Short paper

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Activation of virus-specific major histocompatibility complex class II-restricted CD8+ cytotoxic T cells in CD4-deficient mice

Acute enteritic or respiratory disease is a consequence of coronavirus infection in man and rodents. Mouse hepatitis virus, stain A59 (MHV-A59) causes acute hepatitis in mice and rats and induces a response of major histocompatibility complex (MHC) class II-restricted CD4+ cytotoxic T cells, protecting mice against acute infection. In the present study we show that MHV-A59 infection of mice that lack a functional CD4 gene activates effector cells of the CD8+ phenotype. These cytotoxic T cells lyse virus-infected target cells in a MHC class II-restricted fashion. The results indicate that CD8+ T cells have the potential to utilize MHC class II as restriction element, illustrating that the immune system can effectively deal with evading microorganisms, such as viruses which down-regulate MHC class I.

1 Introduction

A rather strict association exists between the expression of CD4/CD8 accessory molecules and the MHC restriction of mature TcR αβ-expressing T cells [1]. CD8+ T lymphocytes recognize antigen in the context of MHC class I molecules, whereas CD4+ T cells recognize antigen associated with MHC class II molecules. Exceptions of the strict association between expression of the co-receptor and MHC class I or II restriction have been previously described [2–7].

Here we describe another exception of mismatch between co-receptor and MHC restriction. We have analyzed mouse hepatitis virus strain A59 (MHV-A59)-infected CD4-deficient mice for the induction of cytotoxic activity. Earlier studies showed that intraperitoneal infection with MHV-A59 causes acute hepatitis in mice and rats [8], and induces a response of MHC class II-restricted CD4+ cytotoxic T cells (CTL) [9, 10]. Transfer studies using virus-specific CD4+ CTL clones have shown that these cells are able to protect mice against a lethal virus challenge. In order to study the role of CD4 in the protection against MHV-A59, mice that lack a functional CD4 gene ($CD4^{-/-}$ mice) were infected with MHV-A59. The CD4-deficient mice were able to generate cytotoxic T cells that were able to lyse the MHV-A59-infected target cells in a MHC class II-restricted fashion. In contrast to the cells of the wild-type mice (CD4) the cells from the mutant mice were of the CD8 phenotype. Thus, MHV-A59 infection induces the activation of virus-specific MHC class II-restricted CD8+ T cells in CD4-deficient mice. The characteristics and role of these cells in protection against an acute infection is determined and discussed. Furthermore, we discuss the thymic selection of these cells and the possible existence of a subset of class II-restricted CD8+ T cells in normal mice.

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Abbreviation: **MHV:** Mouse hepatitis virus

Key words: CD8+ T cells / Major histocompatibility complex class II-restricted / Mouse hepatitis virus

2 Materials and methods

2.1 Mice

Specific pathogen-free (including seronegative for MHV) mice used in this study were homozygous or heterozygous for the disrupted CD4 gene, and have been previously described [11]. All mice were used between 6 and 10 weeks of age.

2.2 Virus and immunization

The virulent hepatotropic MHV strain A59 and the avirulent temperature-sensitive mutant ts342 of MHV-A59, were propagated on Sac(−) cells and virus stocks were prepared as described [12, 13]. For immunization experiments, mice were injected intraperitoneally with 10^4 PFU ts342 and boosted 10 days later with 5×10^4 PFU wild-type MHV-A59.

2.3 Generation of virus-specific CTL in bulk culture

Spleen cells (1×10^8) from immunized mice were isolated and stimulated in bulk culture with 5×10^7 irradiated (3000 rad) MHV-A59-infected syngeneic spleen cells (MOI of 0.3) in 50 ml IMDM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM glutamine, antibiotics and 2-ME (2×10^{-5} M) for 5 days. To separate $CD4^-CD8^-$ from $CD4^-CD8^+$ effector cells, the cultures of *in vitro* stimulated spleen cells derived from MHV-A59 primed $CD4^{-/-}$ mice, were stained with FITC-conjugated anti-CD8 mAb (53-6.7) followed by cell sorting.

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2.4 Cytotoxicity assay

CTL assays were performed as described [14]. In short, 2.5×10^3 ^{51}Cr-labeled target cells were added to varying numbers of effector cells and incubated for 5–6 h at 37 °C and 5% CO_2. As target cells were used LB15.13, an H-2^b-expressing tumor cell line (MHC class I^+II^+) and G4 [10], a transfectant of the MHV-non-permissive H-2^b T cell lymphoma EL4 expressing the receptor for MHV-A59 [15] (MHC class I^+II^-). Virus-infected target cells were prepared by infection of cells with MHV-A59 at a multiplicity of infection (MOI) of 50 for 5 h at 37 °C prior to the 1-h labeling with ^{51}Cr. The percentage of specific lysis was calculated as (cpm experimental release-cpm spontaneous release)/(cpm total release-cpm spontaneous release) × 100. Spontaneous release was below 20% of total release; standard deviations were below 10%.

2.5 Adoptive transfer experiments

Eight-week-old, pathogen-free, C57BL/6 mice were injected i.v. either with 5×10^6 MHC class II-restricted MHV-A59-specific $CD8^+$ CTL (> 95% $CD8^+$) derived from $CD4^{-/-}$ mice or with 5×10^6 MHC class II-restricted MHV-A59-specific $CD4^+$ CTL (> 85% $CD4^+$) derived from $CD4^{+/-}$ mice. Cells were restimulated twice *in vitro* with MHV-A59-infected syngeneic spleen cells. One day after transfer of cells, mice were inoculated i.p. with 1×10^4 PFU MHV-A59 ($250 \times LD_{50}$). Control mice received PBS 1 day before the MHV-A59 infection. Infected mice were monitored up to 20 days.

3 Results and discussion

To study the role of CD4 in the protection against MHV-A59, CD4-deficient mice ($CD4^{-/-}$) [11] were infected with MHV-A59. Like wild-type mice [16], $CD4^{-/-}$ mice survive a lethal MHV-A59 infection only when they are first inoculated with a temperature-sensitive mutant (ts342) of MHV-A59 (data not shown). Spleen cells of MHV-A59-infected $CD4^{-/-}$ mice proliferate specifically against inactivated MHV-A59 (stimulation index = 6.8 ± 1.7), although the response is lower than that of $CD4^{+/-}$ mice (stimulation index = 29.7 ± 8.7). In addition, the sera of $CD4^{-/-}$ mice contain virus-specific neutralizing antibodies 3 weeks after boosting with wild-type MHV-A59, although these antibody titers are about tenfold lower than those of control mice (data not shown). Together these data show that a protective immunity is induced in $CD4^{-/-}$ mice.

In order to investigate the nature of this protective immunity in more detail, the cytotoxic T cell activity of MHV-A59-primed CD4-deficient mice was tested. Spleen cells from primed $CD4^{-/-}$ mice, stimulated for 6 days *in vitro* with irradiated infected splenocytes, lyse MHV-A59-infected MHC class II-positive LB15.13 target cells, but not virus-infected MHC class II-negative G4 targets (Fig. 1A). The cytotoxic activity of heterozygous mice did result in a similar pattern (Fig. 1B), showing that both $CD4^{-/-}$ as well as $CD4^{+/-}$ mice respond to MHV-A59 infection in a MHC class II-restricted fashion. Since

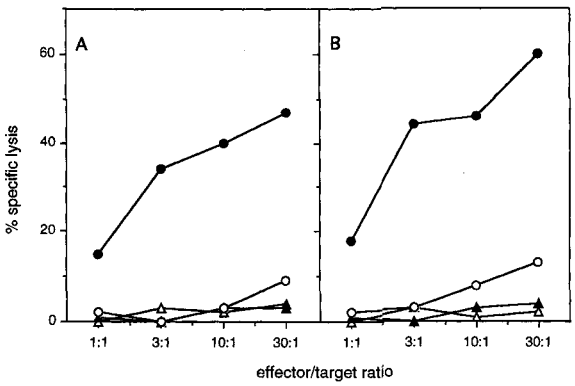

Figure 1. MHV-specific MHC class II-restricted cytotoxic activity of *in vitro* stimulated spleen cells of $CD4^{+/-}$ and $CD4^{-/-}$ mice. *In vitro* stimulated spleen cells from MHV-A59-primed $CD4^{+/-}$ (A) and $CD4^{-/-}$ (B) mice were tested against MHV-A59-infected (▲) and uninfected (△) G4 (MHC class I^+II^-) and MHV-A59-infected (●) and uninfected (○) LB15.13 target cells (MHC class I^+II^+), at the effector/target ratios indicated in the figure.

$CD4^{-/-}$ mice have been shown to mount normal $CD8^+$ class I-restricted antiviral responses after infection with lymphocytic choriomeningitis virus (LCMV) or vaccinia virus [11], it was surprising that these mice responded in an MHC class II-restricted fashion.

$CD4^{-/-}$ mice have been shown to have a significant number of $CD4^-CD8^-$ αβ T cells, which are functional MHC class II-restricted helper T cells [17, 18]. To determine whether the $CD4^-CD8^-$ or the $CD4^-CD8^+$ cells were

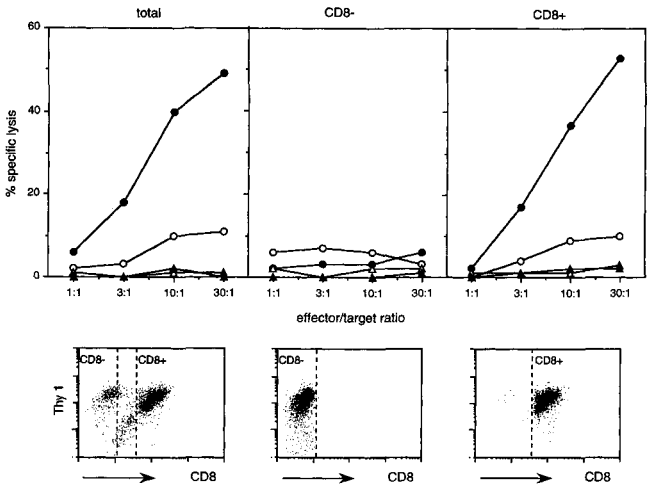

Figure 2. The $CD8^+$ effector cells are responsible for the virus-specific MHC class II-restricted cytotoxicity. *In vitro* stimulated spleen cells derived from MHV-A59-primed $CD4^{-/-}$ mice were tested either unseparated (total) or after sorting into $CD4^-CD8^-$ ($CD8^-$) or $CD4^-CD8^+$ ($CD8^+$) cells against MHV-A59-infected (▲) and uninfected (△) G4 and MHV-A59-infected (●) and uninfected (○) LB15.13 target cells. The effector cells (consisting of > 90% $CD4^-CD8^+$ and ± 5% $CD4^-CD8^-$) were stained with FITC-anti-CD8 (53-6.7) mAb and tested directly or after sorting. The flow cytometric analysis of the unseparated and the sorted effectors are indicated below the figures; they show that the separated populations are > 99% pure. Sorting was performed on clear $CD8^-$ and $CD8^+$ cells, therefore, two dotted lines are present in the flow cytometric analysis of the total effector population.

responsible for the MHC class II-restricted cytotoxicity, sorting experiments were performed. After labeling with FITC-anti-CD8, $CD8^-$ and $CD8^+$ cells were sorted. As can be seen in Fig. 2, none of the two sorted populations lysed MHV-A59-infected G4 (class I^+ II^-) targets. However, the $CD8^+$ population was able to lyse infected LB15.13 (class I^+ II^+) cells. These data show that the $CD8^+$ T cells of the $CD4^{-/-}$ mice mediate the cytolytic activity. The fact that only the MHC class II-positive targets were lysed demonstrates that the $CD8^+$ cells utilize the MHC class II molecule as a restriction element. To test this more directly, we performed antibody inhibition experiments. During the cytotoxic assay, anti-MHC class II mAb (M5/114, $I\text{-}A^b$) [19] were added and a complete inhibition of the cytotoxic activity of the sorted $CD8^+$ effector cells against MHV-infected LB15.13 was observed (Fig. 3). Anti-MHC class I antibodies R1-21-2 [20] had no effect. In conclusion, MHV-A59-infected CD4-deficient mice generate $CD8^+$ CTL, which are not MHC class I-, but MHC class II-restricted.

Adoptive transfer of the polyclonal MHV-A59-specific MHC class II-restricted $CD8^+$ T cells (> 95 % $CD8^+$) showed that three out of four mice were protected against a lethal challenge of MHV-A59 (Table 1). Polyclonal $CD4^+$ CTL derived from $CD4^{+/-}$ mice (> 85 % $CD4^+$) protected two out of four mice against a challenge with MHV-A59. Although we cannot exclude the contribution of the double-negative cells, the data suggest that MHC class II-restricted $CD8^+$ T cells play a pivotal role in the protection against MHV-A59 infection.

Infection of mice with the coronavirus MHV-A59 does not lead to a normal MHC class I-restricted cytotoxic response as observed with other viruses [21–23]. In normal mice $CD4^+$ class II-restricted cells are the main effector cells against this virus [9]. In this study we show that $CD4^{-/-}$ mice respond with MHC class II-restricted $CD8^+$ CTL. We have postulated that the reason for this MHC class II-restricted response is the fact the MHV-A59 itself precludes an MHC class I-restricted response, by down-regulation of MHC class I expression on MHV-A59-infected cells [9, 10, 24].

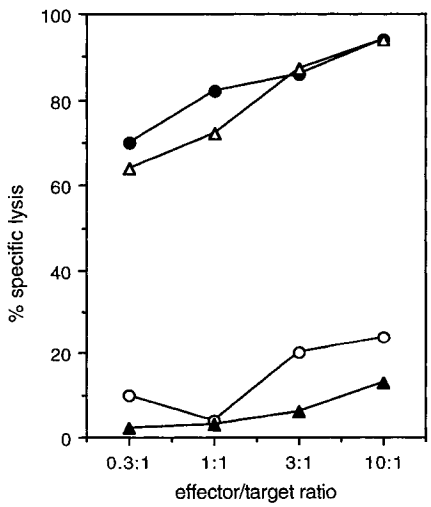

Figure 3. MHC class II-specific mAb inhibit the $CD8^+$ cytolytic activity completely. Three times *in vitro* stimulated MHV-A59 specific $CD4^-CD8^+$ sorted effector cells were tested against MHV-A59-infected (●) and uninfected (○) LB15.13 target cells in the presence of MHC class II-specific mAb M5/114 ($I\text{-}A^{b.d}$), (▲) or MHC class I-specific mAb R1.21.2 (most haplotypes except $H\text{-}2^d$) (△), both at final dilution of 1:4.

Table 1. Virus-specific class II-restricted $CD4^-CD8^+$ T cells protect C57BL/6 mice against a lethal MHV-A59 challenge[a)]

Transfer (cells/origin)	No. of mice	Survivors/ Total
None	4	0/4
$CD8^+$ (95 %)/$CD4^{-/-}$ mice	4	3/4
$CD4^+$ (85 %)/$CD4^{+/-}$ mice	4	2/4

a) C57BL/6 mice were injected i.v. with either 5×10^6 MHC class II-restricted MHV-A59-specific $CD8^+$ CTL (> 95 % $CD8^+$) derived from $CD4^{-/-}$ mice or with 5×10^6 MHC class II-restricted MHV-A59-specific $CD4^+$ CTL (> 85 % $CD4^+$) derived from $CD4^{+/-}$ mice. One day after transfer of cells, mice were inoculated i.p. with 1×10^4 PFU MHV-A59 ($250 \times LD_{50}$). Control mice received PBS 1 day before the MHV-A59 infection. Infected mice were monitored up to 20 days.

One could speculate that these MHC class II-restricted T cells inhibit virus spread by destroying the MHV-infected MHC class II-positive cells during MHV-A59 infection. The infected cells are MHC class II^+ macrophages, Kupffer cells, hepatocytes and B cells ([25] and O. Wijburg et al., manuscript in preparation).

Alloreactive $CD8^+$ T cells recognizing MHC class II antigens have been described [5–7]. Only recently, antigen-specific class II-restricted $CD8^+$ T cells have been described [2]. In this study, however, these $CD8^+$ cells were forced to be class II restricted by the expression of a transgenic TcR αβ, which was isolated from a class II-restricted $CD4^+$ T cell hybridoma. In our study, MHC class II-restricted $CD8^+$ CTL were activated in MHV-A59-infected $CD4^{-/-}$ mice, in which the $CD8^+$ compartment possesses a functional class I-restricted TcR repertoire [11]. The presence of these unusual class II-restricted $CD8^+$ T cells in $CD4^{-/-}$ mice most likely results from the combined absence of CD4 in these mice and a lack of induction of class I-restricted CTL during MHV-A59 infection [9, 10].

The question is whether these $CD8^+$ class II-restricted T cells are only present in $CD4^{-/-}$ mice as a consequence of the CD4 null mutation or whether they are a physiological population of cells present in normal mice. If the class II-restricted $CD8^+$ cells are present only in $CD4^{-/-}$ mice this is probably due to lack of negative selection on MHC class II during thymic development. Cells that have a receptor recognizing MHC class I with slight cross-reactivity for class II antigens, would be negatively selected in the presence of CD4. In the absence of CD4, however, these cells may survive and give rise to $CD8^+$ T cells, which are responsible for the class II-restricted response observed in this study. Alternatively, if these class II-restricted $CD8^+$ T cells are present in wild-type mice, they are probably positively selected by MHC class II molecules. In previous experiments, using wild-type mice, no evidence for the existence of class II-restricted $CD8^+$ CTL was found [10].

However, we cannot exclude the presence of such cells, since $CD8^+$ class II-restricted T cells might be overruled by large populations of $CD4^+$ T cells, and therefore would be hard to detect using the standard assays for CTL activity.

Regardless of whether the observed class II restriction is physiological or a consequence of the use of $CD4^{-/-}$ mice, it is striking that the immune system does not employ virus-specific class I-restricted cells. This supports our hypothesis that the virus interferes with presentation of its antigens on class I molecules. Activation of the class II-restricted CTL, even in the $CD4^{-/-}$ mice, illustrates, the plasticity of the immune response when dealing with evasive mechanisms employed by microorganisms. This suggests that during infection with viruses that either down-regulate MHC class I or reduce the amount of $CD4^+$ T cells, such as HIV, $CD8^+$ class II-restricted T cells may play a significant role.

The authors thank W. van Eden (Utrecht) and C. Melief (Leiden) for critically reading the manuscript. This work was supported by the Netherlands Organization for Scientific Research (NWO), grants 900-502-125 and 900-792-147.

Received December 29, 1994; in revised form February 6, 1995; accepted February 8, 1995.

5 References

1 Swain, S. L., *Immunol. Rev.* 1994. *74:* 129.
2 Kirberg, J., Baron, A., Jakob, S., Rolink, A., Karjalainen, K. and von Boehmer, H., *J. Exp. Med.* 1994. *180:* 25.
3 De Bueger, M., Bakker, A. and Goulmy, E., *Eur. J. Immunol.* 1992. *22:* 875.
4 McKisic, M. D., Sant, A. J. and Fitch, F. W., *J. Immunol.* 1994. *147:* 2868.
5 Vidovic, D., Juretic, A., Nagy, A. and Klein, J., *Eur. J. Immunol.* 1981. *11:* 499.
6 Shinohara, N. and Kojima, M., *J. Immunol.* 1984. *132:* 578.
7 Spits, H., Yssel, H., Thompson, A. and de Vries, J. E., *J. Immunol.* 1983. *131:* 678.
8 Wege, H., Siddell, S. and ter Meulen, V., *Curr. Top. Microbiol. Immunol.* 1982. *99:* 164.
9 Boog, C. J. P., Heemskerk, M. H. M., Schoemaker, H. M. and Spaan, W. J. M., *J. Cell. Biochem.* 1994. 18: 351.
10 Heemskerk, M. H. M., Schoemaker, H. M., Spaan, W. J. M. and Boog, C. J. P., *Immunology* 1995. (*in press*).
11 Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W., Kündig, T. M., Sambhara, S. R., Narendran, A., Arabian, A., Wakeham, A., Paige, C. J., Zinkernagel, R. M., Miller, R. G. and Mak, T. W., *Nature* 1991. *353:* 180.
12 Spaan, W. J. M., Rottier, P. J. M., Horzinek, M. C. and van der Zeijst, B. A. M., *Virology* 1981. *108:* 424.
13 Koolen, M. J. M., Osterhaus, A. D. M. E., Steenis, G., Horzinek, M. C. and van der Zeijst, B. A. M., *Virology* 1982. *125:* 393.
14 de Waal, L. P., Kast, W. M., Melvold, R. W. and Melief, C. J. M., *J. Immunol.* 1983. *130:* 1090.
15 Lorber, M. I., Loken, M. R., Stall, A. M. and Fitch, F. W., *J. Immunol.* 1982. *128:* 2798.
16 Heemskerk, M. H. M., Schoemaker, H. M., Alphen, H. E., van der Zee, R., Joosten, I., Spaan, W. J. M. and Boog, C. J. P., *Adv. Exp. Med. Biol.* 1994. *342:* 407.
17 Locksley, R. M., Reiner, S. L., Hatam, F., Littman, D. R. and Killeen, N., *Science* 1993. *261:* 1448.
18 Rahemtulla, A., Kündig, T. M., Narendran, A., Bachmann, M. F., Julius, M., Paige, C. J., Ohashi, P. S., Zinkernagel, R. M. and Mak, T. W., *Eur. J. Immunol.* 1994. *24:* 2213.
19 Bhattacharya, A., Dorf, M. E. and Springer, T. A., *J. Immunol.* 1981. *127:* 2488.
20 Koch, S., Koch, H., Robinson, P. and Hämmerling, G., *Transplantation* 1983. *36:* 177.
21 Askonas, B. A., Taylor, P. M. and Esquivel, F., *Ann. NY Acad. Sci.* 1988. *532:* 230.
22 Lehmann-Grube, F., Moskophidis, D. and Lohler, J., *Ann. NY Acad. Sci.* 1988. *532:* 238.
23 Byre, J. A. and Oldstone, M. B. A., *J. Virol.* 1984. *51:* 682.
24 Bergmann, C., McMillan, M. and Stohlman, S., *J. Virol.* 1993. *67:* 7041.
25 Coutelier, J. P., Godfraind, C., Dveksler, G. S., Wysocka, M., Cardellichio, C. B., Noël, H. and Holmes, K. V., *Eur. J. Immunol.* 1994. *24:* 1383.