Production of protective gamma interferon by natural killer cells during early mouse hepatitis virus infection

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Gamma interferon (IFN-γ) plays a major role in the protection against lethal infection with mouse hepatitis virus A59. IFN-γ production reaches a maximum level 2 days after viral inoculation, especially in liver immune cells. Among these cells, natural killer cells are the major producers of this cytokine. Transfer experiments indicated that the protective role of IFN-γ is mediated through a direct effect on cells targeted by the virus rather than through indirect activation of T lymphocytes.

Mouse hepatitis virus (MHV) strain A59 (MHV-A59) is a murine coronavirus, both hepatotropic and neurotropic (reviewed by Godfraind & Coutelier, 1998; Barthold & Smith, 2007). The protective anti-MHV immune response involves a wide range of cell populations and molecules. Both CD4⁺ and CD8⁺ cells are required for the survival of mice after MHV infection (Sutherland et al., 1997; Williamson & Stohlman, 1990). With MHV strains like JHM and MHV3, CD8⁺ cells seem more protective than CD4⁺ against hepatitis (Kyuwa et al., 1996; Lamontagne et al., 2001), whereas virus-specific cytolytic CD4⁺ cells may control MHV-A59 (Wijburg et al., 1996). On the other hand, natural killer (NK) cells prevent MHV-A59 replication and decrease the associated hepatitis (Bukowski et al., 1983). Similarly, fulminant hepatitis induced by MHV3 correlates with NK cell impairment (Lehoux et al., 2004). Moreover, protection against neurological infection with MHV-A59 results from local recruitment and activation of NK cells (Trifillo et al., 2004).

Gamma interferon (IFN-γ) is required for the protection against lethal infection with MHV-JHM and -A59 (Kyuwa et al., 1998, 2002; Schijns et al., 1996, 1998; Smith et al., 1991). Both viral replication and hepatocellular injury are increased after infection of IFN-γ receptor-deficient mice with MHV-A59 (Schijns et al., 1996). This cytokine is also critical for the protection of the central nervous system (Trifillo et al., 2004).

Both T lymphocytes and NK cells produce IFN-γ in the course of viral infections. The cellular origin of MHV-induced IFN-γ has not been extensively analysed, although a few studies have indicated that either T cells (Bergmann et al., 2004; Pearce et al., 1994) or NK cells (Daniels et al., 1996) might produce this cytokine. Moreover, control of MHV pathogenicity can be mediated by an IFN-γ effect on either T cells (Wijburg et al., 1996) or macrophages (Lucchiari et al., 1991). The purpose of this work was to investigate further the cellular origin and protective action of IFN-γ after infection with MHV-A59.

129/Sv mice bred at the Ludwig Institute for Cancer Research (Brussels) by G. Warnier were infected when they were 8–12 weeks old by intraperitoneal injection of approximately 10⁴ TCID₅₀ of MHV-A59 grown in NCTC 1469 cells. Bleeding was performed under diethyl ether anaesthesia. Spontaneous IFN-γ production was assessed by ELISA in the serum at different times after infection, using a commercial kit (CytoSet). As shown in Fig. 1(a), IFN-γ production was observed in C57BL/6 mice (Fig. 1b; P = 0.0159 and 0.0317 for data at 2 and 4 days, respectively, versus controls).

MHV-induced early IFN-γ response was confirmed by RT-PCR analysis, using the spleen of infected mice. An early expression of IFN-γ message, analysed as described previously (El Azami El Idrissi et al., 1998), was found in 129/Sv mice, with the strongest message at 2 days after infection (Fig. 1c).

IFN-γ-producing cells were analysed by flow cytometry. Liver immune cells, dispersed through a CCellector Complete (Bellco) were isolated by two successive centrifugations on 40% Percoll (Amersham Biosciences) containing 100 U heparin (Leo Pharma) ml⁻¹. After lysis of erythrocytes in ACK lysis buffer (0.15 M NaCl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.3), cells were labelled with a mouse IFN-γ secretion assay detection kit (Miltenyi...
Biotec) and analysed as described previously (Markine-Goriaynoff et al., 2002).

A strong increase in the proportion of liver cells secreting this cytokine was found, in two independent experiments, in infected 129/Sv mice (Fig. 1d). It reached a maximum level 2 days after MHV-A59 inoculation, then quickly decreased. An increase in IFN-γ-producing cells was also found in the liver of MHV-infected C57BL/6 mice (data not shown).

The protective effect of IFN-γ against MHV-A59 was then investigated by using IFN-γ receptor-deficient mice (G129 or IFNGR−/−) (Huang et al., 1993), received by courtesy of Dr Brombacher (Max Planck Institute for Immunobiology, Freiburg, Germany). As reported previously, IFN-γ was required for the survival of mice to MHV-A59 infection. Indeed, while immunocompetent 129/Sv mice survived MHV-A59 inoculation most animals deficient for the IFN-γ receptor quickly died (Fig. 2a). This observation made in two independent experiments, confirms reports by others, which also showed increased viral replication and hepatocellular lesions in these immunodeficient animals (Schijns et al., 1996).

Immunosuppression by 700 rads whole body irradiation strongly increased the sensitivity of 129/Sv animals to MHV infection (Fig. 2b). Full protection was restored by adoptive transfer of 30 \( \times 10^6 \) spleen cells from naive immunocompetent animals (Fig. 2b). This result confirmed that irradiation-sensitive cells, such as lymphocytes were required for the protection against MHV-A59 lethality.

We next analysed whether such an irradiation, which suppresses T lymphocytes, but preserves at least some NK cell functions (Kiessling et al., 1977; Lotzova et al., 1983), might prevent the protective IFN-γ response induced by MHV infection. As shown in Fig. 2(c), irradiation of 129/Sv mice before MHV-A59 infection did not decrease their ability to produce IFN-γ (P=0.200).

Protection by IFN-γ might therefore be due either to a direct effect on cells infected by the virus or to T-lymphocyte stimulation. To discriminate between these hypotheses, 129/Sv (IFNGR+/+) and G129 (IFNGR−/−) mice immunosuppressed by 700 rads whole body irradiation were reconstituted by intravenous injection of 30 \( \times 10^6 \) spleen cells freshly obtained from non-irradiated mice. Reconstitution of irradiated IFNGR+/+ mice with spleen cells from either IFNGR+/+ or IFNGR−/− animals conferred protection against MHV-A59, showing that protective cells did not need stimulation by IFN-γ (Fig. 2d). In contrast, most irradiated IFNGR−/− animals succumbed to MHV-A59, irrespective of the origin of the spleen cells that were transferred, indicating that IFN-γ-responding T cells were not sufficient to confer protection (Fig. 2d). A similar effect was found in two independent experiments.

The cellular origin of this protective IFN-γ was analysed ex vivo by flow cytometry on liver cells freshly obtained 2 days after MHV-A59 infection. In addition to IFN-γ labelling, cells were marked with either a fluorescein-labelled anti-mouse CD49b (DX5) antibody (ref. 553857; BD Biosciences) or a fluorescein-labelled anti-NK1.1 antibody (ref. 553164; BD Biosciences) and a biotinylated anti-mouse TCRβ antibody (ref. 553169; BD Biosciences), followed by streptavidin-peridinin chlorophyll-a protein (Streptavidin-PerCP; BD Biosciences).

In 129/Sv mice, NK cells, defined by CD49b expression represented about a sixth of total immune liver cells, and T cells...
lymphocytes around 50% (Fig. 3a). In contrast, a large majority (72.8%) of IFN-γ-producing cells were NK cells, while T lymphocytes represented only 26% of them (Fig. 3a). Similar results were obtained in two independent experiments.

NK/T cells are major cytokine-producing cells in mice acutely infected with various pathogens and could therefore be involved in MHV-induced IFN-γ production. This cell population has been defined by the expression of the NK1.1 marker that is present in C57BL/6, but not in 129/Sv mice, whereas CD49b has been demonstrated as a poor marker for NK/T cells (Pellicci et al., 2005). Since IFN-γ production kinetics were similar in 129/Sv and C57BL/6 mice, we analysed the putative role of NK/T cells in C57BL/6 animals. In these mice, 10–15% of liver

![Fig. 2. Mechanisms of IFN-γ protection against MHV-A59. (a) Survival after MHV-A59 infection of 10 129/Sv (IFNGR+/+) or G129 (IFNGR−/−) mice. (b) Survival after MHV-A59 infection of 9–10 control animals or irradiated 129/Sv mice reconstituted with 30×10^6 spleen cells or untreated. (c) Serum IFN-γ 2 days after MHV-A59 infection of four control or irradiated 129/Sv mice (irradiation 1 day before infection). Results are mean ± SEM. (d) Survival of eight irradiated 129/Sv (IFNGR+/+, circles) or G129 (IFNGR−/−, squares) mice, reconstituted with 30×10^6 129/Sv (open symbols) or G129 (closed symbols) spleen cells 2 days before MHV-A59 infection.]

![Fig. 3. Cellular origin of IFN-γ: (a) Proportion of T and NK cells in total cells and in IFN-γ-producing cells from pools of three 129/Sv mice 2 days after MHV-A59 infection. (b) Proportion of IFN-γ-producing cells in cell populations defined by NK1.1 and TCRb expression. Results are mean ± SEM for three independent groups of two C57BL/6 mice (controls and 2 days after MHV-A59 infection). (c) Proportion of NK cells and IFN-γ-producing cells in the liver of groups of four 129/Sv mice (controls and 2 days after MHV-A59 infection with and without anti-ASGM1 treatment). Results are mean ± SEM. (d) Serum IFN-γ in groups of four 129/Sv mice 2 days after MHV-A59 infection, performed 1 day after anti-CD4, anti-CD8 or anti-ASGM1 treatment. Results are mean ± SEM.]
immune cells were NK cells and 2–5% were NK/T cells (data not shown). The proportion of NK cells secreting IFN-γ after MHV-A59 infection was strongly enhanced when compared with NK cells from control animals (Fig. 3b, \(P=0.0012\)). IFN-γ production by T lymphocytes was also enhanced, but the difference did not reach significance \((P=0.1290)\). In contrast, the infection did not modify the proportion of NK/T cells that produced this cytokine, which was already high in control animals \((P=0.7273)\). Similar results were obtained in three independent experiments.

The preponderant role of NK cells in MHV-A59-induced IFN-γ production was confirmed by their in vivo depletion in 129/Sv mice after treatment with 1.5 mg polyclonal rabbit anti-asialoganglioside-GM1 (ASGM1) immunoglobulins, as described previously (Markine-Goriaynoff et al., 2002). This treatment resulted in a sharp decrease of liver NK cells, identified by flow cytometry with an anti-CD49b antibody, whereas T-cell population was not suppressed (55.7% in pooled cells from treated animals versus 30.7% in untreated mice). The significant \((P=0.0286)\) reduction in NK cells obtained after anti-ASGM1 injection into MHV-infected 129/Sv mice was correlated with a similarly significant \((P=0.0286)\) decrease in IFN-γ-producing cells (Fig. 3c).

Moreover, in vivo treatment of 129/Sv mice with anti-ASGM1 antibody before MHV infection largely suppressed the enhancement of plasma IFN-γ levels induced by the virus (Fig. 3d; extremely significant difference between MHV-infected mice without treatment and with anti-ASGM1, \(P=0.0001\)). Such an effect of NK cell depletion was found in two independent experiments. T lymphocytes were depleted with GK1.5 anti-CD4 (Dialynas et al., 1983) or 53/6.72 anti-CD8 monoclonal antibody, obtained from ATCC (Ledbetter & Herzenberg, 1979). In vivo efficiency of these antibodies has been extensively reported previously (Coulie et al., 1985; Coutelier, 1991; El Azami El Idrissi et al., 1998). This treatment was not followed by modification in IFN-γ levels (Fig. 3d, no significant difference between MHV-infected mice without treatment and with treatment with anti-CD4 and anti-CD8 antibody, \(P=0.4108\) and 0.7690, respectively).

Together, our results indicate that liver NK cells are a major source of IFN-γ after infection with MHV-A59 when the virus is inoculated in a way that leads mostly to hepatitis. Similar results were obtained in different mouse strains, including 129/Sv, C57BL/6 and BALB/c (data not shown). This is in agreement with previous findings of IFN-γ production by NK cells after infection by MHV and by other viruses (Daniels et al., 2001). IFN-γ secretion by NK cells has also been shown after infection with another mouse nidovirus, lactate dehydrogenase-elevating virus (LDV) (Markine-Goriaynoff et al., 2002), although LDV does not induce hepatitis, and its tropism is more restricted than that of MHV. Moreover, the bulk of MHV-induced IFN-γ production depends on NK cells, and not on T lymphocytes, which contrasts with the cytokine production in the central nervous system observed 6 days after infection with the JHM neurotropic strain of the virus (Pearce et al., 1994). Finally, although a significant proportion of NK/T cells produced IFN-γ, this was not enhanced by MHV-A59 infection. Therefore, the cellular origin of IFN-γ induced by MHV-A59 is closer to that reported after murine cytomegalovirus than after lymphocytic choriomeningitis virus infection. This probably explains the high susceptibility to MHV of mice treated with an anti-ASGM1 antibody (Bukowski et al., 1983). Moreover, previous studies have shown that increased susceptibility to MHV of mice depleted from their NK cells or unable to respond to IFN-γ correlated with enhanced virus replication and liver histopathologic lesions (Bukowski et al., 1983; Schijns et al., 1996, 1998).

Although IFN-γ is required for mouse protection against MHV, the production of this cytokine is not sufficient, as demonstrated in mice rendered susceptible by irradiation despite a normal IFN-γ response. Thus, both T-helper lymphocytes (Williamson & Stohlman, 1990; Wijburg et al., 1996) and IFN-γ are needed for an adequate antiviral defence. This protective effect of IFN-γ might be exerted directly on viral replication in target cells or through activation of antiviral T lymphocytes. The former hypothesis is supported by our adoptive transfer experiments, which indicated that the ability of immune cells to restore resistance of irradiated animals to MHV was independent of their responsiveness to IFN-γ. In contrast, IFN-γ receptor-deficient animals could not be protected by a similar transfer of immune cells able to respond to this cytokine. Interestingly, after infection with MHV-JHM, CD8 lymphocytes were found to suppress MHV-JHM replication at least in oligodendroglia through IFN-γ production (Bergmann et al., 2004). This cytokine was also involved in the demyelination induced by this neurotropic virus (Templeton & Perlman, 2008). IFN-γ may thus have different effects, depending on the organ affected by MHV infection and/or on the virus strain.

The protective role of IFN-γ after MHV-A59 infection might be similar to its effect on hepatocyte infection by other viruses such as hepatitis B virus (Wieland et al., 2003). It remains to be determined whether other NK cell functions, like perforin-mediated cytolysis, which are also involved in liver protection against murine cytomegalovirus (Loh et al., 2005), play a role in the defence against MHV as well. Moreover, possible interactions between MHV-triggered IFNs and NK cell activating molecules like Ly49H (Daniels et al., 2001) and NKG2D (Walsh et al., 2008) should be investigated further. Therefore, MHV-A59 infection might provide a useful mouse model for comparing the induction of a protective liver NK-cell response and the mechanisms by which IFN-γ may prevent liver infection induced by different viruses.
Acknowledgements

The authors are indebted to J. Van Snick for critical reading of this manuscript, to M.-D. Gonzales, T. Briet, N. Ouled Haddou, J. Van Broeck and A. Tonon for expert technical assistance. This work was funded by the Fonds National de la Recherche Scientifique (FNRS), Fonds de la Recherche Scientifique Médicale (FRSM), Loterie Nationale, Fonds de Développement Scientifique (UCI), the State-Prime Minister's Office – S.S.T.C. (interuniversity attraction poles, grant no. 44) and the 'Actions de recherche concetrées' from the Communauté française de Belgique – Direction de la Recherche scientifique (concerted actions, grant no. 04/09-318), Belgium. G. T. is a FRIA fellow. J.-P. C. is a research director with the FNRS.

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