

## **Antiviral Activity of Tumor Necrosis Factor (TNF) Is Mediated via p55 and p75 TNF Receptors**

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### **Summary**

The antiviral nature of tumor necrosis factor (TNF) is generally well accepted. TNF appears to induce multiple antiviral mechanisms, and to synergize with interferon (IFN)- $\gamma$  in promoting antiviral activities. We infected TNF receptor (TNFR)-deficient mice with the virulent murine pathogen, ectromelia virus (EV), and observed that otherwise resistant mice were susceptible to lethal infection. To study the molecular basis of the antiviral action of TNF, mice were infected with a recombinant vaccinia virus encoding murine TNF (VV-HA-TNF). In normal mice, the replication of VV-HA-TNF was highly attenuated. In contrast, mice in which the TNFR type 1 (p55) or the TNFR type 2 (p75) were genetically disrupted showed a moderate defect in their capacity to clear the TNF-encoding virus. The contribution of both TNF receptors to the control of VV-HA-TNF was confirmed by the enhanced replication of VV-HA-TNF in mice deficient for both p55 and p75. These observations were corroborated by infecting TNFR-deficient mice with EV. For both infections, the p55 and p75 TNFRs were necessary to maintain normal levels of resistance. Thus, the antiviral activity of TNF is mediated via both TNFRs *in vivo*. Furthermore, these studies establish that TNF is an important component of the host response to a natural virus infection.

Many studies have demonstrated the antiviral potential of TNF *in vitro*. Since recombinant TNF became available 10 yr ago, it has been reported that TNF treatment of some cell types reduced the infectivity of a number of viruses (1–3) and synergized with IFN- $\gamma$  to promote antiviral function (1, 4).

Various studies of the effects of TNF on virus infection have also illustrated antiviral activity *in vivo*. For instance, the coexpression of TNF and viral proteins from a recombinant vaccinia virus (VV) lead to rapid attenuation of the infection in mice (5). When mice were treated with TNF before murine cytomegalovirus infection, the antiviral effects of IFN- $\gamma$  were enhanced (6). However, the overall picture obtained from anti-TNF treatment of virus-infected mice is not clear and may reflect virus-specific differences with respect to TNF sensitivity. In some cases, anti-TNF treatment correlated with increased virus replication (7) and reduced survival of murine cytomegalovirus-infected mice (8). Other studies have reported no effects of anti-TNF treatment on influenza virus (9) or lymphocytic choriomeningitis virus (LCMV; 10) replication in mice, although inflammation was reduced in some infections (9, 11).

Perhaps the most compelling support for an antiviral role for TNF comes from the demonstration that different virus families encode factors which target TNF-dependent activities. Several of the poxviruses encode soluble versions of TNF receptors (12–14) and importantly, disruption of the viral TNFR gene in myxoma virus resulted in reduced virulence *in vivo* (13). Adenoviruses have been shown to encode multiple genes that can block the cytotoxic effects of TNF (15). A further anti-TNF strategy appears to be encoded in the genomes of molluscum contagiosum virus and equine herpesvirus type 2, both of which contain homologues of death effector domains involved in p55-mediated signaling (16). Overexpression of these viral genes inhibited TNF-induced apoptosis. However, despite *in vitro* and *in vivo* evidence of TNF-mediated antiviral activity, direct evidence of a physiological role for TNF in the control of virus infection is rare.

The activities of TNF are mediated by binding to two receptors: TNFR1, or p55, and TNFR2, or p75 (for review see Aggarwal and Natarajan, reference 17). In line with the majority of TNF-triggered events, most studies *in vitro* suggested the dominance of p55 and no role for p75 in host antiviral activity (17, 18). However, no defect was

observed in the antiviral responses of TNFR1-deficient mice infected with VV or LCMV (19).

The studies described here aim to establish whether TNF plays a determining role in the physiological control of a virus infection and to define the receptor specificity of the antiviral activity of TNF.

## Materials and Methods

**Mice.** Mice bearing genetic mutations in TNFR1/p55 (20) and TNFR2/p75 (21) have been previously described. Double TNFR-deficient mice were generated by the appropriate intercrosses of p55 and p75 mutant mice (22, 23). All TNFR-deficient mice used in these studies were hybrids between C57BL/6 and 129/Sv (B6 × 129). Control mice were (B6 × 129)F2. The derivation of p75<sup>-/-</sup> and p55<sup>-/-</sup>p75<sup>-/-</sup> mice has been described elsewhere (21–23). All mice were bred and housed under specific pathogen-free conditions at the Animal Breeding Establishment, John Curtin School of Medical Research (Canberra, Australia). Genotypes of the mutant and wild-type mice were confirmed by PCR. Mice used in these studies were between 6 and 12 wk of age. Previous data show that both strains contribute to resistance to ectromelia virus (EV) and that B6 × 129 are more resistant than either parent (Ruby, J., unpublished results). Both B6 and 129 strains also contribute to resistance to VV and increased resistance to VV is seen in B6 × 129.

**Viruses.** The construction of the recombinant vaccinia viruses, VV-HA-TNF and VV-HA-TK (herpes simplex virus thymidine kinase gene), has been previously reported (5, 24). Both viruses also encode the hemagglutinin (HA) gene from influenza virus (A/PR-8). Stocks of rVV were prepared as crude stocks from CV-1 cells. EV (Moscow strain) was prepared as a crude stock from BSC-1 cells.

**Infection of Mice and Assay of Virus Replication.** To study replication of VV-HA-TNF or VV-HA-TK, female mice were inoculated intravenously with 10<sup>7</sup> PFU of rVV in 200 μl saline. After 3 d, mice were killed and ovaries were harvested for titration of virus using a plaque assay on 143B cells (25). Mice treated with soluble (s)TNFR received a fusion protein of human TNFR2/p75 linked to the Fc portion of human IgG1 (26). Mice were inoculated intraperitoneally with 300 μg sTNFR on days 0, 1, and 2 after infection and with VV-HA-TNF on day 0. Mice were killed and the ovaries collected on day 3. To investigate EV replication, male mice were infected in the right hind footpad with 5 × 10<sup>3</sup> PFU of EV in a volume of 20 μl using a 30 gauge needle. Mice infected with EV were killed 7 d after infection and liver, spleen, and inoculated foot (amputated at the first joint) were harvested. EV was titrated using BSC-1 cells (27). The limit of sensitivity of the infectious plaque assay is 100 PFU. Values below the level of plaque assay sensitivity were assigned a value of 50 PFU.

## Results

**Residual TNF-mediated Antiviral Activity in p55<sup>-/-</sup> Mice.** It has been previously shown that overexpression of TNF by an rVV caused an attenuated infection in mice (5). To determine whether the enhanced clearance of rVV encoding the gene for murine TNF (VV-HA-TNF) was dependent on p55, mice deficient for this receptor were infected with VV-HA-TNF. In three separate experiments, a small but significant ( $P < 0.05$ ) increase in VV-HA-TNF replica-

**Table 1.** Replication of rVV in p55<sup>-/-</sup> Mice

	Virus recovered (log <sub>10</sub> PFU)*	
	VV-HA-TNF	VV-HA-TK
Wild type	1.8 ± 0.1	6.9 ± 0.0
p55 <sup>-/-</sup>	2.8 ± 0.5 <sup>§</sup>	6.3 ± 0.1
p55 <sup>-/-</sup> + sTNFR <sup>‡</sup>	4.2 ± 0.4 <sup>  </sup>	ND

\*Mice were infected with 10<sup>7</sup> PFU of rVV intravenously. Virus replication was assessed in the ovaries of mice 3 d after infection. Data shown represent mean titers ± SEM of virus present in total ovarian tissue of 5–10 individual mice. The limit of detection of the plaque-forming assay was 100 PFU and samples below this level were assigned a value of 50 (1.7 log<sub>10</sub>) PFU. Data are representative of three separate experiments.

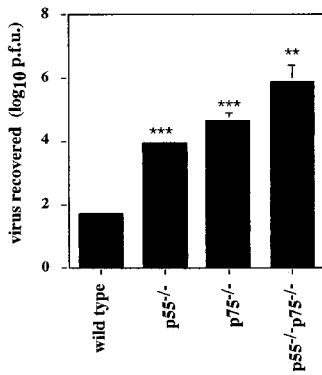
<sup>‡</sup>Mice treated with sTNFR received 300 μg intraperitoneally on days 0, 1, and 2 after infection.

<sup>§</sup> $P < 0.05$ , compared to wild-type mice infected with VV-HA-TNF, Student's *t* test.

<sup>||</sup> $P < 0.05$ , compared to p55<sup>-/-</sup> mice infected with VV-HA-TNF.

tion was measured in p55-mutant compared to wild-type mice (Table 1). However, the increased replication of VV-HA-TNF in p55<sup>-/-</sup> mice represented only partial reversal of attenuation since the yield of the control virus construct, VV-HA-TK, in these mice was three orders of magnitude higher (Table 1). The finding that VV-HA-TNF replication was limited in p55<sup>-/-</sup> mice was surprising given that p55 has been shown to mediate the antiviral activity of TNF in vitro (18). Despite the lack of p55, TNF was able to activate antiviral mechanisms which resulted in the rapid resolution of VV infection. The attenuation of VV in this model was dependent on TNF since treatment of p55-mutant mice with sTNFR resulted in significantly increased replication of VV-HA-TNF ( $P < 0.05$ ; Table 1). Thus, the TNF-dependent inhibition of VV-HA-TNF replication was not solely mediated by p55.

**Both p55 and p75 TNFRs Contribute to the Attenuation of VV-HA-TNF.** To determine the relative roles of p55 and p75 in the attenuation of VV-HA-TNF, mice bearing mutations in either or both TNFRs were infected with VV-HA-TNF. The yields of virus recovered from TNFR-deficient mice indicated that TNF bound both p55 and p75 to initiate antiviral mechanisms (Fig. 1). Although the levels of virus were undetectable in the ovaries of wild-type mice 3 d after infection with VV-HA-TNF, elevated replication occurred in either p55- or p75-mutant mice ( $P < 0.001$ ). The contribution of both receptors to the attenuation of VV-HA-TNF was confirmed by the increase in virus replication in mice deficient for both TNFRs compared to wild-type mice ( $P < 0.01$ ). Indeed, the attenuation of VV-HA-TNF was completely abrogated in the double TNFR<sup>-/-</sup> mice, as the rVV replicated to the same extent as the control virus, VV-HA-TK (Table 1 and Fig. 1). There is no evidence from these experiments to suggest that the antiviral activity of TNF was mediated by mechanisms other than



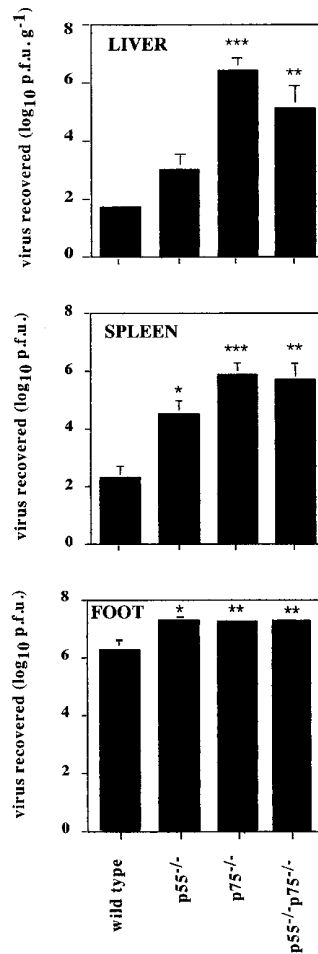
**Figure 1.** Replication of VV-HA-TNF in TNFR mutant mice. Groups of five female mice of the strains indicated were infected intravenously with  $10^7$  PFU of VV-HA-TNF. The virus was recovered from the ovaries of mice 3 d after infection. Data represent the mean titer  $\pm$  SEM for the total ovarian tissue of five individual mice and are representative of four separate experiments. The limit of detection of the plaque-forming assay was 100 PFU and samples below this level were assigned a value of

50 ( $1.7 \log_{10}$ ) PFU. \*\* $P < 0.01$ , compared to wild-type mice, Student's *t* test. \*\*\* $P < 0.001$ , compared to wild-type mice.

via p55 or p75. These data indicate that both p55 and p75 are necessary and sufficient to limit the replication of VV-HA-TNF. In addition, the replication of VV-HA-TNF was significantly greater in p75<sup>-/-</sup> and p55<sup>-/-</sup>p75<sup>-/-</sup> mice compared to p55<sup>-/-</sup> mice ( $P < 0.05$ ; Fig. 1).

**Impaired Antiviral Responses in p55<sup>-/-</sup> and p75<sup>-/-</sup> Mice.** As described above, the overexpression of TNF could affect antiviral activity. Therefore, we sought evidence of a role for TNF in the physiological control of virus infection. Various strains of TNFR mutant mice were infected with the natural murine pathogen EV. The mice used in these studies were genetically resistant to EV; both C57BL and 129 backgrounds contribute to resistance to EV and B6  $\times$  129 mice were more resistant than either parent strain (Ruby, J., unpublished data, and Karupiah, G., personal communication). Consistent with the resistant phenotype, no virus was detected in the livers of wild-type mice 7 d after infection (Fig. 2). In contrast, virus was demonstrated in the livers of mice with mutations in either TNFR. The level of EV recovered from the livers of p55<sup>-/-</sup> mice was consistently higher than that in wild-type mice, although the difference was not statistically significant. However, significantly higher yields of EV were obtained from the livers of p75<sup>-/-</sup> ( $P < 0.001$ ) and p55<sup>-/-</sup>p75<sup>-/-</sup> mice ( $P < 0.01$ ) relative to wild-type mice (Fig. 2). Furthermore, EV replicated to a significantly greater extent in the livers of p75<sup>-/-</sup> mice compared to p55<sup>-/-</sup> mice ( $P < 0.01$ ; Fig. 2).

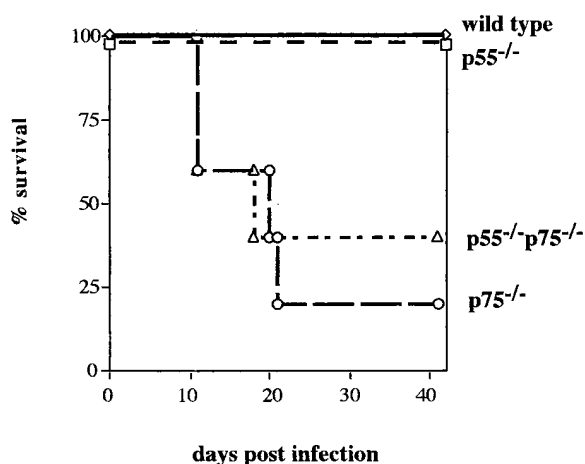
Increased levels of virus replication were also measured in the spleens of TNFR mutant mice, compared to control mice, 7 d after infection (Fig. 2). In this organ, significantly higher EV titers were measured in p55<sup>-/-</sup> ( $P < 0.05$ ), p75<sup>-/-</sup> ( $P < 0.001$ ), and p55<sup>-/-</sup>p75<sup>-/-</sup> mice ( $P < 0.01$ ) compared to wild-type mice. Very high titers of virus were obtained from the inoculated feet of all strains of mice, consistent with the persistence of EV at this site (28, 29). However, TNF did play a noticeable role in the control of virus replication at this site with significantly higher replication in p55<sup>-/-</sup> mice ( $P < 0.05$ ) and each of the p75-deficient strains ( $P < 0.01$ ; Fig. 2). Observations of morbidity of the TNFR mutant mice reflected the yields of virus recovered. The most dramatic effects were seen in p55<sup>-/-</sup> p75<sup>-/-</sup>



**Figure 2.** Replication of EV in TNFR<sup>-/-</sup> mice. Groups of five male mice of the strains indicated were infected with  $5 \times 10^3$  PFU of EV (Moscow) in the right hind footpad. The mice were killed 7 d after infection and virus was assayed in the liver, spleen, and inoculated foot. Data are representative of the mean virus titer  $\pm$  SEM of the tissues shown for five individual mice. For the liver, titers are expressed per gram of tissue. Virus was recovered from the entire spleen and foot. Morbidity of these mice is discussed in the text. Similar data were obtained in a duplicate experiment in which mice were infected with  $10^4$  PFU of EV (data not shown). The limit of detection of the plaque-forming assay was 100 PFU and samples below this level were assigned a value of 50 ( $1.7 \log_{10}$ ) PFU. \* $P < 0.05$ , in comparison with wild-type mice, Student's *t* test. \*\* $P < 0.01$ , in comparison with wild-type mice. \*\*\* $P < 0.001$ , in comparison with wild-type mice.

mice which showed signs of severe morbidity (ruffled coat, hunched appearance) 2 d after infection. At the time of harvest, the popliteal lymph node draining the inoculation site remained intact only in the control mice. Thus, defects in either TNFR introduced susceptibility to EV.

To further test the antiviral role of the two TNFRs, survival of TNFR mutant mice infected with EV was monitored. Wild-type mice recovered from infection with high doses ( $5 \times 10^3$  PFU) of the virulent Moscow strain of EV. Prominent swelling of the inoculated footpad was observed in the wild-type mice. However, the footpad resumed a normal appearance  $\sim 14$  d after infection. There were no other visible signs of morbidity in these mice. In contrast, clinical signs of susceptibility to EV, ranging from atrophy of the infected foot to skin lesions and even mortality, were seen in the TNFR mutant mice. Like the wild-type mice, all p55<sup>-/-</sup> mice survived infection with EV (Fig. 3). However, the reduced resistance of these mice was indicated by the symptoms of mousepox observed. Within 10 d of infection, the inoculated foot of all p55<sup>-/-</sup> mice was severely atrophied. Tail lesions were apparent in some (20%) mice after 17 d, but these were resolved at the termination of the experiment (42 d after infection). Mice lacking p75 were significantly more susceptible to EV, with most (80%) mice succumbing to lethal infection in the period from 10 to 21 d



**Figure 3.** Survival of TNFR<sup>-/-</sup> mice infected with EV. Groups of five male mice of various strains were infected with  $5 \times 10^3$  PFU EV in a volume of 20  $\mu$ l via the footpad. The strains of mice infected were C57BL/6  $\times$  129 (wild-type control,  $\diamond$ ; p55<sup>-/-</sup>,  $\square$ ; p75<sup>-/-</sup>,  $\circ$ ; and p55<sup>-/-</sup>p75<sup>-/-</sup>,  $\triangle$ ). Similar observations were made in a duplicate experiment. Morbidity and mortality is discussed in the text.

after infection (Fig. 3). Morbidity and mortality of p55<sup>-/-</sup>p75<sup>-/-</sup> mice was consistent with the increased susceptibility of p75<sup>-/-</sup> mice. EV infection was lethal for the majority (60%) of p55<sup>-/-</sup>p75<sup>-/-</sup> mice, which died between 10 and 17 d after infection (Fig. 3). Atrophy of the inoculated hind foot was evident in all mice within 8 d of infection. At the termination of the experiment, the surviving mice showed generalized skin lesions.

## Discussion

Using two different systems of virus infection of mice, we have demonstrated that both p55 and p75 TNFR mediate the antiviral function of TNF in vivo. In an experimental model, the overexpression of TNF during infection with VV was previously shown to lead to rapid elimination of the virus (5). This effect was partially dependent on p55 since the attenuation of VV was reversed to some extent in p55<sup>-/-</sup> mice. However, a component of the antiviral mechanism was independent of p55 since treatment of these mice with sTNFR caused further reversal of the attenuation. The antiviral effects of TNF were totally inhibited in mice lacking both TNFRs; in these mice VV-HA-TNF replicated to the same extent as the control virus. Hence, both p55 and p75 were required to activate TNF-dependent antiviral activity. This conclusion was corroborated by studies of the natural murine pathogen, EV, in TNFR<sup>-/-</sup> mice. The capacity of TNFR mutant mice to resolve a normally self-limiting infection was impaired. The increased replication of EV in the target organs (liver and spleen) of p55<sup>-/-</sup> mice and the clinical symptoms and morbidity consistent with mousepox indicated that p55 plays a role in resistance to this virus. However, the importance of p75 in the antiviral response was highlighted by the increased susceptibility of p55<sup>-/-</sup>p75<sup>-/-</sup> mice; the lack of both TNFRs resulted in

high morbidity and mortality in otherwise resistant mice. Together, these studies indicate that both TNFRs are necessary and sufficient to mediate the antiviral effects of TNF.

Previously, a normal immune response to VV or LCMV was reported (19) in the TNFR1/p55<sup>-/-</sup> mice used in this study. We also found that the replication of the control VV construct was not increased in p55<sup>-/-</sup> mice (Table 1). VV exhibits only low virulence in mice, and lack of IFN- $\gamma$  or IFN- $\alpha/\beta$ , or loss of function of CD8 T cells, is the only factor shown to increase virulence of wild-type VV in resistant mice (25, 27, 30, 31). Consistent with a less important role for TNF in the host response to VV, the sTNFR homologue is truncated in the VV genome (32). Based on the data from this and other studies, it is possible to compare the relative importance of antiviral cytokines. For instance, while TNF clearly plays a determining role in resistance to EV, it is less critical than IFN- $\gamma$ . When resistant B6 mice were infected with a similar dose of EV, 100% of mice treated with neutralizing anti-IFN- $\gamma$  antibodies died within 7 d of infection (27). Although EV was lethal for 60–80% of mice deficient for both TNFRs, the survival period was greatly extended, to  $\sim$ 20 d.

The majority of TNF-dependent functions have been attributed to signal transduction via TNFR1/p55 (17). In contrast, a minor or accessory role has been proposed for TNFR2/p75, with many studies supporting a ligand-passing role for p75 (19, 33, 34). In this scenario, p75 acts to lower the effective concentration of TNF necessary to activate p55 by binding TNF and passing it to p55. However, a distinct role for p75 in mediating cytotoxicity has been suggested in addition to an accessory role (34). In particular, TNF-dependent activation-induced cell death of T cells may be triggered by p75 (21, 35). The use of TNFR-specific antibodies has suggested that both receptors were required for the cutaneous inflammatory response after intradermal injection of TNF (36) and that cytotoxicity was greatly enhanced when both TNFRs were activated (37). These cases suggest that cooperative signaling of TNFR may occur independently of a ligand-passing mechanism. The formation of p55/p75 heterocomplexes has been proposed as an alternative mechanism to describe cooperative signaling between TNFRs (38). In TNF-treated cells, TNFR-specific mAb coprecipitated both p55 and p75. This finding suggests a possible mechanism for the interaction of signaling molecules downstream of either receptor.

A constant observation in mice infected with VV-HA-TNF or EV was that the defect in the antiviral response of p75<sup>-/-</sup> mice was greater than that of p55<sup>-/-</sup> mice. Although it is well established that the soluble 17-kD form of TNF binds p55 with higher affinity, it has recently been proposed that the membrane-bound 26-kD TNF binds predominantly to p75 (39). It is possible that the dominant role played by p75 in the antiviral studies we report reflects an important role for membrane-bound TNF in activating antiviral activity. In this regard, TNF expressed from cells infected with VV-HA-TNF remained cell-associated 24 h after infection and only reached maximal levels in the supernatant after 48 h (5), when lysis of the infected cells occurred.

This contrasts with maximal supernatant levels of secreted cytokines, e.g., IL-2 from similar VV constructs within 12 h of infection (24). Furthermore, the impaired response of p55<sup>-/-</sup>p75<sup>-/-</sup> mice to virus infection was not different from that of p75<sup>-/-</sup> mice. Thus, there was no evidence of synergistic or cooperative interaction between the two TNFRs to support a ligand-passing role for p75 or a role for p55-

p75 complex formation in signaling antiviral activity. These findings suggest that p75 plays an independent role in signaling antiviral activity and that p75 appears to be more important than p55 in the control of poxvirus infection. We are currently attempting to address the question of whether different antiviral mechanisms are mediated via each of the TNFRs.

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