CD40 ligand has potent antiviral activity

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For B cells to make antibodies against most antigens, they require help from T cells. T cell help is delivered as two signals to the B cell, one of which is via CD40 and the other can be through receptors for any of a variety of soluble cytokines. We have constructed recombinant vaccinia viruses that express the ligand for CD40 and have shown that the growth of these viruses is dramatically controlled *in vivo*, even in mice that lack T or B cells. In this paper, we also describe our attempts to analyse the CD40 ligand-mediated antiviral activity by studying the clearance of these viruses in mice that are deficient in important antiviral mechanisms. Thus, the antiviral activity of CD40L may represent a surprising and potent effector mechanism of T cells activated during a virus infection.

Signalling of B cells via the constitutively expressed CD40 molecule is an important component of the T-cell help required for an antibody response[1]. The ligand for CD40 (CD40L or gp39) has been recently cloned[2] and shown to be induced on the surface of T cells as a consequence of antigen recognition. The interaction between CD40L and its receptor on the surface of B cells has been shown to be an essential step in triggering B cells to make antibody[3]. The ligation of CD40 provides the contact-dependent component of T-cell help, while other critical signals are provided by soluble cytokines, such as interleukins (IL) 4 and 5 (ref. 1). Experiments using membranes prepared from either activated T-cells[4] or cells transfected with CD40L (refs 2,5), or soluble gp39 (ref. 6), and recombinant cytokines have shown that these two signals are sufficient to stimulate resting B cells to undergo proliferation and differentiation, and to switch from IgM production to other immunoglobulin classes. The essential role of the CD40L–CD40 interaction in the generation of antibody responses has recently been demonstrated *in vivo*. Mice treated with a neutralizing monoclonal antibody to CD40L were unable to mount primary or secondary antibody responses to T-cell-dependent antigens[3]. Furthermore, defective expression of CD40L in humans has been shown to correlate with the hyper-IgM X-linked immunodeficiency syndrome[7]. This disease is characterized by normal or high levels of immunoglobulin-M (IgM), but other isotypes (IgG, IgA and IgE) are present at very low or undetectable levels, indicating a failure in the ability of T cells to provide the appropriate signals to activate B cells. A similar phenotype has now been described in CD40-deficient mice[8].

We have expressed the genes for a number of cytokines in recombinant vaccinia viruses as a means of studying the immunobiology of these factors *in vivo*[9]. By adopting this approach to studying the role of CD40L, we have been able to demonstrate that this molecule has strong antiviral activity, in addition to its previously known function in the activation of B cells.

Results

Recombinant vaccinia viruses encoding CD40L

To determine whether the provision of CD40L and IL-4 in the presence of antigen would stimulate a specific antibody response *in vivo*, we constructed recombinant vaccinia viruses (vv) encoding these factors. A recombinant virus was constructed that encoded both CD40L and murine IL-4 (vv-IL4-CD40L) and a second construct encoded CD40L and the haemagglutinin gene of influenza virus A/PR-8 (vv-HA-CD40L). CD40L was expressed at high density on the surface of cells infected with viruses encoding this gene, as plasma membranes prepared from such cells were very efficient stimulators of B-cell proliferation (data not shown). The membrane-induced proliferation of B cells was blocked in the presence of CD40-γ_1 fusion protein[10] (data not shown). Virus-encoded

Table 1 Levels of total immunoglobulin and virus-specific IgG in sera of normal or athymic, nude mice infected with vaccinia virus encoding CD40L

Virus	Normal mice		Athymic mice
	Total Ig	vv-specific IgG	Total Ig
vv-HA-TK	6.2	4.3 ± 0.1	5.2 ± 0.2
vv-HA-IL-4	6.2 ± 0.1	4.1	*
vv-HA-CD40L	6.2	3.6 ± 0.2	5.1 ± 0.4
vv-IL-4-CD40L	6.2 ± 0.1	3.8 ± 0.2	5.0 ± 3.0

Groups of five female CBA/H or Swiss outbred nude mice were infected with 10^7 or 5×10^6 PFU, respectively, of recombinant vaccinia virus i.v. Serum was collected from normal mice after 30 days and from nude mice after 6 days. Total serum immunoglobulin or virus-specific IgG levels were measured using ELISA assays[33]. Data are represented as the means of the $\log_{10}$ titre ± s.e.m.
*Infection of nude mice with the control virus, vv-HA-IL4, was lethal within 6 days of infection, owing to the increased pathogenicity of this virus (D. Sharma, A. Ramsay, D. Maguire and I.A.R. manuscript submitted), and the mice died from symptoms of IL-4 toxicity.

CD40L stimulated IgE production by B cells when IL-4 was also present. Three days after the addition of murine B cells to L cells infected with vv-IL4-CD40L, negligible IgE was detected in the medium (<4 ng ml^{-1}); however, after seven days, IgE secretion was significantly increased (26.5 ng ml^{-1}). Only background levels of IgE (<4 ng ml^{-1}) were present in media from B cells cultured with L cells infected with vv-HA-CD40L or the control viruses, vv-HA-IL4 or the construct with thymidine kinase (TK), vv-HA-TK, after three or seven days.

To investigate whether vv-IL4-CD40L could replace the signals provided by T helper (T_H) cells, which stimulate B cells to produce antibody, athymic, nude mice were infected with the virus constructs and their antibody levels measured after six days. There was no significant difference between the levels of total serum immunoglobulin of nude mice infected with vv-IL4-CD40L, vv-HA-CD40L, or vv-HA-TK (Table 1). Similarly, equivalent levels of serum Ig were measured after infection of normal mice with each construct (Table 1). Given our findings

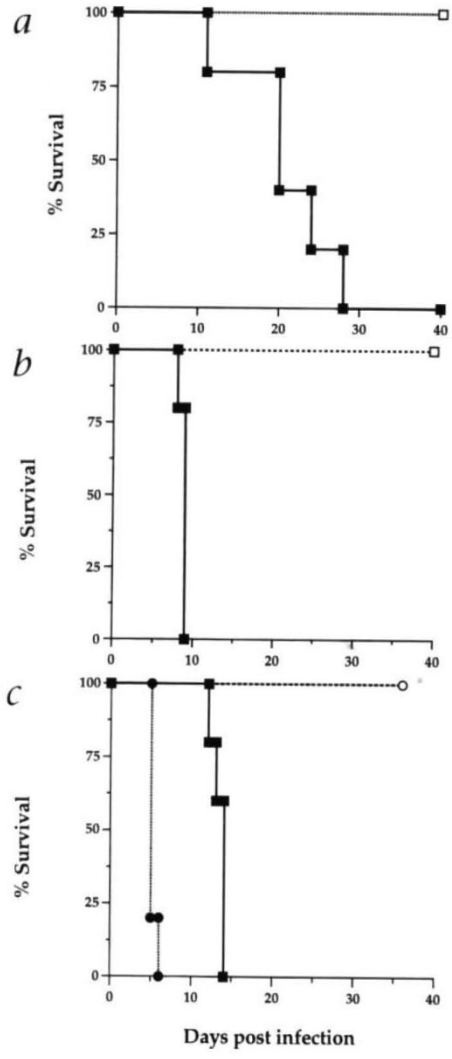

Fig. 1 Survival of immunodeficient mice infected with recombinant vv expressing CD40L; *a*, nude mice, *b*, sublethally irradiated mice (650 rad), *c*, SCID mice. Groups of 5 female mice were infected i.v. with 5 x 10^6 PFU of vv-HA-CD40L (□), vv-HA-TK (■), vv-IL4-CD40L (○) or vv-HA-IL4 (●).

in vitro, it was surprising that infection of normal mice with the CD40L-encoding viruses led to reduced levels of virus-specific IgG, compared with levels in mice infected with either control virus 30 days post infection (Table 1).

The poor antibody responses were consistent with reduced growth of the CD40L-encoding viruses *in vivo*. In fact, these viruses were attenuated to the extent that immunocompromised mice could survive a normally lethal infection. The survival of various immunodeficient mice infected with viruses encoding CD40L or control constructs is represented in Fig. 1. Although infection with either control virus invariably killed mice with severe combined immunodeficiency disease (SCID mice)[11] (Fig. 1*c*), all mice infected with vv-IL4-CD40L survived indefinitely, with no signs of morbidity. The same patterns of survival and lack of morbidity were observed in sublethally irradiated (650 rads) CBA/H mice (Fig. 1*b*) and athymic, nude mice (Fig. 1*a*) infected with the CD40L-encoding viruses. Again, irradiated or nude mice could not resolve infections of either control virus.

Viruses encoding CD40L were rapidly cleared from the organs of nude mice (Fig. 2). Only negligible quantities of virus were recovered from the ovaries four days post infection (p.i.). In contrast, control viruses grew to high titres in the ovaries. The kinetics of virus growth indicate that the clearance mechanism was induced very early since, after only 24 hours, the titres of virus recovered from the ovaries were markedly lower compared with those from mice infected with the control viruses. Indeed, we have no evidence that the CD40L-encoding viruses ever replicate to the same extent as control viruses *in vivo*. This finding contrasts with the clearance of a recombinant vaccinia virus encoding IL-2 (vv-HA-IL2), which was also shown to be attenuated in nude mice[12]. The clearance of vv-HA-IL2 was dependent on the recruitment and expansion of a population of interferon-γ (IFN-γ)-secreting natural killer-like cells[13,14]. In this case, the titres of vv-HA-IL2 and vv-HA-TK recovered from the ovaries were consistent until two to three days after infection. Thus, the antiviral mechanism induced by CD40L is unlikely to involve the recruitment of a host effector cell population.

The antiviral activity of CD40L expressed by recombinant vaccinia virus was neutralized when infected mice were treated with antibody to CD40L (Fig. 3*a*). Four days after infection, we observed a 1,000-fold increase in the growth of vv-HA-CD40L in the ovaries of mice that received a single dose (250 μg) of anti-CD40L. The same treatment had no detectable effect on the growth of the control virus. The anti-CD40L antibody also blocked the generation of primary and secondary antibody responses in mice immunized with T-dependent-type antigens[3].

Host-derived TNF or IFN-γ in the antiviral activity of CD40L

Several of the cytokines produced by activated T cells have potent antiviral activity. Both IFN-γ and TNF directly activate antiviral mechanisms *in vitro*, which limit virus replication in infected cells or induce resistance in uninfected cells. In addition, there is strong synergy between IFN-γ and TNF in their antiviral activity[15]. The antiviral action of CD40L is unclear; it is difficult to propose antiviral mechanisms based on the reported distribution of CD40. CD40 is expressed on the surface of B cells[16], follicular dendritic cells[17], thymic epithelium[18] and some tumour cells[16]. Our finding that CD40L-expressing viruses are rapidly cleared in SCID mice indicates that CD40 expressed on B cells is not necessary for the antiviral activity of CD40L. How-

ever, it is interesting that CD40 has been recently shown to be induced on the surface of monocytes incubated with granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-3, IFN-γ or CD40L[19]. After incubation with CD40L, monocytes were tumoricidal, although the mechanism was unknown. It has also been shown recently that IFN-γ activated macrophages produce nitric oxide (NO), which is likely to be an important antiviral effector mechanism *in vivo*[20]. Mice treated with a NO inhibitor were unable to resolve an otherwise non-lethal infection with ectromelia virus. The observation that plasma membranes of activated T_H cells induced NO secretion by IFN-γ-treated splenic macrophages[21] added credence to the possibility that CD40L–CD40 interaction on activated macrophages may stimulate antiviral activity. Although the role of such a mechanism in the CD40L-mediated effects we report is unknown, the induction of NO does not appear to be an important component. First, the host's production of IFN-γ is not critical to the clearance of CD40L-encoding viruses. This contrasts with the clearance of vv-HA-IL2, which is clearly dependent on IFN-γ[14], and, indeed, the resolution of normal vaccinia and ectromelia viruses in immunocompetent mice, for which IFN-γ is essential[22]. In mice in which the IFN-γ receptor (IFN-γR) has been disrupted[23], vv-HA-CD40L grew to a greater extent than in their wild-type littermates (Fig. 3*b*). However, the virus yield from the IFN-γR-deficient mice infected with vv-HA-CD40L was 1/100th of that from mice infected with vv-HA-TK, despite the essential role for IFN-γ in the control of vaccinia virus. The most compelling evidence for an IFN-γ-independent mechanism in the clearance of vv-HA-CD40L was obtained from nude mice that were infected with vv-HA-CD40L and treated with neutralizing monoclonal antibody to IFN-γ[22]. All athymic nude mice infected with vv-HA-CD40L at 10^7 plaque-forming units (PFU) intravenously (i.v.) and treated with anti-IFN-γ monoclonal antibody survived infection with no signs of morbidity. Conversely, all mice infected with vv-HA-TK and treated with anti-IFN-γ died within 12 days (results not presented). Thus, the increased virus titres in the IFN-γR-deficient mutant mice demonstrate that the clearance of vv-HA-CD40L is partly dependent on IFN-γ; however, the survival data clearly show that CD40L induced an IFN-γ-independent mechanism sufficient to allow the mice to resolve the infection. Secondly, the incubation of IFN-γ-treated murine macrophages (RAW264.7 cells or peritoneal macrophages from normal or virus-infected mice) with plasma membranes from vv-HA-CD40L infected cells, did not stimulate the production of NO (results not presented).

Tumour necrosis factor (TNF) is a very potent antiviral agent. When TNF was expressed by recombinant vaccinia virus, the virus infection was rapidly cleared by mice[24]. Also, several poxviruses have evolved strategies designed to inhibit the action of TNF, for example, myxoma virus encodes a se-

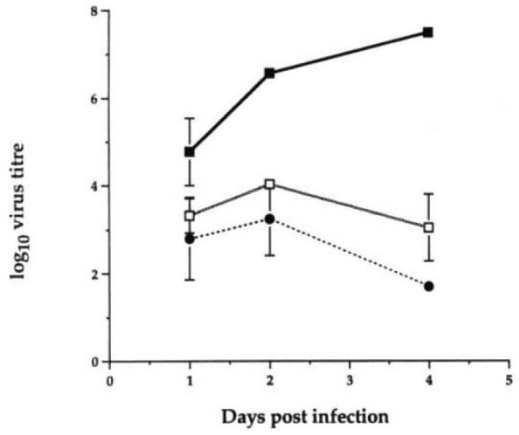

Fig. 2 Kinetics of virus clearance from the ovaries of nude mice infected with recombinant vaccinia virus expressing CD40L. Female Swiss outbred nude mice (5 per group) were infected i.v. with 5 x 10^6 PFU of vv-HA-CD40L (□), vv-HA-TK (■) or vv-IL4-CD40L (●). Mice were killed 1, 2 and 4 days following infection, and the data are shown as mean titres ± s.e.m. of virus recovered from the total ovarian tissue[13].

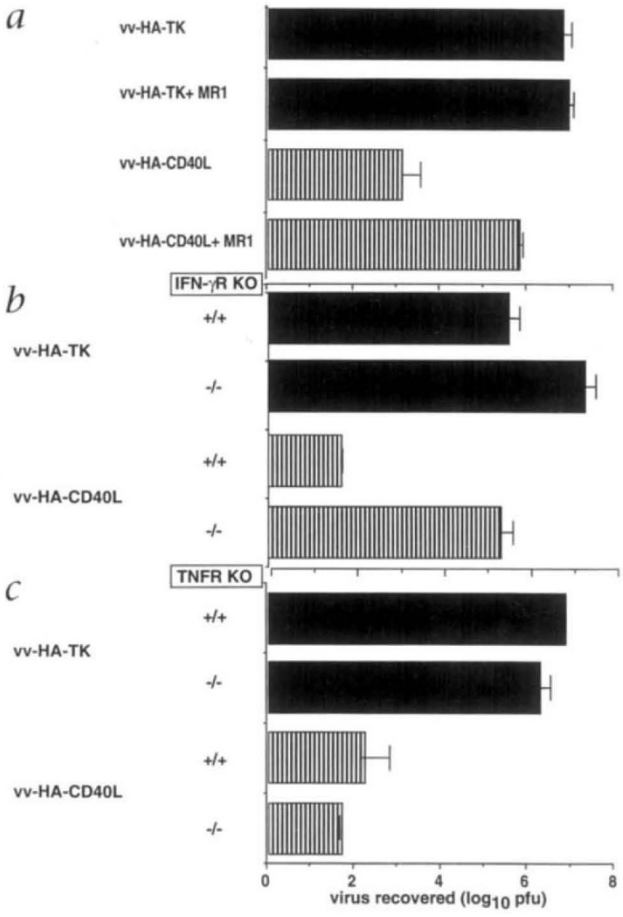

Fig. 3 *a*, Growth of recombinant vaccinia virus encoding CD40L in the ovaries of mice treated with anti-CD40L. Groups of 4 female CBA/H mice were infected with 10^7 PFU of vv-HA-CD40L (striped bars) or vv-HA-TK (solid bars) i.v. Some groups were also treated with a single dose of 250 μg anti-CD40L (MR1[3]; Pharmingen, San Diego) i.p. immediately after infection. *b*, Growth of vv-HA-CD40L or vv-HA-TK in IFN-γ receptor-deficient mice[23] or *c*, TNF receptor 1 (ref. 26) gene targetted mice. Groups of 5 female wild-type (+/+) or gene knockout (-/-) mice were infected with 10^7 PFU of vv-HA-CD40L (striped bars) or vv-HA-TK (solid bars) i.v. The mice were killed 4 days after infection and virus growth was assessed in the ovarian tissue[13]. Data are shown as mean titres ± s.e.m. of virus recovered from the total ovarian tissue.

creted protein (T2), which is homologous to the TNF receptor (TNFR)[25]. In the case of myxoma virus infection, the antiviral role of TNF has been clearly demonstrated, as the deletion of T2 resulted in attenuation of the virus *in vivo*[25]. We have questioned the role of TNF in the antiviral activity of CD40L by studying the growth of vv-HA-CD40L in different models of TNF deficiency. First, mice rendered genetically defective for type 1 TNF receptor (TNFR1)[26] were able to clear vv-HA-CD40L as rapidly as their wild-type littermates (Fig. 3*c*), maintaining the striking attenuation of the CD40L-encoding virus. Second, TNF antagonists that were shown to neutralize other TNF-mediated effects in mice did not inhibit the attenuation of vv-HA-CD40L (see Methods; results not presented). Taken together, these observations do not support a significant role for TNF in the antiviral activity of CD40L.

CD40L has antiviral activity *in vitro*

Evidence that CD40L can act in a directly antiviral manner was obtained *in vitro*. L929 cells were infected with herpes simplex virus (HSV-1) and treated with CD40L, which was prepared as membranes from fibroblasts infected with vv-HA-CD40L. The CD40L membrane preparation inhibited the replication of HSV-1 to the same extent as recombinant murine TNF that was titrated for optimal antiviral activity in this assay (Table 2). The anti-HSV activity of the CD40L preparation was present at dilutions at which the B-cell-stimulating activity was no longer present. Similar levels of anti-HSV activity were also observed in chick embryo fibroblasts (data not shown).

Discussion

It is clear that cytokines such as IFN-γ and TNF are very important components of the antiviral effector mechanisms available to the host[9]. Here we report that another product of activated T cells, CD40L, has potent antiviral activity. Analysis of the growth of recombinant viruses encoding CD40L in various mouse models suggests that the CD40L-mediated antiviral activity is not critically dependent on the major host antiviral strategies. The kinetics of virus clearance in immunodeficient mice indicates that the antiviral mechanism of CD40L is rapidly activated and independent of lymphocytes. Furthermore, in mice that were deficient in the receptors for the antiviral cytokines, TNF and IFN-γ, virus expressing CD40L was still attenuated. Independence from TNF was clearly demonstrated in TNFR1-deficient mice and in mice treated with neutralizing agents to TNF. However, the attenuated growth of vv-HA-CD40L appeared to be reversed to some extent in IFN-gR deficient mice. The interpretation of this finding is complicated by the fact that the clearance of vaccinia virus is absolutely dependent on IFN-γ. Since mice in which the IFN-γ response was neutralized were still able to survive the infection, we suggest that a component of the CD40L-induced antiviral mechanism is independent of IFN-γ. Thus, the clearance of CD40L-encoding viruses does not appear to be critically mediated via either TNF or IFN-γ. CD40L was also shown to inhibit the replication of herpes simplex virus *in vitro*, suggesting that CD40L may be directly antiviral.

In vitro studies have shown that after activation virtually all $CD4^+$ and a proportion of $CD8^+$ T cells expressed CD40L[27] and, the ligand may be constitutively expressed on mast cells and basophils[28]. We are currently investigating the expression of CD40L during virus infection which should help to elucidate its role. The defective expression of CD40L in patients with X-linked hyper-IgM syndrome is associated with a very high incidence of infection. Not all clinical findings in these patients are clearly linked to antibody deficiencies. In one survey, 12% of patients had pneumonia due to *Pneumocystis carinii*, an infection usually associated with T-cell immunodeficiencies[29]. Furthermore, some defects observed in these patients are not reversible by treatment with intravenous immunoglobulin, in contrast to other humoral immunodeficiencies. The findings presented in this paper suggest that CD40L may also act as a potent antiviral effector mechanism.

Methods

Recombinant vaccinia viruses. Recombinant vaccinia viruses were constructed according to standard procedures[9]. The construction of vv-HA-TK and vv-HA-IL-4 has been described elsewhere[12] (D. Sharma, A. Ramsay, D. Maguire & I.A.R., manuscript submitted). The cDNA encoding CD40L was kindly provided by Marilyn Kehry (Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut). The cDNA was completely sequenced after PCR amplification from a D10.G4.1 cDNA library. The CD40L coding sequence was excised from pCDSRα[30] with *Eco*RI and inserted into the *Hin*cII site of the plasmid vector pBCBO7. An *Eco*RI fragment, comprising the CD40L coding sequence immediately downstream of a P7.5 vaccinia virus promoter, was cut from this vector and ligated into pFB-TK. The resultant plasmid was used in a marker rescue with vv-HA-PR8, which carries the haemagglutinin (HA) gene of influenza virus A/PR/8-34, or vv-IL4, which encodes the murine IL-4 gene and is described elsewhere (D. Sharma, A. Ramsay, D. Maguire and & I.A.R., manuscript submitted). *In vitro* homologous recombination with vv-HA-PR8 gave rise to vv-HA-CD40L, which contained the genes for CD40L and thymidine kinase (TK) of herpes simplex virus (HSV), as a selectable marker, in the *Hin*dIII F region, and the HA gene in the *Hin*dIII J region. Alternatively, vv-IL4-CD40L was obtained from homologous recombination with vv-IL4. The correct position of the inserted

Table 2 Growth of HSV-1 (log_{10} PFU) in L929 cells after pretreatment with membranes expressing CD40L

Treatment	Multiplicity of infecting virus 10	1.0	0.1
nil	5.5	4.6	3.5
CD40L1:256	4.4	3.5	2.3
1:1000	4.7	3.5	2.3
1:2000	5.1	4.6	2.9
control1:256	5.5	5.1	3.8
1:1000	5.6	4.9	3.9
1:2000	5.3	4.7	3.2
TNF 100 ng ml^{-1}	4.8	3.5	2.3

Murine L929 cells were seeded into six-well plates (1×10^6 cells per well) in medium containing membranes prepared from 143B cells infected with vv-HA-CD40L or vv-HA-TK at the indicated concentrations or TNF or left untreated. After 24 hours of pretreatment with membranes, recombinant murine TNF (kindly provided by G. Adolf, Boehringer Ingelheim, Vienna) or no treatment, medium was removed and L929 cells were infected with HSV-1 at the multiplicities shown for 1 hour. Infected cells were then incubated in fresh medium for 48 hours, after which HSV was titrated by plaque assay. The results shown are representative of two separate experiments.

genes was confirmed by restriction analysis and Southern blotting. Expression of HA was confirmed by immunofluorescence and the biological activity of IL-4 was confirmed in proliferation assays using CT.4S cells. The biological activity of vaccinia virus-encoded CD40L was established in B-cell proliferation assays[10] using plasma membranes[4] prepared from 143B cells infected with vaccinia virus encoding CD40L or control constructs after overnight infection at 5 PFU per cell. CD40L expressed by cells infected with recombinant vaccinia virus also induced B cells to secrete IgE in the presence of IL-4 (see text).

Preparation of cell membranes expressing CD40L. Membranes were prepared[31] from human 143B cells, which were infected overnight with vv-HA-CD40L or vv-HA-TK at a multiplicity of 5. Residual vaccinia virus in the membrane preparations was inactivated by psoralen-UVA treatment. Inactivation of vaccinia virus was confirmed by plaque assay and biological activity of the membranes was standardized in a B-cell proliferation assay[10].

TNF antagonists. A polyclonal antibody to murine TNF was raised in rabbits, and IgG was partially purified using sodium sulphate. An inoculum of 0.3 ml intraperitoneally (i.p.) protected mice from bacterial lipopolysaccharide challenge. SCID mice were infected with 10^7 PFU of vv-HA-TK or vv-HA-CD40L intravenously (i.v.) and injected i.p. with 0.3 ml antibody. Mice were killed 3 days later. Although no difference in the quantities of virus recovered from the ovaries of anti-TNF treated or untreated mice was found, the induction of reactive nitrogen intermediates in the serum in response to the infection were inhibited by anti-TNF treatment (M. Rolph, pers. commun.). Soluble TNF receptors (human p80 linked to the Fc portion of human IgG1) were a gift from M. Widmer and M. Spriggs, Immunex Corp., Seattle, Washington. TNFR1 and wild-type mice were infected with 10^7 PFU of vv-HA-TK or vv-HA-CD40L i.v. and injected daily with 100 µg soluble TNFR. Mice were killed after 3 days, and virus infectivity in the ovaries was titrated. This dosage of soluble TNFR inhibited lethal endotoxemia[32] and in our laboratory abrogated the antiviral activity of human TNF in mice.

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