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Natural antiviral activity of mouse macrophages against encephalomyocarditis virus

Dario Soldateschi, Diana Boraschi* and Aldo Tagliabue

Laboratory of Immunopharmacology, Sclavo Research Center, Via Fiorentina 1, 53100 Siena, Italy

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Summary

Resident mouse peritoneal cells (PC) express a significant antiviral activity against encephalomyocarditis virus (EMCV) in vitro, as judged by decreased virus yield from infected mouse embryo fibroblasts (MEF). This natural antiviral activity of PC was not due either to enhanced lysis of virus-infected cells, as these were protected from lysis rather than destructed by PC, or to interferon (IFN) production, as no direct correlation between IFN and anti-EMCV activity was found. Among PC, macrophages (Mφ) appear to be responsible for the anti-EMCV activity, which was indeed attributable to a Thy 1.2-negative, adherent mononuclear cell. Moreover, Mφ-defective C3H/HeJ mice showed a significant impairment of anti-EMCV activity, whereas Mφ of mice defective for natural killer (NK) activity (bg/bg, SJL/J) or for mature T cells (nu/nu) possessed an intact antiviral capacity.

encephalomyocarditis virus, interferon, macrophages, natural antiviral activity

Introduction

Encephalomyocarditis virus (EMCV) infection in the mouse is characterized by viremia [3,8] followed by rapid viral replication in various organs. Protection from EMCV infection could be obtained by in vivo administration of interferon (IFN) [22] or IFN inducers such as poly-I : poly-C and transfer RNA [20,21]. Functionally intact macrophages (Mφ) were found to be necessary for establishment of protection in vivo [20–22]. Moreover, Mφ activators such as *Corynebacterium acnes* and *Mycobacterium tuberculosis* also induced protection against EMCV in vivo [9,12,13]. The importance of Mφ as antiviral effector cells has been further indicated in other experimental viral

* To whom correspondence should be addressed

infections where either elicited or unstimulated peritoneal Mφ have been found able to exert antiviral activity in vitro [11,17,23]. To further clarify the role of Mφ in protection against EMCV, we have investigated the ability of unstimulated mouse Mφ to reduce EMCV infection of mouse embryo fibroblasts (MEF) in vitro. Results obtained indicate that, indeed, Mφ are capable of exerting a natural anti-EMCV activity, which does not depend on IFN production.

Materials and Methods

Mice

C3H/HeN mice (Charles River Breeding Laboratories, Calco Italy), A/J, C3H/HeJ, C57BL/6J bg/bg, C57BL/6J bg/+ and SJL/J mice (Jackson Laboratories, Bar Harbor, ME) were bred in our animal facilities. Mice of both sexes were used between 8 and 16 weeks of age, without any significant variation of results. Specific pathogen-free male C3H/BOM nu/nu and nu/+ mice (Bomholtgaard, Denmark) were used upon arrival.

Tissue culture, viruses and culture reagents

Mouse embryo fibroblasts (MEF) were prepared from C3H/HeN embryos. L cells, clone 929, were kindly provided by Dr. G.B. Rossi, Istituto Superiore di Sanità, Rome. Cells were grown in Eagle's MEM (GIBCO, Glasgow, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sera-Lab, Crawley Down, Sussex, U.K.), 1% non-essential amino acids (GIBCO), 2 mM L-glutamine (GIBCO), 200 units/ml penicillin (Farmitalia, Milan, Italy) and 200 μg/ml streptomycin (Squibb, Rome, Italy) (hereafter referred to as culture medium). EMCV, kindly supplied by Dr. R.H. Kiefer (Flow Laboratories, McLean, VA), derives from ATCC lot 6. After two passages in brains of suckling mice, the virus was expanded in cell cultures. The EMCV lot used in this work was obtained from infected L929 monolayers and had a titre of 2.1×10^7 PFU/ml. Vesicular stomatitis virus (VSV), Indiana strain, was also propagated in L929 monolayers.

Effector cells

PC were obtained by peritoneal washings of normal, unstimulated mice as previously described [2]. Peritoneal Mφ were purified by adherence for 1 h to microexudate-coated plastic following the method of Ackerman and Douglas [1], adapted to mouse cells [24]. Isolated adherent cells (>98% viable as judged by trypan blue dye exclusion) were routinely checked for Mφ morphology on smears prepared by cytocentrifugation (Cytospin centrifuge, Shandon Southern, Camberley, U.K.) and stained with Diff-Quik (Harleco, Gibbstown, NJ). Nonadherent PC were obtained by depletion of adherent cells on plastic for 2 h followed by passage on Sephadex G-10 columns for 90 min, as described by Letvin et al. [10]. Leukocytes from spleen (SpL) and mesenteric lymph nodes (MnL) were prepared by gentle teasing of the organs in culture medium. Depletion of Thy 1.2-positive cells from PC was performed by incubating 10^7 PC in 0.5 ml of a 1 : 200 dilution of monoclonal anti-Thy 1.2 antibodies

(New England Nuclear, Boston, MA) for 30 min at 4°C. Cells were then washed once and resuspended in low-toxicity rabbit complement (Low-Tox M, Cedarlane Laboratories, Hornby, Ontario, Canada) at a final dilution of 1 : 14 for 45 min at 37°C. After two washes, cells were resuspended in culture medium without readjustment of the concentration.

Antiviral activity assay

Secondary MEF were grown to confluency (about 1.5×10^5 cells/cm^2) in 10 mm diam. wells of tissue culture plates (Cluster[48], Costar, Cambridge, MA), and infected for 1 h at room temperature with EMCV at 4×10^{-4} PFU/cell, unless otherwise stated. After washing of MEF monolayers to remove unadsorbed virus, effector cells were added to duplicate wells in 0.5 ml culture medium with 2% FBS. Control wells of infected MEF without effector cells were included in each experiment. After 24 h of incubation at 37°C in moist air with 5% CO_2, supernatants of duplicate wells were harvested, pooled and centrifuged 10 min at $1200 \times g$ to remove cellular debris. Virus yield and IFN content were tested either immediately or after short storage at -80°C. Identical results were obtained by running the antiviral assay either in culture medium alone or containing Hepes 20 mM (which maintained neutral pH up to the end of the assay), thus excluding the possibility that pH variation in culture could be responsible for EMCV yield reduction [6].

Cytolysis assay

MEF monolayers in 10 mm wells were infected with EMCV as described above, in the presence of 2 μCi/well sodium chromate ($Na_2\ ^{51}CrO_4$, sp. act. 250–500 mCi = 9.25–18.5 GBq/mg Cr; The Radiochemical Centre, Amersham, U.K.). Cells were then incubated with PC at different attacker to target (A : T) ratios. Control wells included infected MEF incubated without PC and uninfected MEF incubated with and without PC. After 6–24 h of culture, aliquots of supernatants were harvested from duplicate wells to measure radiolabel release. Percent of net cytolysis was determined according to the formula: $100 \times (a-b)/c$; where a is the radioactivity in sample supernatants, b the spontaneous release of uninfected MEF and c the total release from cells lysed with 1% SDS.

Virus titration

Serially diluted (log_5) supernatants from infected cultures were added to triplicate confluent monolayers of L929 cells in 6.4 mm wells of tissue culture plates (Cluster[96], Costar). After 1 h at room temperature, cultures were washed to remove the unadsorbed virus and overlayed with 0.1 ml of phenol red-free MEM containing 1% carboxymethyl-cellulose, high viscosity (Sigma Chemical Co., St. Louis, MO) and 2%. FBS. After 24 h at 37°C in moist air with 5% CO_2, cultures were fixed with a fixative solution of 33% absolute ethanol and 1.3% formaldehyde in saline, then stained with crystal violet 0.5%. After gentle rinsing of the plates in tap water to remove excess stain, microplaques were counted with the aid of a magnifying glass.

IFN titration

IFN was titrated by the dye-uptake method of Epstein and McManus [4], modified as follows. After virus inactivation by ultraviolet (UV) irradiation, test supernatants were serially diluted and added in 50 μl volumes to duplicate L929 monolayers, previously established in 4.5 mm wells of tissue culture plates (Cluster[96] half area, Costar). After overnight incubation at 37°C in humid air with 5% CO_2, supernatants were removed and L929 cells were challenged with VSV for 24 h. After fixation of residual cells in fixative solution and staining with crystal violet, the dye was eluted with ethanol 50% in distilled water and its absorbance at 560 nm wavelength was determined spectrophotometrically with an automated eight-channel Multiskan Titertek apparatus (Flow). IFN titre was defined as the reciprocal of the dilution giving 50% dye uptake. A laboratory standard, calibrated against the NIH reference mouse IFN G002-902-026, was introduced in every titration. Titres were expressed in international units (IU). The IFN detected in culture was characterized as IFN-α based on pH 2 stability, complete neutralization with a rabbit antiserum anti-mouse IFN-α + β (Enzo Biochem, New York, NY) and lack of neutralization with a rabbit anti-mouse IFN-β (Lee BioMolecular, San Diego, CA).

Statistical analysis

Data in Figs. 1 and 3 are presented as means ± S.E.M. of replicate determinations. Statistical significance was assessed by Student's *t*-test.

Results

Resident PC of normal mice significantly reduced EMCV yield from infected MEF cultures. Antiviral activity of PC began to be evident after 11 h, but was maximal only after 19 h of coculture with infected cells (Fig. 1, left). Cocultivation of PC with infected MEF resulted in the protection of the latter cells, as judged by ^{51}Cr release from prelabeled MEF. In fact, radiolabel release from virus-infected MEF was strongly decreased in the presence of PC (Fig. 1, right), with kinetics comparable to those of virus yield reduction. Comparable kinetics data were obtained with MEF infected at higher virus multiplicity (4×10^0 PFU/cell), whereas PC did not affect ^{51}Cr release from uninfected MEF (data not shown). This would therefore suggest that the anti-EMCV activity of PC was not principally due to lysis of virus-infected cells.

The antiviral activity of PC against EMCV was best evident on MEF infected at low virus multiplicity. At higher multiplicity of infection, the anti-EMCV effect was less pronounced, though still evident (Fig. 2).

To clarify the role of IFN in the antiviral activity of PC, the production of IFN in culture was measured and compared to anti-EMCV activity. As shown in Fig. 3, no direct correlation was found between antiviral activity and IFN production in cocultures of PC and virus-infected MEF at several A : T ratios. Indeed, IFN production appeared to inversely correlate to the PC-induced antiviral activity, as higher amounts of IFN were detected at A : T ratios causing low anti-EMCV activity. Furthermore, leukocytes from other anatomical sites (SpL, MnL) were devoid of significant antivi-

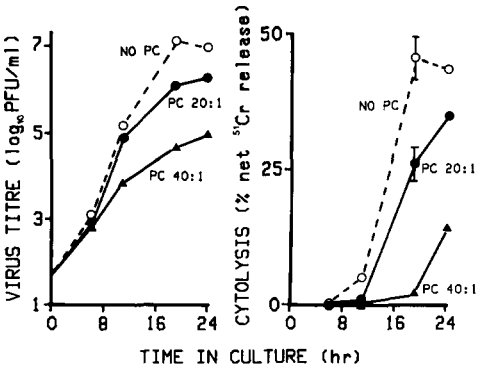

Fig. 1. Time course of the natural anti-EMCV activity of PC. MEF monolayers infected with 4×10^{-4} PFU/cell of EMCV were cocultured with PC from C3H/HeN mice at A : T ratios of 20 : 1 and 40 : 1 for 6–24 h. Left panel: EMCV titre in supernatants of cultures of infected MEF alone (o---o), with PC 20 : 1 (●——●) or with PC 40 : 1 (▲——▲). Right panel: radioactivity release from ^{51}Cr-labeled infected MEF cultured alone or in the presence of PC 20 : 1 and 40 : 1. Total releasable radioactivity was 3600 ± 88 cpm. Bars represent S.E.M. for duplicate cultures. S.E.M. below 2.5% are not reported. Statistical analysis: PC 40 : 1 statistically different ($P<0.01$) from no PC, except at 6 h. PC 20 : 1 statistically different ($P<0.05$) from no PC, except at 6 h. PC 40 : 1 statistically different ($P<0.05$) from PC 20 : 1 at 19 and 24 h.

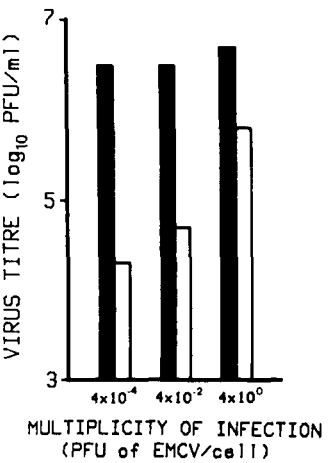

Fig. 2. Effect of the multiplicity of virus infection on the natural anti-EMCV activity of PC. MEF monolayers were infected with EMCV at different multiplicity of infection, then cultured alone (▬) or in the presence of PC from C3H/HeN mice at an A : T ratio of 40 : 1 (▭), before determination of the virus titre in culture supernatants.

ral activity against EMCV, although able to produce high levels of IFN upon coculture with infected MEF (Fig. 4). Neither MEF nor effector cells could absorb or consume IFN in culture (Table 1). Further evidence suggested that IFN or other soluble factors were not directly involved in the mechanism of natural anti-EMCV activity. In fact, UV-inactivated supernatants from cocultures of PC and infected

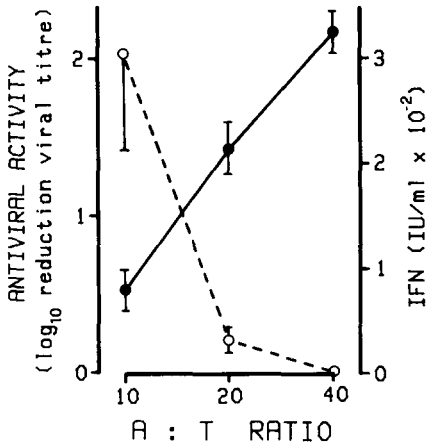

Fig. 3. Natural anti-EMCV activity and IFN production by PC. EMCV-infected MEF cultured with PC of C3H/HeN mice at different A : T ratios. Culture supernatants were then tested for both viral titre (●——●) and IFN activity (○---○). Data are the means ± S.E.M. of 7 separate experiments. Statistical analysis: antiviral activity: each point statistically different ($P<0.05$) from others; IFN production: A : T ratio 10 statistically different ($P<0.05$) from others; ratio 20 not different ($P>0.05$) from ratio 40.

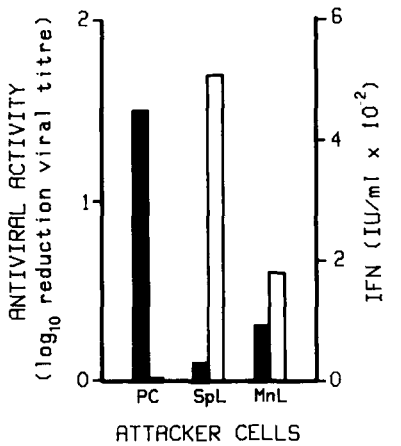

Fig. 4. Natural anti-EMCV activity and IFN production by leukocytes from different anatomical sources. EMCV-infected MEF were cocultured with cells from the peritoneal cavity (PC), spleen (SpL) or mesenteric lymph nodes (MnL) of C3H/HeN mice at an A : T ratio of 20 : 1, prior to determination of viral titre (▬) and IFN activity (▭) in culture supernatants.

MEF (A : T ratio 40 : 1; reduction of virus titre 3.2 $\log_{10}$) were unable to reduce virus yield when incubated with infected MEF for 24 h (data not shown).

Characterization of the cell responsible for the anti-EMCV activity of PC was performed. After depletion of Thy 1.2-positive cells by means of specific monoclonal antibodies and complement, PC (at an A : T ratio of 40 : 1) still reduced the EMCV

TABLE 1

IFN consumption by MEF and Mφ[a]

Cells	Residual IFN titre (log_{10} IU/ml)	
	4 h	20 h
None	2.0	1.9
MEF	2.2	1.7
Mφ	2.1	1.7

[a] IFN (100 IU), produced upon stimulation of Mφ with EMCV, was incubated in duplicate wells for 4 or 20 h either alone or in the presence of MEF ($1.5 \times 10^5/cm^2$) or Mφ ($3 \times 10^6/cm^2$). Supernatants were then harvested and tested for IFN content.

titre in infected MEF of 2.2 log_{10}, as compared to a reduction of 2.7 log_{10} induced by control PC exposed to complement alone (data not shown). Moreover, the data in Fig. 5 show that nonadherent PC (obtained after adherence to plastic and passage on Sephadex G-10) were devoid of antiviral activity. Conversely, the anti-EMCV activity of peritoneal Mφ purified on microexudate-coated plastic was about double that of unseparated PC (that contained approximately 50% of Mφ). Thus, the effector cell of anti-EMCV activity in PC appeared to be an adherent Thy 1.2-negative mononuclear cell, possibly a Mφ. The anti-EMCV activity of Mφ was not attributable to adsorption or uptake of virus by effector cells. In fact, the data in Table 2 show that the virus titre was never reduced by incubation with increasing numbers of Mφ for different times. Comparable results were obtained over a wide range of virus multiplicities, from 8×10^0 to 8×10^{-4} PFU/Mφ (data not shown).

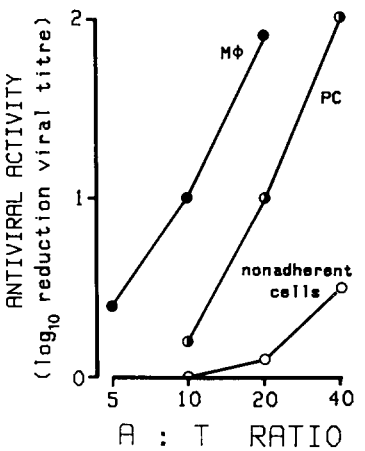

Fig. 5. Natural anti-EMCV activity by subpopulations of PC. EMCV-infected MEF were cocultured at different A:T ratios either with unseparated PC of C3H/HeN mice (◑) or with adherence-purified peritoneal Mφ (●), or nonadherent PC (○). Culture supernatants were then assayed for viral titre.

TABLE 2

Virus uptake by Mφ[a]

N. of Mφ	Virus titre (log_{10} PFU/ml)	
	4 h	20 h
None	6.8	6.6
3×10^6	7.0	6.5
1.5×10^6	7.0	6.6
0.75×10^6	7.0	6.6

[a] Different numbers of Mφ were incubated with EMCV (8×10^0PFU/cell) for either 4 or 20 h, before determination of virus titre.

Analysis of antiviral activity by PC from different mouse strains further indicated that Mφ, rather than T or NK cells, are responsible for the anti-EMCV effect. In fact, cells of Mφ-defective C3H/HeJ mice showed impaired antiviral capacity as compared to their normal C3H/HeN counterpart (Fig. 6, upper left). In contrast, two mouse strains defective for NK activity, i.e. C57BL/6Jbg/bg (Fig. 6, upper right) and SJL/J

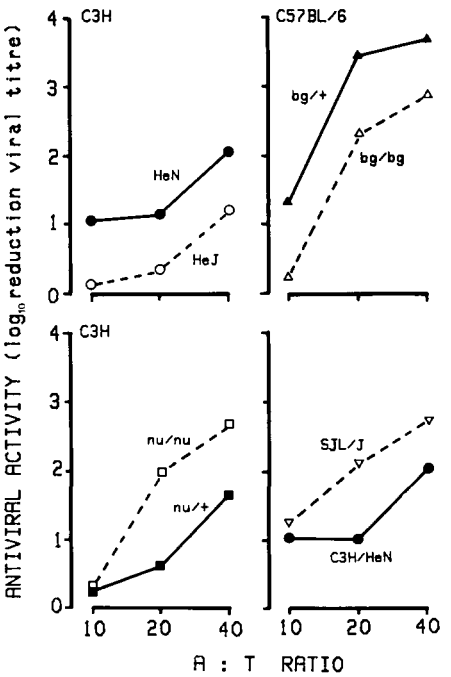

Fig. 6. Natural anti-EMCV activity by PC from different mouse strains. EMCV-infected MEF were cocultured at different A : T ratios with PC from different mouse strains, before assessment of viral titre in culture supernatants. Upper left: C3H/HeN (●——●) and C3H/HeJ mice (○---○). Lower left: C3H/BOM nu/+ (■——■) and nu/nu mice (□---□). Upper right: 8-week-old C57BL/6 bg/+ (▲——▲) and bg/bg mice (△---△). Lower right: 8-week-old C3H/HeN (●——●) and SJL/J mice (△---△).

mice (Fig. 6, lower right), possessed a very significant anti-EMCV activity. Finally, C3H nu/nu mice, genetically deficient of mature T cells, did not show any impairment of antiviral activity with respect to their normal nu/+ littermates (Fig. 6, lower left).

Discussion

The role of Mφ in the natural resistance against viruses has been widely reported [14]. In vitro, unstimulated Mφ and monocytes have been found able to exert a significant antiviral activity in certain virus systems [11,18,23]. However, only activated Mφ have been reported to be important in antiviral activity against EMCV both in vivo and in vitro [13,15,16,22]. The data presented in this paper show that unstimulated mouse peritoneal Mφ also possess a strong anti-ECMV activity, as judged by reduced virus yield from infected fibroblasts. The natural anti-EMCV antiviral activity of peritoneal cells (which contained 50% Mφ) was completely accounted for by Mφ, as nonadherent cells were devoid of activity, whereas adherence-purified peritoneal Mφ were active against EMCV twice as much as unseparated PC. Further evidence indicating the Mφ as the effector cell of natural anti-EMCV activity was provided by data obtained with the Mφ-defective mouse strain C3H/HeJ [25], which showed a significant impairment in the expression of antiviral activity. In contrast, mice defective for mature T cells (C3H nu/nu) did not show any decrease of anti-EMCV activity, which was rather enhanced as compared to that of their normal heterozygous littermates. The possibility that NK cells may play a role in the effector mechanism of natural anti-EMCV activity was investigated with NK-deficient mouse strains. Indeed, C57BL/6J bg/bg mice possessed very significant levels of anti-EMCV activity, though lower than that of their high NK bg/+ counterparts. That NK cells do not have a relevant role in the expression of the natural anti-EMCV activity was undoubtedly indicated by the high antiviral activity exerted by cells of the low NK strain SJL/J, which markedly exceeded that of the high NK strain C3H/HeN. The lack of involvement of T and NK cells in the natural antiviral activity was further stressed by the finding that depletion of Thy 1.2-positive cells from PC did not affect their anti-EMCV activity.

It has been reported that Mφ can exert antiviral activity by different mechanisms, possibly depending on the host–virus system examined. In fact, Mφ antiviral activity can be expressed as uptake and digestion of virus particles [14], IFN production [19], or lysis of infected cells [11,17]. As far as EMCV is concerned, previous data indicated the in vitro antiviral activity of PC from mycobacteria-sensitized mice as depending on the production of an antiviral molecule, possibly identical to IFN-γ [16]. In contrast, the natural anti-EMCV activity of unstimulated Mφ described in this report appeared to be independent of IFN production. Indeed, in cocultures of Mφ and virus-infected fibroblasts it was possible to detect the presence of IFN, although this was characterized as IFN-α after neutralization with specific antisera. However, no direct correlation was ever observed between anti-EMCV activity and IFN production. Rather, IFN production appeared to inversely correlate with antiviral activity, as IFN was detectable only concomitantly with low or absent anti-EMCV activity, but

not in the presence of strong virus-reducing activity. Since it was found that cells in culture did not adsorb or consume IFN, the observed inverse relationship between IFN and antiviral activity is possibly attributable to the reduction of EMCV (which acts as IFN inducer) due to the Mφ antiviral activity. Thus, the IFN production in culture might be considered only a secondary event, not relevant in controlling the initial viral infection. That anti-EMCV activity of Mφ might be independent of IFN has also been suggested in vivo by data showing that the poly-I : poly-C-induced Mφ-mediated protection against EMCV in mice did not correlate with IFN production [20]. Moreover, protection from systemic EMCV infection was attained in mice treated with the Mφ activator *Corynebacterium acnes*, which on the other hand was found able to suppress, rather than enhance, virus-induced IFN production both in the intact mouse [5] and in isolated peritoneal cells [7]. Thus, mechanisms distinct from IFN production possibly play a more prominent role in the anti-EMCV activity of Mφ. In addition, it can be reasonably excluded that the Mφ antiviral activity might be mediated through enhanced lysis of infected fibroblasts. In fact, radioactivity release from prelabeled cells indicated that normal Mφ may exert their antiviral activity by protecting fibroblasts from viral lysis, rather than by destructing EMCV-infected cells.

The natural anti-EMCV activity of Mφ in vitro was highly significant when fibroblasts had been infected with low multiplicity of virus, but became less evident with a higher multiplicity of infection. Indeed, also in vivo the presence of intact Mφ has been reported to increase both survival after sublethal EMCV infection and survival time after lethal virus inocula, but it was not sufficient to eradicate the infection [22]. It might thus be suggested that the natural anti-EMCV activity of Mφ represents a mechanism of surveillance able to eliminate low numbers of EMCV particles at the beginning of infection. This natural anti-EMCV activity would therefore broaden the spectrum of natural antiviral resistance mechanisms exerted by Mφ.

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