specific for CD4) and 53-6.7 (specific for CD8), followed by exposure of the cells to a magnetic field in a column and evaluation by flow cytometry. CD4$^+$ ($>$93% pure), CD4$^-$ (no CD4$^+$ cells detectable), CD8$^+$ ($>$96% pure), and CD8$^-$ (no CD4$^+$ cells detectable) splenocytes were cultured in conditioned medium at a concentration of 3 $\times$ 10^6 for 48 h. Cytokine levels were determined as described in the legend of A and B.
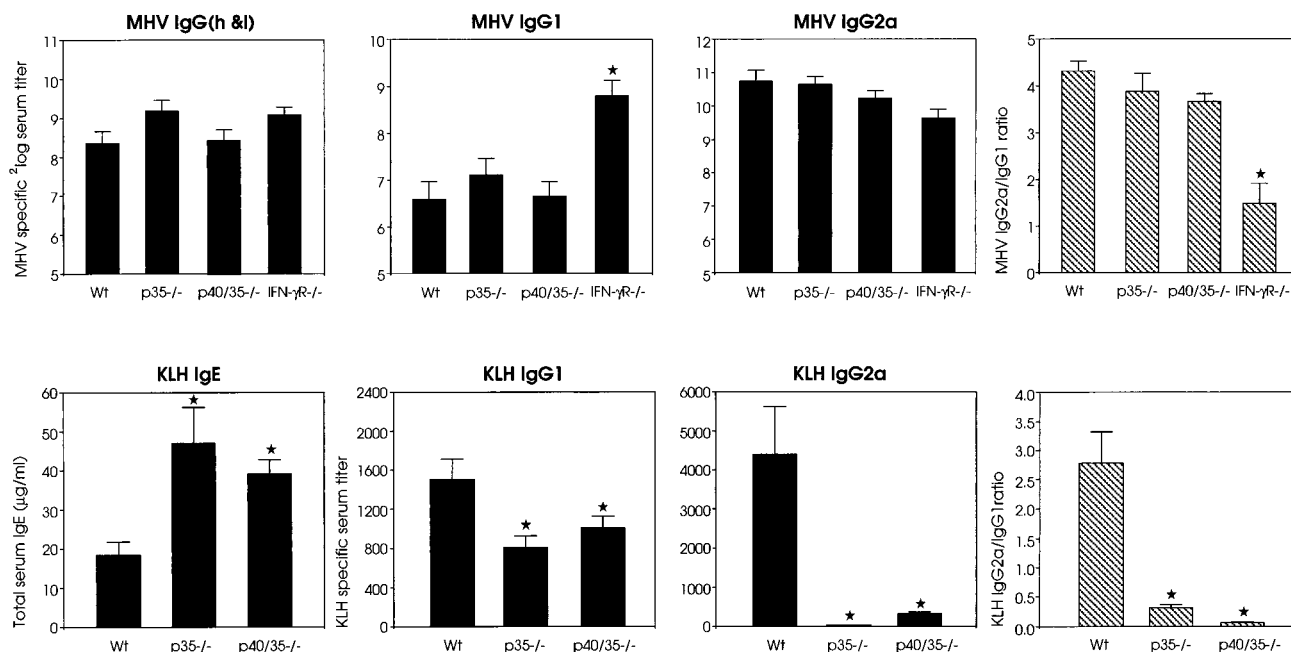
FIGURE 4. Serum levels of Ig subclasses specific for MHV (*top panels*) and KLH (*bottom panels*) in IL-12-deficient, IFN-γR$^{-/-}$, and wild-type mice 14 days after infection with MHV and 7 days after the second priming with KLH. Groups of 6 to 18 mice of the indicated genotype were infected with MHV (10^7 PFU/animal), as described in Figure 2. Infection-surviving animals were bled from the retro-orbital plexus on day 14, and their individual sera analyzed for virus-specific total IgG heavy and light (h & l) chains, IgG1, and IgG2a by ELISA using twofold serial dilutions of sera on 96-well plates coated with Ag equivalent with 10^6 PFU/100 μl/well. Other groups of wild-type and IL-12-deficient mice were immunized three times in biweekly intervals with KLH absorbed to aluminum hydroxide (17). Their sera were analyzed for KLH-specific IgE, IgG1, and IgG2a (17). Ab titers are expressed as means $\pm$ SEM. Statistical significance of differences between values from wild-type mice vs data from mutant mice is indicated by an asterisk.

Depletion of both CD4 and CD8 splenocytes isolated at day 4 after infection, using microbeads, almost completely abolished IFN-γ production (not shown), indicating that only α/β TCR-positive T cells are responsible for IFN-γ synthesis. Immunomagnetic selection of CD4$^+$ and CD8$^+$ cells before seeding in conditioned medium identified CD4$^+$ cells as the major source of IFN-γ-producing cells (Fig. 3C). Together, these data indicate that during coronaviral infection, CD4$^+$ T cells develop into Th1 type cells, despite the absence of endogenous IL-12 function.

Virus-infected mice lacking IL-12 produce an IgG isotype profile characteristic for a type 1 cytokine response

In murine systems, the levels of Ag-specific IgG1 and IgG2a are normally correlated with IL-4 and IFN-γ production, respectively (25). MHV-infected IL-12$^{-/-}$ mice produced the same levels of virus-specific serum IgG2a and IgG1 as wild-type mice (Fig. 4, *upper panels*). Only IFN-γR$^{-/-}$ mice that survived the infection showed a reduced serum IgG2a/IgG1 ratio, indicative for defective IFN-γ function (16, 19). In contrast, IL-12-deficient mice, immunized with KLH (absorbed to aluminum hydroxide), showed a reduced KLH-specific serum IgG2a/IgG1 ratio and increased IgE responses (Fig. 4, *lower panels*), indicative of a type 2-dominated cytokine pattern.

Neither IFN-γ neutralization in IL-12-deficient mice nor IL-12 neutralization in IFN-γR$^{-/-}$ mice prevents the generation of Th1 cells after MHV infection

Having established that a polarized type 1 cytokine expression profile, with a high IFN-γ/IL-4 ratio, after MHV infection prevails in gene-targeted IL-12-deficient and in IFN-γR-deficient mice (4), we next determined whether the absence of both IL-12 and IFN-γ would affect the antiviral cytokine response. We therefore depleted

IFN-γR$^{-/-}$ mice of endogenous IL-12 using mAb C17.8 (1 mg/animal injected 5 h before and 24 h after MHV infection), before assessing splenic IFN-γ and IL-4 production. In vivo neutralization of IL-12 in MHV-infected IFN-γR$^{-/-}$ mice had no apparent effect on the virus-induced IFN-γ production and failed to promote IL-4 synthesis (Fig. 5). The type 1 pattern of cytokine responses was also not affected in IL-12p35$^{-/-}$ mice treated with the IFN-γ-neutralizing mAb DB-1 (Fig. 5). The efficacy of DB-1 was suggested by increased liver pathology in most Ab-treated IL-12p35$^{-/-}$ animals.

Thus, these data suggest that both IL-12 and IFN-γ are not essential for developing a Th1 cytokine profile during coronaviral infection.

Discussion

The typical Th2-biased cytokine profile normally observed in IL-12-deficient mice does not develop during MHV hepatitis, as we show in this study; their immune responses unexpectedly tend toward Th1 responsiveness. IL-12 function also appears unnecessary for the induction of a polarized Th1-type response to inactivated coronavirus, suggesting that viral replication is not required. Similarly, after infection with attenuated pseudorabies virus, an HSV-1-related α-herpesvirus, we measured substantial production of IFN-γ, but no IL-4 in both normal and IL-12-deficient mice (Schijns et al., unpublished observations). Our observation of an IL-12 redundance for IFN-γ production supports Orange and coworkers (14), who noted unimpaired late IFN-γ production in mice injected with IL-12-neutralizing Abs after infection with lymphocytic choriomeningitis virus and MCMV. However, the neutralizing anti-IL-12 Abs reduced early IFN-γ production by NK cells after MCMV infection, resulting in diminished antiviral defense (14).
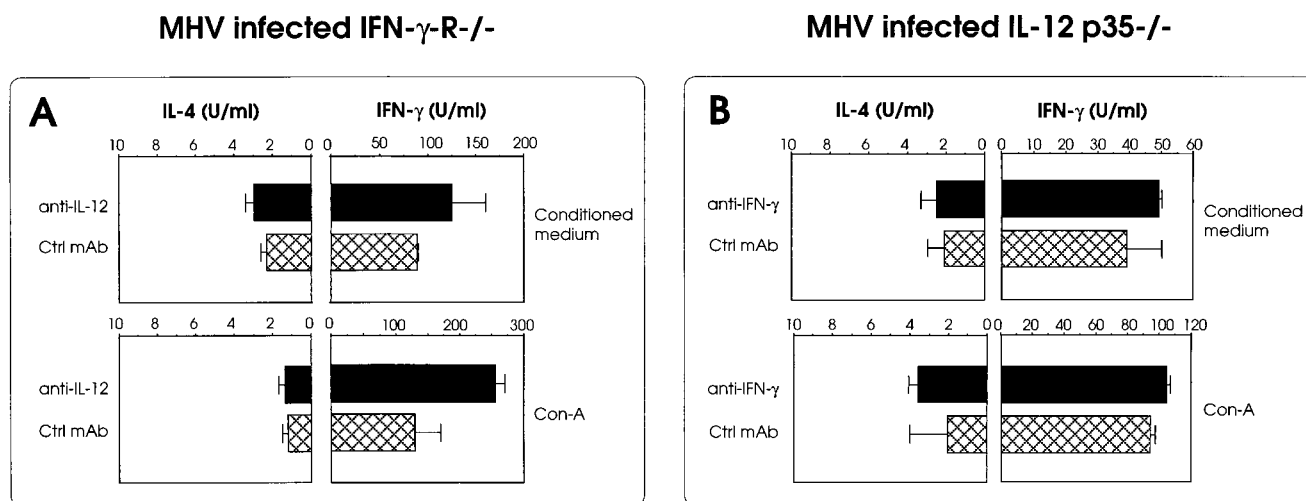
FIGURE 5. Splenic cytokine responses following MHV infection after in vivo neutralization of IL-12 in IFN-γR$^{-/-}$ mice and IFN-γ depletion in IL-12-deficient animals. *A*, Splenocytes were isolated 4 days after infection with 10^7 PFU MHV from IFN-γ$^{-/-}$ mice treated with 1 mg anti-IL-12 mAb C17.8 injected 5 h before and 24 h after infection. Control animals received 1 mg of irrelevant IgG2a. *B*, Splenocytes were isolated 4 days after with 10^7 PFU MHV from IL-12p35$^{-/-}$ mice treated with 5000 neutralizing units of anti-IFN-γ mAb DB-1 (4) injected 2 h before and 24 h after infection. Control animals received 1 mg of irrelevant control mAb. A quantity amounting to 3×10^6 cells was cultured in 1 ml conditioned medium for 48 h, and the levels of IFN-γ and IL-4 were assessed by ELISA, as described in the legend of Figure 3. Data are expressed as mean ± SEM.

Our observations that neither depletion of IFN-γ in IL-12$^{-/-}$ mice nor neutralization of IL-12 in IFN-γR$^{-/-}$ mice affects the virus-elicited Th1 cytokine secretion pattern suggest that other cytokines or host factors compensate the lack of endogenous IL-12 and IFN-γ. IFN-α, a characteristic product in most viral infections, has been suggested to favor the development of Th1 cells (recently reviewed in Ref. 26). Interestingly, mice deficient for IFN-regulatory factor-1, an activator for type I IFNs and IFN-inducible genes, exhibit strongly impaired Th1 responses (27). In contrast, IFN-α/β has been shown to inhibit endogenous IL-12 synthesis and associated IFN-γ production (13). Moreover, IFN-α is unable to induce Th1 development in a TCR-transgenic system (28). Alternatively, the recently discovered IFN-γ-inducing factor is a strong IL-12-independent inducer of IFN-γ and Th1 cell development (29). It is induced in IL-12-deficient mice infected with *Mycobacterium tuberculosis* (30), but has not yet been examined during viral infections.

IFN-γ responses, and most likely Th1-type responses, dominate most viral infections, also in genetically susceptible BALB/c mice (see Refs. 31–33), which are predisposed toward developing Th2-type responses following various parasitic infections (reviewed in Ref. 1). Even after virus infections at mucosal surfaces, known to favor a type 2 cytokine profile (34), IFN-γ production prevails (33); additional low levels of IL-4 may be induced, e.g., following mucosal influenza or respiratory syncytial virus infections (35, 36). Virus-induced IL-4 production in the absence of IL-12 has not been investigated before. Our data show that increased IL-4 responses, which are normally observed in IL-12$^{-/-}$ mice in various antigenic systems, are suppressed or absent after infection of IL-12$^{-/-}$ mice with a hepatotropic coronavirus (an RNA virus) or a neurotropic pseudorabies virus (a DNA virus).

Antiviral Ab responses generally show an isotypic bias toward IgG2a (37, 38), a major complement-fixing and FcR-binding Ig associated with IFN-γ production (16, 19), which is in line with the predominance of Th1-type responsiveness. In this study, we demonstrated that even in the absence of physiologic IL-12, antiviral IgG2a (and IgG1) responses were not altered, conforming to the observation that no shift in cytokine response had occurred.

IL-12 gene deficiency dramatically increases the susceptibility to *Leishmania major* infection (10). In contrast, this defect did not alter the pathogenesis of MHV hepatitis, an unexpected finding in view of the increased susceptibility of IFN-γR$^{-/-}$ mice to MHV infection (4). IFN-γR$^{-/-}$ mice resemble IL-12$^{-/-}$ with respect to antiviral cytokine responses. Similar to IL-12-deficient mice, they did not produce IL-12p40 serum protein (unpublished data), and their splenocytes were defective in IL-12p40 mRNA expression (4). Moreover, their levels of IL-4 were not increased and their levels of IFN-γ normal. The latter is nonfunctional due to the genetic lack of IFN-γ receptor signaling, which results in increased susceptibility and reduced IgG2a synthesis (4). The exact antiviral effector mechanism of physiologic IFN-γ, however, remains to be established. rIFN-γ exerted a pronounced in vitro and in vivo antiviral activity against MHV, suggesting induction of an antiviral state in the target cells (4). However, an IFN-γ-induced immunoregulatory activity contributing to viral resistance cannot be excluded. In transfer studies, a pivotal role in protection against coronavirus-induced hepatitis has been demonstrated for CD4$^+$, but not for CD8$^+$ class I-restricted T cells (39). These antiviral Th cells exerted virus-specific MHC class II-restricted cytotoxicity and IFN-γ secretion, but not IL-4 synthesis (31).

Collectively, our data suggest that the unaltered resistance of IL-12-deficient mice is due to an unimpaired IFN-γ response, most likely derived from activated CD4$^+$ T cells regulating subsequent antiviral responses. Endogenous IL-12 is not required for antiviral defense in a coronaviral hepatitis, and most likely for viral infections in general. In addition, the data establish the development of antiviral Th1 cells during viral hepatitis in the absence of IL-12 and/or IFN-γ. Identification of the mechanisms and viral components directing immunity toward type 1 cytokine responsiveness would be of interest for prophylactic or therapeutic intervention aiming either at the induction of cell-mediated immunity or immune deviation away from type 2-dominated cytokine responses.

Acknowledgments

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