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Theiler's virus strain-dependent induction of innate immune responses in RAW264.7 macrophages and its influence on viral clearance versus viral persistence

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Infection of susceptible mice with the DA strain of Theiler's murine encephalomyelitis virus (TMEV) induces a persistent central nervous system infection accompanied by demyelination that resembles multiple sclerosis. In contrast, Theiler's GDVII strain does not persist, because infected animals either clear the virus or die. Previously, the authors have shown that *in vitro* infection of RAW264.7 macrophages displays a similar strain-dependent outcome, resulting in the establishment of a persistent infection with the DA strain and clearance of the GDVII strain. Here, the authors show that when RAW264.7 cells were infected with both strains, the antiviral response triggered by the GDVII virus interfered with the DA virus' ability to induce a persistent infection. Treatment of cells with 2-aminopurine, a protein kinase R inhibitor, increased GDVII virus yields in contrast to DA virus yields. By comparing the antiviral activity of RAW264.7 macrophages against TMEV, it was found that GDVII-infected macrophages mounted a five times more potent antiviral response than the DA-infected ones, indicating that there are strain-dependent differences in the induction of host innate immune responses. Measurements of interferon (IFN) production confirmed this finding. In addition, it was found that the macrophages' antiviral response is dependent on the multiplicity of infection. The antiviral activity resulting from GDVII-infected macrophages could be partially neutralized with antibodies against IFN-α or IFN-γ, but not with an anti-IFN-β antibody. Because only a partial neutralization was reached, the authors speculate that apart from the investigated IFNs, other cellular factors contribute to the observed antiviral activity. Taken together, these results demonstrate the importance of host innate immune responses in determining the balance between viral clearance and viral persistence. *Journal of NeuroVirology* (2007) **13,** 47–55.

Keywords: innate immunity; interferons; macrophage; persistent infection; picornavirus; TMEV; viral clearance

Introduction

Theiler's murine encephalomyelitis viruses (TMEV) are picornaviruses belonging to the *Theilovirus* species within the *Cardiovirus* genus that cause subtype-dependent neurological diseases in mice. The neurovirulent subtype (e.g., strain GDVII) infects the central nervous system gray matter, resulting in acute encephalomyelitis with a high case mortality. In the few mice that survive, the virus is cleared by the immune system (Lipton, 1980). In contrast, the demyelinating subtype (e.g., strain DA) causes a biphasic disease, first resulting in a similar disease as induced by the neurovirulent subtype, but with a low case mortality. In a second phase, however, the virus spreads from the gray to the white matter and causes a demyelinating disease resembling multiple sclerosis (Lipton, 1975). In opposition to the neurovirulent strains, the demyelinating strains persist throughout

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the animals' life time, which is believed necessary to induce the multiple sclerosis-like disease (Lipton *et al*, 1991; Rodriguez *et al*, 1996).

In order to establish a persistent infection, viruses have to deal with host immune responses aimed at clearing the pathogens. Interferons (IFNs) are among the cytokines that are produced early after a viral infection and exert potent antiviral activities (Goodbourn *et al*, 2000). They are divided into three groups, designated as type I and type II and the recently discovered IFN-λ group (Ellermann-Eriksen, 2005). The type I IFNs comprise a broad group of cytokines, with IFN-α/β being the most extensively studied ones, and are produced by most cells types following viral infection (Biron and Sen, 2001; Malmgaard, 2004). Type II IFN consists of only one member, IFN-γ, which was originally thought to be exclusively produced by natural killer cells and activated T cells. Only in recent years did it become evident that IFN-γ can also be produced by other cell types, including monocytes/macrophages (Gessani and Belardelli, 1998; Frucht *et al*, 2001).

Although type I and type II IFNs exhibit no structural homology and use different signaling receptors, they share the ability to induce an antiviral state in neighboring uninfected cells whereby virus replication is blocked or impaired (Goodbourn *et al*, 2000). Mediators of the IFN-induced antiviral activity include the double-stranded RNA-dependent protein kinase (PKR) (Williams, 1999), the 2′,5′-oligoadenylate synthetase/RNase L system (Silverman, 1994) and the Mx proteins (Staeheli *et al*, 1993). In view of the fact that IFNs influence the expression of hundreds of genes (de Veer *et al*, 2001) and that mice triply deficient in PKR, RNase L, and Mx are still able to mount an IFN-induced antiviral response (Zhou *et al*, 1999), it is very likely that other, yet unknown, antiviral pathways exist.

Like other viruses (Goodbourn *et al*, 2000), picornaviruses have developed several strategies to counteract these defenses in order to preserve their replication, such as degradation of PKR (Black *et al*, 1993), induction of the expression of the RNase L inhibitor (Cayley *et al*, 1982), inhibition of the activation of IFN regulatory factor-3 (IRF-3) (Fenster *et al*, 2005) and viral leader (L) protein–mediated antagonism of IFN-α/β production (de Los Santos *et al*, 2006; van Pesch *et al*, 2001; Zoll *et al*, 2002). The L protein of TMEV has also been found to disrupt the nucleocytoplasmic trafficking of cellular proteins, including IRF-3 (Delhaye *et al*, 2004).

We have previously shown that Theiler's DA strain can induce a persistent infection in RAW264.7 macrophages, whereas the GDVII strain is cleared within 4 to 5 days after infection. In both cases the macrophages grew well without obvious cytolytic effect (Steurbaut *et al*, 2006). The purpose of the present study was to investigate the contribution of the innate immune system to this differential outcome.

Results

Replication of the DA strain is impaired by coinfection with the GDVII strain

Because infection of RAW264.7 macrophages with the DA strain resulted in the establishment of a persistent infection, whereas the GDVII strain was eliminated after a couple of days (Steurbaut *et al*, 2006), we first investigated what the outcome would be of a double infection of these cells with both TMEV strains. Therefore, 2.5×10^4 RAW264.7 cells/well in 96-well plates were infected with 10 plaque-forming units (PFU)/cell of the GDVII or DA strain alone and with the combination of both viruses. The evolution of the infection was followed in function of time by microscopic evaluation of the cells' condition and by plaque assay (Figure 1). No marked differences regarding the amount of cells or cytopathic effects were observed between the mono infection (GDVII or DA) and the double infection (GDVII and DA) (results not shown). Nevertheless, and consistent with our previous results, the DA strain reached higher viral titers than the GDVII strain, which cannot be attributed to differences in binding kinetics, given their equal replication efficiencies during the first replication cycle (Steurbaut *et al*, 2006). Remarkably, infective titers from the double infection were comparable with those obtained after mono infection with the GDVII strain. Although we didn't investigate whether the residual infectivity was due to the GDVII strain, the DA strain, or the combination of both, this result indicates that the replication of the DA strain is severely hampered by coinfection with the GDVII strain. When the GDVII strain was added 24 to 48 h prior to infection with the DA strain, even lower titers or no infectivity at all were registered, indicative for the induction of an antiviral immune response triggered by the GDVII strain (results not shown). From

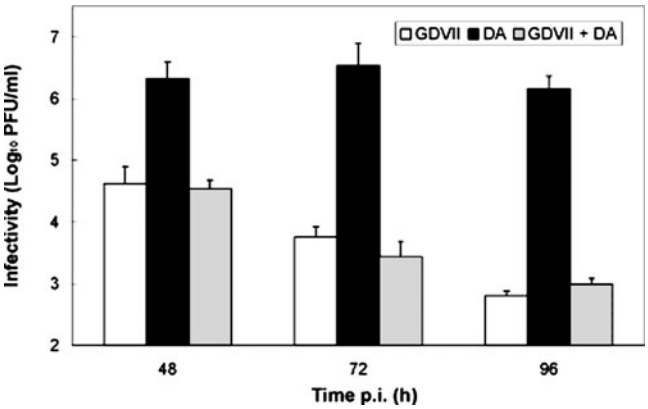

Figure 1 Suppression of DA replication by the GDVII strain. RAW264.7 cells, seeded at 2.5×10^4 cells/well in 96-well plates, were infected with the GDVII or DA strain alone and with the combination of both viruses at an m.o.i. of 10 PFU/cell for each virus strain. Virus titers were determined at 48, 72, and 96 h p.i. by plaque assay. Data are the mean result of duplicate samples from two independent experiments ± standard deviation.

this, it can be concluded that the DA's ability to induce a persistent infection can even be abrogated by coinfection with the GDVII strain.

Recovery of GDVII infectivity with 2-aminopurine
IFNs are innate immune response molecules that contribute to viral clearance by transcriptional activation of effector proteins that display strong antiviral activities (Brierley and Fish, 2002). To evaluate the involvement of IFNs in the restriction of GDVII replication and the GDVII-mediated interference of DA replication shown in the previous experiment, we made use of 2-aminopurine (2-AP) that blocks the induction of IFN genes through inhibition of PKR (Hu and Conway, 1993; Marcus and Sekellick, 1988). Various concentrations of 2-AP ranging from 0.25 to 25 mM were tested beforehand on the RAW264.7 cells to determine the highest, noncytotoxic concentration, being 1 mM (results not shown). RAW264.7 cells, seeded at 2.5×10^4 cells/well in 96-well plates, were preincubated with or without 1 mM 2-AP 4 h prior to infection and thereafter infected with the GDVII or DA strain at a multiplicity of infection (m.o.i.) of 10. The well contents, consisting of cells and supernatants, were harvested at 48, 72, and 96 h post infection (p.i.) and submitted to plaque assay. Table 1 shows that 2-AP treatment didn't affect GDVII virus yields at 48 h p.i., but 10.5- and 31.2-fold yield increases were obvious at 72 and 96 h p.i., respectively, in comparison with untreated TMEV-infected control cells. For the DA strain, no gain of infectivity could be demonstrated at any time with 2-AP. On the contrary, decreased DA virus yields were actually noted with 2-AP compared to the untreated cells.

The finding that 2-AP induced a strain-specific effect on TMEV replication suggests that there are differences in innate immune response against the GDVII and DA strain.

Culture supernatants from GDVII-infected RAW264.7 cells are more potent in restricting viral replication than culture supernatants from DA-infected ones
To assess if there are quantitative differences in the induction of antiviral responses by the GDVII and DA strain, we compared the degree of antiviral protection mediated by culture supernatants from GDVII- and DA-infected macrophages. RAW264.7 cells were either infected with 10 PFU/cell of the GDVII or DA strain, or treated with phosphate-buffered saline (PBS) to serve as mock-infected controls. Based on a preliminary experiment in which the degree of antiviral protection was studied as a function of the time (results not shown), culture supernatants were collected at 48 h p.i., i.e., the time corresponding with the highest degree of induced antiviral protection. Subsequently, the supernatants were subjected to ultrafiltration to render them virus-free and added to IFN-responsive L929 monolayer cells. After 24 h of priming and removal of the supernatants, the cells were challenged with the DA strain and the degree of protection against viral infection was assessed by plaque assay. As shown in Figure 2, supernatants from GDVII- and DA-infected RAW264.7 cells exhibited a different resistance against subsequent infection. Whereas the GDVII supernatant (dish A2) mediated a complete protection against the challenging DA strain, because no plaques were found, only a 50% reduction of the amount of plaques relative to the PBS control (dish B1) was witnessed with the DA supernatant (dish B2). The GDVII supernatant's 50% plaque reduction level was reached with a 1:5 dilution of this sample, indicating that the GDVII supernatant is five times more potent than the DA supernatant in conferring antiviral protection.

Strength of the antiviral response is m.o.i dependent
In order to investigate the influence of the m.o.i. on the potency of the induced antiviral response, RAW264.7 cells were infected at an m.o.i. of 0.1, 1, and 10 PFU/cell. Culture supernatants were obtained and tested for their antiviral potential in the same way as described above. PBS-treated cells served as mock-infected controls. The results are also displayed in Figure 2. In contrast with the supernatant from cells infected with the GDVII strain at 10 PFU/cell, a reduced or no protective effect at all was seen with an m.o.i. of 1 and 0.1, respectively (compare dishes A2, A3, and A4). Supernatants from DA-infected cells at an m.o.i. of 0.1 (dish B4) and 1 (dish B3) yielded no antiviral activity.

IFN secretion by TMEV-infected RAW264.7 macrophages
Because IFNs are secreted in response to viral infections and induce an antiviral state in neighboring uninfected cells, we quantified the amount of the type I IFNs α and β, as well as the type II IFN γ, in culture supernatants from TMEV-infected RAW264.7 cells. The macrophages were either infected with 10 PFU/cell GDVII or DA, or PBS mock-infected. Supernatants were collected from 8 to 96 h p.i. and analyzed using IFN-specific enzyme-linked immunosorbent assays (ELISAs). Results are given for the TMEV-infected samples only (Table 2), because all PBS controls generated signals below the experimental detection limit of 30 pg/ml. During the first 48 h p.i., IFNs could not be registered in the infected samples, except for the GDVII supernatant at 48 h p.i., where an IFN-α concentration of 130 pg/ml was measured. This concentration increased to 305 pg/ml during the following 48 h. For the DA strain, IFN-α was detectable at 72 and 96 h p.i., but the concentrations were, respectively, 5- and 3.8-fold lower than those found for the GDVII strain. In GDVII supernatants, IFN-β was detectable at 72 h (53 pg/ml) and 96 h (93 pg/ml), whereas in DA supernatants, this cytokine was only measurable at 96 h p.i. and with 59 pg/ml again lower in concentration compared to

Table 1 Influence of 2-AP on TMEV virus yields in function of time

Time p.i. (h)	*2-AP (1 mM)*	*GDVII*		*DA*	
		Log_{10} PFU/ml	*Ratio*	*Log_{10} PFU/ml*	*Ratio*
48	+	5.28 ± 0.13	1.18 ± 0.19	6.05 ± 0.06	0.24 ± 0.02
	−	5.21 ± 0.18		6.69 ± 0.02	
72	+	5.03 ± 0.02	10.50 ± 1.67	6.07 ± 0.01	0.09 ± 0.04
	−	4.01 ± 0.10		7.11 ± 0.14	
96	+	4.66 ± 0.19	31.23 ± 4.36	6.35 ± 0.07	0.55 ± 0.12
	−	3.17 ± 0.24		6.61 ± 0.04	

Note. RAW264.7 cells, seeded at 2.5 $\times 10^4$ cells/well in 96-well plates, were incubated 4 h prior to infection in the presence (+) or absence (−) of 1 mM 2-AP. The cells were infected with the GDVII or DA strain at an m.o.i. of 10 and virus titers were determined at 48, 72, and 96 h p.i. by plaque assay. Data are the mean result of duplicate samples from two independent experiments ± standard deviation.

Table 2 Quantification of IFNs in culture supernatants from TMEV-infected RAW264.7 cells

Time p.i (h)	*IFN-α*		*IFN-β*		*IFN-γ*	
	GDVII	*DA*	*GDVII*	*DA*	*GDVII*	*DA*
8	ND(a)		ND		ND	
24	ND		ND		ND	
48	130 ± 12	ND	ND		ND	
72	246 ± 17	49 ± 9	53 ± 9	ND	34 ± 5	ND
96	305 ± 14	80 ± 12	93 ± 8	59 ± 17	40 ± 18	ND

Note. Culture supernatants from 2.5 × 10^4 RAW264.7 cells infected with 10 PFU/cell of strain GDVII or DA were collected at several points in time and assayed for the presence of IFNs using IFN-specific ELISAs. Values, expressed in pg/ml, are the mean result of duplicates from two independent experiments. (a), Not detectable: <30 pg/ml; $^*P < .05$ (unpaired Student's *t* test).

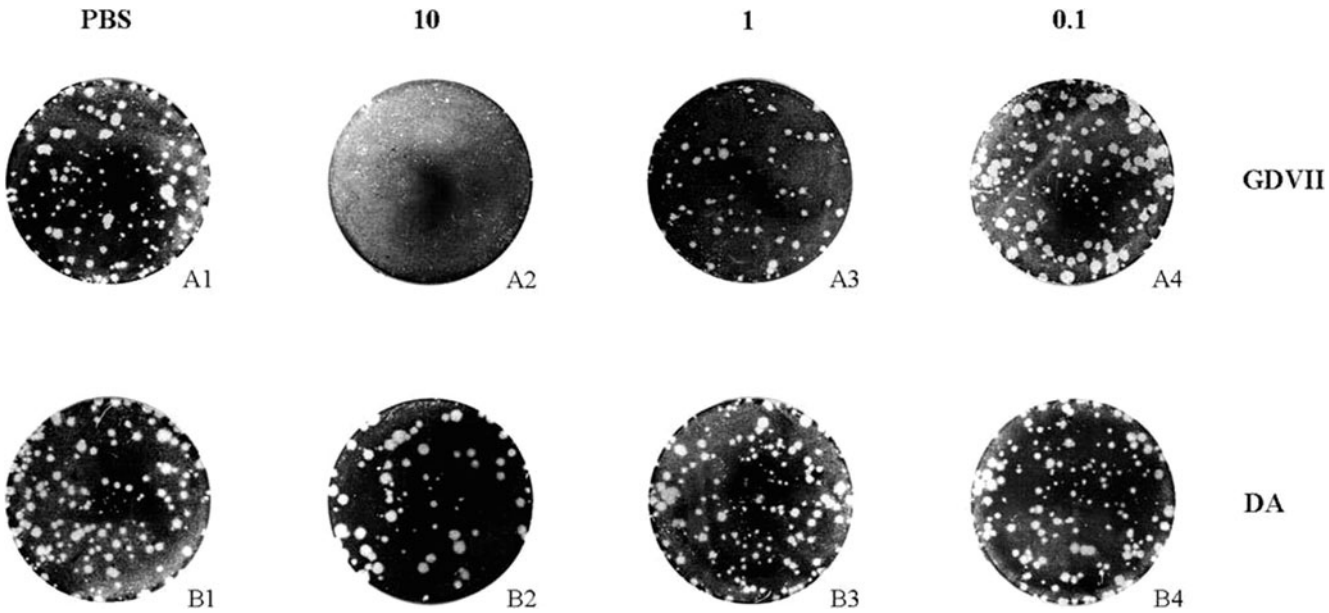

Figure 2 Antiviral effect of culture supernatants from TMEV-infected RAW264.7 macrophages. Culture supernatants from GDVII- or DA-infected RAW264.7 cells were obtained and tested for their potential to confer protection against a challenging virus as described in Materials and methods. The effect of the m.o.i. on the potency of the antiviral response was investigated by comparing culture supernatants from PBS mock-infected cells with supernatants from cells infected with 0.1, 1, and 10 PFU/cell. The experiment has been performed twice and representative dishes are shown.

the corresponding GDVII sample. For IFN-γ, low levels were detected in GDVII supernatants at 72 and 96 h p.i., whereas this cytokine could not be measured in DA supernatants. In conclusion, we found that GDVII-infected cells produced more IFN than DA-infected cells, and this for the three IFNs tested.

Partial neutralization of antiviral activity with antibodies against IFNs

To confirm the involvement of IFNs in the observed antiviral activity, neutralizing antibodies against IFN-α, -β, and -γ were tested for their capacity to reduce the antiviral protection mediated by GDVII-infected RAW264.7 culture supernatants. Culture supernatants from 10 PFU/cell GDVII-infected RAW264.7 cells were obtained as described above and treated with 250 and 1500 units (U) of either anti-IFN-α, -β, and -γ alone or with the combination of these three antibodies. The differently treated supernatants were again placed on L929 monolayers for 24 h, after which the cells were infected with the DA strain to assess the degree of infectivity. The amount of plaques obtained after priming with untreated PBS mock-infected RAW264.7 supernatants was used as a reference and equated with 100% infectivity (Table 3).

In agreement with our previous results (Figure 2), incubation with untreated GDVII supernatants reduced the infectivity to 2% $\pm$ 2%, indicative of a near-absolute antiviral protection. Treatment of supernatants with anti-IFN-α antibodies increased the infectivity approximately 20% to 30%, whereas anti-IFN-β antibody treatment had no or only a moderate effect on the infectivity. In contrast, treatment with antibodies against IFN-γ also resulted in an increase of infectivity of about 25% to 30%. However, no cumulative effect was observed with the combination of the three antibodies. The fact that the antiviral activity could not be completely neutralized didn't result from the use of too low antibody concentrations, because comparable amounts of infectivity were obtained with 250 and 1500 U of each antibody, indicating that the antibodies were present in excess. In addition, the intrinsic neutralizing capacity of each IFN-antibody was verified by spiking PBS supernatants with 0.25 ng/ml of IFN-α, -β, or -γ and treating them with 1500 U of the respective antibody, followed by a similar priming and challenge procedure as for the GDVII supernatants. Whereas IFN-spiked, non-antibody-treated controls displayed a certain amount of antiviral activity, each IFN-spiked and antibody-treated sample reached 100% infectivity, proving that neutralization was complete (Table 3). These data point towards other factor(s) that contribute to the antiviral response of RAW264.7 cells against Theiler's GDVII strain.

Furthermore, by testing the antiviral potential of IFN-α, -β, and -γ in a concentration series, we found that for each IFN, at least 100-fold higher concentrations than those found with ELISA (Table 2) were needed to mimic the complete antiviral effect mediated by GDVII supernatants (Table 3).

Discussion

Effective viral clearance is imperative for the prevention of a persistent infection and requires both vigorous innate and adaptive immune responses.

Table 3 Antiviral activity and neutralization of antiviral activity with anti-IFN antibodies

Culture supernatant	*IFN (ng/ml)*	*anti-IFN*	*antibody (U)*	*% infectivity*
	—	—		2 ± 2
	—	α	(250)	24 ± 4
	—		(1500)	22 ± 8
	—	β	(250)	5 ± 4
	—		(1500)	1 ± 1
GDVII				
	—	γ	(250)	30 ± 1
	—		(1500)	27 ± 2
	—	$\alpha + \beta + \gamma$	(250)	25 ± 5
	—		(1500)	26 ± 4
	—	—		100 ± 1
	—	$\alpha + \beta + \gamma$		102 ± 3
	α 0.25	—		43 ± 2
	0.25	α	(1500)	104 ± 5
	2.5	—		7 ± 1
	25	—		0 ± 0
	β 0.25	—		22 ± 3
PBS	0.25	β	(1500)	103 ± 4
	2.5	—		0 ± 0
	γ 0.25	—		54 ± 6
	0.25	γ	(1500)	97 ± 3
	2.5	—		14 ± 3
	25	—		1 ± 1
	250	—		0 ± 0

Note. Culture supernatants from mock-infected (PBS) and 10 PFU/cell GDVII-infected RAW264.7 macrophages were obtained as described in Materials and methods. Supernatants were either untreated (—) or treated with IFN and/or neutralizing antibodies against IFN (using 250 or 1500 U of each antibody) and subsequently brought on L929 cells for 24 h. After removal of the supernatants, the cells were challenged with the DA strain and the infectivity was determined by plaque assay. Results were expressed relative to the infectivity resulting from priming with untreated mock-infected supernatants, set at 100%. The mean result from three independent experiments ± standard deviation is shown.

Conversely, a failing or less operational immune system would pave the way for viral persistence, therefore a comprehensive understanding of the immune responses that contribute to the clearance of TMEV is of major importance. Several studies have provided evidence that TMEV clearance is mediated by $CD4^+$ as well as $CD8^+$ T cell–adaptive immune responses, depending on the mouse strain (Lin *et al*, 2004; Mendez-Fernandez *et al*, 2003). Although less studied, innate immune responses also play an important role in the control of TMEV infection, as evidenced by the fact that the L protein of both the DA and GDVII strain counteracts the transcription of genes coding for type I interferon, as well as other cytokines and chemokines (Paul and Michiels, 2006) and that a mutation of this L protein impairs the DA's ability to persist (van Pesch *et al*, 2001).

Because macrophages represent the main viral reservoir during the late disease (Lipton *et al*, 1995; Rossi *et al*, 1997), we previously studied TMEV infection of RAW264.7 macrophages and found that the DA strain establishes a persistent infection in these cells with the continuous production of infectious virus, whereas for the GDVII strain, no infectivity could be demonstrated anymore 4 to 5 days p.i. (Steurbaut *et al*, 2006). The loss of GDVII infectivity didn't result from a decrease of the number of viable cells, and neither from a higher intrinsic susceptibility of this strain to viral degradation (S. Steurbaut, B. Rombaut, and R. Vrijsen, unpublished data). These results suggested that the RAW264.7 cells actively participate in the abrogation of a GDVII infection, whereas they are unable to clear a DA infection.

Here we show that the GDVII strain interferes with the ability of the DA strain to induce a persistent infection in the RAW264.7 macrophages, because a double infection of these cells with both the GDVII and DA strain drastically lowered virus titers when compared to infection with the DA strain alone, while equaling those found after a GDVII mono infection. For this reason, competition for a receptor/coreceptor seems an unlikely explanation for this observation, because one would then expect titers in between those obtained after mono infection with the GDVII and DA strain in the case of the double infection. In addition, early replication kinetics of the DA and GDVII strain show a similar profile, demonstrating equal replication efficiencies during the first replication cycle (Steurbaut *et al*, 2006).

Our interpretation is that the GDVII strain triggers a potent antiviral response that also limits the replication of the DA strain during a double infection. This hypothesis is strengthened by the finding that culture supernatants from GDVII-infected RAW264.7 cells conferred five times more protection against a subsequent infection than supernatants from DA-infected cells. From this, it follows that the GDVII-infected macrophages mount a stronger antiviral response than the DA-infected ones, rendering the latter less effective in clearing the infection and hence favoring the establishment of a persistent infection.

Interestingly, Roos *et al* (1982) suggested a correlation between low-level IFN expression and maintenance of a persistent infection with the DA strain in L929 cells. It is thus conceivable that a weak antiviral response could contribute to viral persistence, whereas a high antiviral response will likely restrict and/or clear the infection. Consequently, the strength of the innate antiviral response might be a critical factor for the outcome of the infection. In this respect, the m.o.i. also seems to be of importance, because we found that the potency of the antiviral response is m.o.i. dependent, with less antiviral activity triggered by a decreased m.o.i.. Moreover, the induction of antiviral defenses requires the sensing of viral factors, e.g., dsRNA, by cellular mediators, such as toll-like receptors or PKR (Malmgaard, 2004). Due to the restricted replication of TMEV in macrophages as compared to other cells (Obuchi *et al*, 1999; Martinat *et al*, 2002), the macrophages' antiviral defenses may not be sufficiently triggered, which might additionally

enhance the susceptibility of these cells to be the target of a persistent infection.

The addition of 2-AP, a PKR phosphorylation inhibitor (Hu and Conway, 1993), prior to infection of RAW264.7 cells with the GDVII strain, led to a 10.5- and 31.2-fold increase of infectivity at 72 and 96 h p.i., respectively. We hypothesize that this increase might result from the down-regulation of IFNs, because 2-AP has been shown to prevent the induction of IFN-α and IFN-β at the transcriptional level (Marcus and Sekellick, 1988). In contrast, 2-AP didn't result in a prolonged replication and higher virus titers in RAW264.7 cells infected with the DA strain. This differential, TMEV strain–dependent outcome of 2-AP treatment might be related with the L protein. From data by Paul and Michiels (2006), it appears that quantitative differences exist regarding the suppression of immediate-early IFN genes by the L proteins of the DA and GDVII strain. Although they don't show results for the GDVII strain, DA mutants carrying the L protein from the GDVII strain seem to induce less IFN suppression as compared to the DA strain. Therefore, one could imagine that 2-AP additionally down-regulates the induction of IFN genes in the case of a GDVII infection, whereas suppression is nearly maximum in the case of a DA infection and cannot be further increased with 2-AP.

In line with the above findings, results from IFN-α, -β, and -γ ELISA measurements showed that GDVII cell culture supernatants contained more of each of these cytokines than DA supernatants. These quantitative differences became apparent for IFN-α at 48 h p.i. and at 72 h p.i. for IFN-β and IFN-γ. IFN-α was the most prominently produced interferon and IFN-γ could only be detected in GDVII supernatants.

To evaluate the contribution of IFN-α, -β, and -γ to the antiviral response of RAW264.7 macrophages against TMEV, culture supernatants from GDVII-infected cells were treated with neutralizing antibodies against these cytokines. Treatment with antibodies against IFN-α or IFN-γ abrogated their antiviral potential with about 20% to 30%, whereas anti-IFN-β treatment had no substantial effect. Surprisingly, when combined together, no increase in infectious titers was observed as compared to treatment with anti-IFN-α or anti-IFN-γ alone. The reason for this remains to be determined.

Using neutralizing antibodies against IFNs, we could only abolish maximally 30% of the antiviral response mounted by the infected RAW264.7 macrophages. This contrasts with results from Martinat *et al* (2002), who showed that bone marrow-derived monocytes/macrophages mount an antiviral response against Theiler's DA virus that was fully neutralized with a hyperimmune IFN-α/β antiserum. The seemingly contradiction could be attributed to the use of different TMEV strains and/or qualitative differences in the nature of the antiviral response mounted by bone marrow-derived monocytes/macrophages and RAW264.7 cells, because the intriguing possibility exists that the antiviral activity generated by GDVII-infected RAW264.7 macrophages is mainly mediated by other IFNs and/or by other (unknown) antiviral molecules. Our findings that a complete antiviral effect, as observed with GDVII conditioned supernatants, could only be obtained with IFN concentrations largely in excess of the amounts produced by the cells, and that this antiviral activity was already maximal at 48 h p.i., whereas IFN production as measured by ELISA was rather restricted at that time, favor the latter possibility.

Taken together, we found that the outcome of a TMEV infection, in terms of viral clearance versus the induction of a persistent infection, correlates with the cells' responsiveness to induce antiviral innate immune responses. Understanding of the factors that contribute to elimination of the GDVII strain might also improve knowledge of the mechanism by which the DA strain persists, because this could be due to interference with host innate immune responses resulting in the incomplete clearance of this strain.

Materials and methods

Cells and viruses

RAW264.7 cells, a mouse monocyte-macrophage cell line originally established from a tumor induced by Abelson murine leukemia virus (Raschke *et al*, 1978) and kindly donated by Thomas Michiels (Christian de Duve Institute of Cellular Pathology, UCL, Belgium), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 2.5% fetal bovine serum.

L929 mouse fibroblasts, purchased from ATCC, were grown in minimal essential medium (MEM) supplemented with Earle's salts, nonessential amino acids, 1 mM sodium pyruvate, and 5% horse serum.

BHK-21 cells (ATCC), for the cultivation of TMEV strains GDVII and DA, were grown in Glasgow MEM supplemented with 2% tryptose phosphate broth and 10% newborn calf serum.

All medium components were purchased from Invitrogen.

After plaque purification, stocks of TMEV strains GDVII and DA, originally received from Thomas Michiels, were obtained after three cell passages and concentrated by ultracentrifugation in a TFT 70.38 rotor (Centrikon) for 1 h 30 min at 130, 000 $\times$ g_{av} and 4°C.

Quantification of interferons

The amount of IFN-α, IFN-β, and IFN-γ present in cell culture supernatants was determined using mouse IFN-specific ELISAs, purchased from PBL Biomedical Laboratories (IFN-α and -β) and RayBiotech (IFN-γ). Supernatants from 2.5 $\times$ 10^4 RAW264.7 cells/well grown in 96-well plates were collected and assayed for IFNs according to the manufacturers' instructions. The OD_{450} was measured with a Bio-Tek FL600 microplate reader and the IFN concentration in pg/ml was determined by interpolation

of the mean absorbance of duplicates from two independent samples on a standard curve. For each ELISA kit the experimental detection limit was about 30 pg/ml.

Chemicals and antibodies

IFN-α, IFN-β, and IFN-γ were purchased from HyCult Biotechnology, PBL Biomedical Laboratories, and Peprotech, respectively. Neutralizing rabbit polyclonal antibodies against mouse IFN-α, IFN-β, and IFN-γ were purchased from PBL Biomedical Laboratories, Chemicon International, and BioSource Europe, respectively. 2-Aminopurine nitrate was purchased from Sigma.

Plaque assay

The amount of infectious virus was quantified by a standard plaque assay on monolayer L929 cells as described previously (Steurbaut *et al*, 2006). Briefly, samples from 96-well plates, consisting of culture supernatants and cells were harvested, freeze-thawed thrice, and samples of 200 μl or dilutions in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}12H_2O$, and 1.4 mM $NaH_2PO_4{\cdot}H_2O$; pH 7.4) were brought on the cells. Following viral adsorption for 1 h at 37°C in a 5% CO_2 incubator, the cells were covered with an agar overlay. Plaques were counted 3 days later after formaldehyde fixation and crystal violet staining.

Preparation of conditioned cell culture supernatants and anti-IFN antibody treatment: This protocol is a modification of the protocol employed by van Pesch *et al* (2001). Culture supernatants from RAW264.7 cells, seeded at 6×10^5 cells/well in 6-well plates (Costar) and infected with 10 PFU/cell GDVII or DA, and PBS mock-infected controls, were collected after 48 h and centrifuged for 5 min at 2000 $\times g$ to remove cellular debris. Subsequently, the culture supernatants were submitted to two rounds of ultrafiltration to remove virus. First, they were centrifuged for 15 min at 3500 $\times g$ through a Vivaspin 300-kDa filter (Vivascience), followed by a second centrifugation for 10 min at 3000 $\times g$ through an Amicon Ultra 100-kDa filter (Millipore). Removal of virus was checked after ultrafiltration by plaque assay. Because both filter devices were supplied nonsterile, they were sterilized using 70% ethanol, followed by washing twice with DMEM. A volume of 1.5 ml of the different conditioned supernatants was then brought on monolayer L929 cells seeded at 1×10^6 cells in 60-mm Petri dishes (Greiner Bio-One).

In some experiments, 1.5-ml samples of conditioned supernatants were incubated with 250 and 1500 units (U) of neutralizing antibodies against mouse IFN-α, IFN-β, and/or IFN-γ for 1 h at room temperature prior to cell priming. After 24 h of priming at 37°C, the conditioned supernatants were removed and the cells were infected with the DA strain at about 100 to 200 PFU per dish. Plaque assay was used to determine the extent of infection of cells primed with the different conditioned supernatants.

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