TNF Protects Resistant C57BL/6 mice Against Herpes Simplex Virus Induced Encephalitis Independently of signaling via TNFR1 or TNFR2

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ABSTRACT

Tumor necrosis factor (TNF) is multifunctional cytokine that has a role in induction and regulation of host innate and adaptive immune responses. The importance of TNF antiviral mechanisms is reflected by the diverse strategies adopted by different viruses, particularly members of the herpesvirus family, to block TNF responses. TNF binds and signals through two receptors, Tnfrsf1a (TNFR1, or p55) and Tnfrsf1b (TNFR2, or p75). We report here that HSV-1 infection of TNF−/− mice on the resistant C57BL/6 genetic background results in significantly increased susceptibility (p<0.0001, LogRank test) to fatal HSV encephalitis (HSE) and prolonged persistence of elevated levels of virus in neural tissues. In contrast, although virus titers in neural tissues of p55−/− N13 mice were elevated to levels comparable to the TNF−/− mice, the p55+/− N13 mice were as resistant as control C57BL/6 mice (p>0.05). The incidence of fatal HSE was significantly increased by in vivo neutralization of TNF using a soluble TNFR1 receptor (sTNFR) or depletion of macrophages in C57BL/6 mice (p=0.0038 and p=0.0071, respectively). Strikingly, in vivo neutralization of TNF in HSV-1 infected p55−/−p75−/− mice using three independent approaches (soluble p55 receptor, anti-TNF mAb, or in vivo siRNA against TNF) resulted in significantly increased mortality (p=0.005), comparable in magnitude to that in C57BL/6 mice treated with sTNFR1 (p=0.0018). Overall, these results indicate that while TNF is required for resistance to fatal HSE both p55 and p75 receptors are dispensable. Precisely how TNF mediates protection against HSV-1 mortality in p55−/−p75−/− mice remains to be determined.
INTRODUCTION

Early innate and subsequent adaptive immune responses to viral and bacterial pathogens are critically dependent on the TNF superfamily of cytokines. These TNF superfamily cytokines act as effectors of host defense, regulate peripheral lymphoid tissue organogenesis and differentiation of natural killer (NK) cells and lymphoid cells (42, 46). TNF, a multifunctional cytokine produced primarily by activated macrophages (70), functions as a key regulator of leukocyte trafficking by affecting chemokine expression and stimulating antigen presentation by inducing dendritic cell maturation (26, 58). TNF exists in two forms, a precursor 26-kDa membrane bound form (mTNF) and a 17-kDa soluble form, both of which are bioactive (46, 71). TNF and the closely related ligand lymphotoxin-α (LT), bind as homotrimers to two receptors, TNFR1 (p55) and TNFR2 (p75) that are widely expressed on most cell types (71). Activation of p55 generally results in gene activation that leads to induction of inflammatory and cytotoxic responses, while activation of TNFR2 is associated with thymocyte proliferation and T cell activation. In response to TNF binding, LPS and several other stimuli the extracellular domain of both TNFR are released by proteolytic cleavage and these soluble TNFR forms function as inhibitors of TNF signaling (1, 7, 18, 40). TNF has a role in several viral diseases of the CNS including, for example, those caused by HIV, FIV, HSV, Cytomegalovirus, Epstein-barr virus, Sindbis virus and Theiler’s murine encephalomyelitis virus with effects ranging from protective to toxic (31, 52).

Peripheral infection of mice with HSV involves local replication in epithelial tissues followed by rapid dissemination of virus via sensory axons to the corresponding ganglia and...
often the CNS (16). CNS infection in susceptible mouse strains can vary from mild to fatal
encephalitis for virulent HSV strains. The early corneal infiltrate elicited by corneal infection is
composed predominantly of neutrophils, and antibody mediated depletion of neutrophils results
in decreased clearance of virus and enhanced spread to the CNS (63, 66). Production of TNF and
NO, molecules with potent antiviral activity, may contribute to neutrophil mediated clearance of
HSV-1, whereas neutrophil production of IL-12 induces a CD4+ Th1 like responses that mediates
development of herpes stromal keratitis (HSK), an immunopathologic disease (20, 30, 65).
Activated macrophages are also present in the cornea early in infection and are responsible for
the release of antiviral factors like TNF and IFN-α/β. Synergism of TNF with IFN-α can induce
IFN-β, resulting in potent suppression of HSV-1 infection both in vitro in cultured human
fibroblasts and in vivo when expressed ectopically in the cornea (12, 57).

In contrast to the cornea, macrophages rather than neutrophils dominate the early (day 3)
inflammatory infiltrate in the trigeminal ganglion (Tg) after corneal inoculation of HSV (35, 59).
Macrophages were shown to be the primary producers of TNF, IL-12 and iNOS, whereas γδ-
TCR+ T cells produced IFN-γ in the ganglion and both cell types were found in close proximity
to infected neurons suggesting a role in the control of HSV-1 replication (35). Accumulation of
T cells, particularly CD8+ T cells was delayed and occurred coincident with clearance of HSV-1
antigen from the ganglion. We and others previously reported the unexpected observation that
the inflammatory response persisted well into latency with associated production of IFN-γ and
TNF in close juxtaposition to infected neurons (11, 27, 41, 59). In one study, TNF was the major
cytokine produced in the ganglion and the only cytokine detected on the CNS side of dorsal root
entry (DRE) zone (60). These observations imply an important role for IFN-γ and TNF in
control of HSV-1 infection in neurons during acute and latent infection. Utilizing IFN-γ and IFN-
γ receptor null mutant mice, we demonstrated a role for IFN-γ in control of in vivo reactivated
HSV-1 but the results did not support a role for IFN-γ in control of the acute infection (9, 10, 32); a role for IFN-γ in control of HSV-1 latency has been confirmed and extended in recent
studies (21).

Although TNF can potently inhibit HSV-1 in cultured cells, its in vivo role has not been
clearly delineated (13, 24). Local TNF has been reported to both exacerbate HSK and mediate
protection from corneal scarring in ocular mouse models (25, 33). In a prior study, TNF
pretreatment was shown to confer significant protection from lethal intraperitoneal HSV-1
challenge of resistant C57BL/6 mice by a mechanism independent of IFN production or NK cell
activation (55). TNF and IFN-γ have also been shown to be important for macrophage activation
and control of HSV and MCMV replication, independent of T and B cells (29). Further evidence
that TNF signaling pathways are crucial for effective host immune defense against herpesviruses
comes from recent reports that herpesviruses encode genes that target TNF-related cytokines and
or their associated receptors, as an immune evasion strategy (6, 37). Thus, HSV-1 exploits the
herpesvirus entry mediator (HVEM or HveA), a member of the TNFR superfamily, to enter
lymphoid cells via gD binding (38, 50). By antagonizing LIGHT, the lymphotoxin related natural
ligand for HVEM that is involved in T cell activation, HSV-1 could potentially impede T cell
activation (14, 45, 64) and also prevent interaction with BTLA, a known co-inhibitory ligand for
HVEM (17).
To better understand the role of TNF in the host immunity to HSV-1, we compared the outcome of infection in mice lacking TNFR1 (p55\(^{-/-}\)) or both known receptors (p55\(^{-/-}\)p75\(^{-/-}\)) (53, 54) to that in mice deficient for TNF (36), all mice being on the resistant C57BL/6 background. Results from these studies showed that TNF signaling via p55 played a role in control of HSV-1 replication in the eye, ganglion and brainstem and also conferred protection against fatal HSE. Surprisingly, neither p55 nor p75 were required for protection against fatal HSE, which implicates a novel TNF receptor in mediating the protective effects of TNF during HSV-1 infection.

MATERIALS AND METHODS

Mouse Strains

TNF receptor p55 (Tnfrsf1a) null mutant mice backcrossed 13 times to C57BL/6 (p55\(^{-/-}\) N13) were obtained from Amgen Inc. (Thousand Oaks, CA). TNF double receptor knockout (p55\(^{-/-}\)p75\(^{-/-}\)N5) mice, originally derived by Peschon et. al. (53) by crossing a p75\(^{-/-}\)N4 strain to a p55\(^{-/-}\) strain produced with B6 ES cells were obtained from Dr. Lyle Moldawer (University of Florida, Gainsville, FL.) or the Jackson Laboratory (Bar Harbor, ME). TNF\(^{-/-}\) mice (also produced using B6 ES cells) (15) were obtained from DNAX (Palo Alto, CA). C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and 129S6 mice were from Taconic (Germantown, NY).

Virus Stocks and Inoculation of Mice
Master stocks of HSV-1 strain 17+ comprised only of cell-released virus were prepared in, and titered on, mycoplasma-free CV-1 cell monolayers. Single use aliquots of virus in Hanks balanced salt solution (HBSS) supplemented with 2% FBS were stored at –80°C. Mice were inoculated with HSV-1 by corneal scarification. The right cornea of mice, deeply anesthetized by intraperitoneal (IP) injection of Ketamine and Xylazine, were gently scarified using a 27 gauge needle; 10 vertical strokes followed by application of HSV in a volume of 4µl of HBSS followed by another 10 horizontal strokes and gentle massaging of the eye with the eyelid to promote virus uptake. The same virus master stock was used for all experiments reported here. The City of Hope animal care committee approved all animal procedures.

**Determination of NO levels in macrophage cultures**

Resident peritoneal exudate macrophages were obtained by lavage with RPMI medium supplemented with 5% FBS. The cells were washed and plated in 100 cm² tissue culture dish in RPMI-10% FBS. The next day the culture was washed, the adherent cells were removed by scraping in cell dissociation buffer and re-plated at a density of 2.5x10⁵ cells per well in a 96 well plate. Macrophages were activated by treatment with IFN-γ/LPS and 24 h later NO levels in macrophage culture supernatants were determined as nitrite concentration [NO] using the Greiss reagent and quantitated by comparison to a standard curve generated using sodium nitrate (62). Briefly, a 100 µl aliquot of medium from the macrophage cultures was mixed with an equal volume of Greiss reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochlororide, 2.1% phosphoric acid) and after 5 min at room temperature, the absorbance
was read at 540 nm. The data presented are averages ± SEM of duplicate cultures assayed in duplicate and are representative of 3-6 experiments.

Antibody responses to HSV

Blood was collected by cardiac puncture immediately following CO\textsubscript{2} asphyxiation of mice, and serum produced by allowing overnight clotting at 4°C. 0.05% NaN\textsubscript{3} was added to serum and samples were stored at 4°C until ELISA analysis. HSV specific IgG production was determined by ELISA on serum samples obtained at > 28 days PI. Briefly, whole HSV antigen in PBS/NaN\textsubscript{3} (PBSN) was adsorbed to high protein binding polystyrene ELISA plates (Corning, Corning, NY) at 4°C overnight, washed three times with PBSN-0.05% Tween-20 (PBST). Plates were blocked for 2 hr with PBS SuperBlock (Pierce, Rockford, IL) and then incubated with serum samples for 4 h, followed by 2 µg/ml HRP-goat-anti-mouse IgG (Southern Biotech, Birmingham, AL) in PBST for a further 2 h. ELISAs were developed with 1-step Turbo TMB soution (Pierce, Rockford, IL) and read on a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA).

siRNA down regulation of TNF in RAW264.7 macrophage cells.

Three siRNAs were designed to target different sites in TNF mRNA (Mark Belke, IDT). Procedures for siRNA down regulation of TNF in RAW264.7 cells were according to our published detailed protocol (5). Briefly, RAW cells transfected with various concentrations of siRNA targeting TNF (siTNF) were incubated for approximately 18 h and then stimulated with 3
ng/ml LPS for 6 h, with Brefeldin A added for the last 5 h after which TNF was detected by intracellular staining and flow cytometry by conventional methods.

In vivo neutralization of TNF and depletion of macrophages using clodronate

Mice were treated on day 0, 2, 4, 6, and 8 with 30 mg/Kg PEGylated monomeric sTNFRI that binds TNF but is not known to bind LT (22), 250 µg hamster-anti mouse TNF (clone 5B8), that does not bind LT (Dr. Hiko Kohno, Amgen, personal communication), or a total dose of 22 µg of a 27-mer siRNA against TNF (siTNF; IDT, Coralville, IA). The siTNF was delivered as a complex with TransIT TKO (Mirus Bio, Madison, WI) to the peritoneal cavity as we have described previously (5) in 6 doses over 9 days (2 µg day 0 and 4 µg on days 1, 2, 4, 6, and 8). Following injection, the siRNA was distributed by massage throughout the peritoneum. The TNF antibody and sTNFR1 were administered to the peritoneum after dilution in PBS. Liposome-encapsulated clodronate (8.0 ml/Kg) was also given intraperitoneally and equal volume PBS injections were used as the appropriate control as per manufacturer’s recommendation (www.clodronateliposomes.org).

RESULTS

Lack of TNF increases HSE mortality in mice on the C57BL/6 background

We have previously shown that B6 mice lacking either or both TNF receptors are as resistant to fatal HSE as are B6 control mice (43). However, a protective role for TNF is
suggested by results showing that intraperitoneal administration of TNF can protect against fatal
HSE (55). Hence, we compared HSV-1 infection in TNF−/− mice to that in C57BL/6 wild-type
mice. TNF−/− mice were derived using B6 ES cells, which avoids the confounding effects that
would result from substitution of the entire MHC complex with 129-derived DNA if 129 ES had
been used. Mice were inoculated with a dose of HSV-1 previously determined to result in >85%
mortality for susceptible 129S6 and BALB/c mice, compared to <15% mortality for resistant
C57BL/6 mice (43). Survival of TNF deficient mice was significantly lower (8/18) than for either the p55−/− (31/40) or the control C57BL/6 mice (45/49); p=0.02 and p<0.0002, respectively
(Figure 1A). Mice that died of fatal HSE were necropsied and HSV-1 titers were determined in
the eyes, trigeminal ganglia and brainstem. Compared to control C57BL/6 mice, HSV-1 titers
were elevated in all target tissues of TNF−/− and p55−/− mice, with eyes showing the greatest
difference (Figure 1B). Thus, TNF appears to be important for control of HSV-1 in the eye.
Necropsy HSV-1 titers in resistant p55−/− and susceptible TNF−/− mice that died were not
significantly different (Figure 1A), which is contrary to the customary expectation that higher
virus loads in target tissues of mice that succumb to HSV infection would allow distinction
between susceptible and resistant strains. Since necropsy titers did not correlate with fatal HSE,
we confirmed a role for TNF in control of acute HSV-1 replication in the same three strains
impaired for TNF signaling. Mice were inoculated with HSV-1 by corneal scarification and the
Persistence of infectious virus in the eyes, trigeminal ganglia and brainstem was determined at
different times post infection (PI). Compared to B6 mice, HSV-1 persisted to a greater extent in
target tissues, particularly in the inoculated ipsilateral eye for both the TNF−/− and p55−/− mice with
titers tending to be somewhat higher in the TNF−/− than p55−/− mice (Figure 1C). Trigeminal
ganglion and brainstem titers tended to be higher for TNF−/− mice compared to control C57BL/6
and p55\(^+/\) mice, however the trend was not statistically significant (p>0.05) in paired one-tailed T-tests comparing tissue titers over time between TNF\(^{-/-}\) mice and either B6 or p55\(^{-/-}\) mice.

**NO production by peritoneal macrophages**

Macrophages and neutrophils produce nitric oxide (NO), which has been shown to block HSV-1 replication in vitro and in vivo (3, 35, 44). As TNF is involved in induction of NO (51), we determined whether the deficiency in TNF signaling in p55\(^{-/-}\) and TNF\(^{-/-}\) mice impaired TNF and NO production in macrophages. Compared to peritoneal exudates macrophages (PE-MP) from control C57BL/6 mice, NO production was significantly reduced in PE-MP from p55\(^{-/-}\) and TNF\(^{-/-}\) mice in response to in vitro activation with IFN-\(\gamma\) and LPS (Figure 2A). Although HSV-1 infection synergized with IFN-\(\gamma\) for induction of NO in control and p55\(^{-/-}\) mice, overall it reduced the levels of NO produced by B6 PE-MP compared to uninfected PE-MP; the same trend was evident for p55\(^{-/-}\) and TNF\(^{-/-}\) macrophages although the effects were smaller (Figure 2B). Additionally, TNF production was reduced in p55\(^{-/-}\) PE-MP compared to B6 PE-MP and interestingly, HSV-1 infection failed to augment TNF production in PE-MP activated with IFN-\(\gamma\) (Figure 2C). Thus, deficiencies in TNF signaling result in reduced NO production in PE-MP and this could contribute to the greater persistence of HSV-1 in p55\(^{-/-}\) and TNF\(^{-/-}\) mice (Figure 1C).

**HSE Resistance in wild-type C57BL/6 mice is dependent on TNF**
To further investigate the discrepancy that TNF^-/- mice are susceptible while neither TNF receptor appeared to be involved in protection against fatal HSE, we tested the effect of treating C57BL/6 mice with a sTNFR1 preparation capable of neutralizing TNF in vivo during HSV-1 infection. C57BL/6 mice treated with sTNFR1 during the course of acute infection showed a dose dependent increase ($R^2 = 0.964$) in mortality (Figure 3A). Compared to untreated mice, mortality was increased approximately 3-fold ($p<0.01$) after intraperitoneal administration of sTNFR1 at 30 mg/Kg body weight. Although injection of sTNFR1 at 10 mg/Kg increased mortality, the difference did not reach statistical significance with the number of mice tested. Because macrophages are the major producers of TNF (70) we anticipated that their depletion would increase susceptibility to fatal HSE. B6 mice injected intraperitoneally with the liposome-encapsulated macrophage toxin, clodronate (Cl$_2$-MDP) to ablate macrophages in vivo (69) showed a 3-fold increase in mortality ($p<0.005$) (Figure 3B), which is comparable to results obtained with sTNFR1 treatment (Figure 3A).

**Humoral immune responses in mice deficient in TNF or macrophages**

Protective immunity to HSV-1 is thought to depend on primarily on antigen specific cellular Th1 responses as well as antibody responses, both processes involving regulation by TNF that reflects on the efficiency of antigen processing by the host. Total HSV specific IgG was determined by ELISA in pooled sera from 2-3 mice sacrificed at >28 days after infection with HSV-1. HSV-1 specific IgG levels were reduced in p55^-/- and TNF^-/- mice relative to control C57BL/6 mice as shown in Figure 4A, implicating TNF signaling in regulating antibody production. Similar defects in primary antibody responses were noted for TNF^-/- and p55^-/- mice.
challenged with *Leishmania* or immunized with a Schistosome vaccine (61, 72). However, neutralizing TNF or depleting macrophages by treatment with sTNFR1 or the macrophage toxin clodronate, respectively, dramatically increased HSV specific IgG levels in wild type C57BL/6 mice relative to control PBS treated mice (Figure 4B). While these results reveal a role for TNF in regulation of HSV-1 specific IgG production, they do not support a protective role for HSV-specific antibody responses against fatal HSE because mortality was also increased in mice ablated for macrophages or treated with sTNFR1 (Figure 3).

**Depletion of TNF increases HSE mortality equally in wild-type B6 and p55\(^{-/-}\)p75\(^{-/-}\) mice**

The discrepant mortality of TNF\(^{-/-}\) mice and TNF receptor null mutant mice in response to HSV-1 infection raised the possibility that TNF mediated protection against fatal HSE was independent of either TNFR1 or TNFR2. Therefore, we tested the prediction that resistance of TNFR double mutant mice would be sensitive to in vivo TNF depletion. From our previous studies, we knew that cumulative mortality for C57BL/6 mice was indistinguishable from that of p55\(^{-/-}\)p75\(^{-/-}\) mice, hence we tested whether in vivo neutralization of TNF in these mice would increase their susceptibility. Indeed, relative to untreated mice, administration of sTNFR1 increased mortality to the same extent in C57BL/6 and p55\(^{-/-}\)p75\(^{-/-}\) double knockout mice (p=0.0018) (Figure 5A). This result reinforces the conclusion that TNF mediated protection against HSV-1 induced mortality is independent of signaling via the known TNF receptors, p55 and p75. Although, the monomeric sTNFR1 preparation used does not bind LT when tested in vitro, there is a remote possibility that in vivo it might bind LT in addition to TNF, both of which are natural ligands for p55 that have been implicated in mediating resistance to HSV-1 (8, 24)
Consequently, we also tested an anti-TNF mAb that does not bind LT and demonstrated that mortality due to HSE was increased to a similar extent as with sTNFR1 treatment (Figure 5D). Another remote possibility that we considered is that reverse signaling through membrane bound TNF (mTNF) might be elicited by either sTNFR1 or TNF neutralizing antibodies (23, 34, 49). To mitigate these potential confounding effects, we developed a procedure utilizing siRNA for efficient down regulation of TNF in vivo (5) as an alternative highly specific approach for demonstrating TNF mediated protection in HSV-1 infected p55⁻/⁻p75⁻/⁻ mice. The siRNA targeting TNF was designed to react specifically with TNF but not LT. We evaluated three independent siRNAs targeting different sites in the TNF mRNA. The RAW264.7 macrophage cell line was transfected with siTNF or irrelevant siRNA (siIRR), treated or not treated with LPS for six hours to induce TNF production that was measured by intracellular staining and flow cytometry analysis. siTNF site 1 (siTNF-S1) was highly effective and reduced TNF protein level to virtually background level obtained with siIRR transfected RAW264.7 cells (Figure 5B). siTNF-S2 was much less efficient, whereas siTNF-S3 activity was intermediate between siTNF-S1 and siTNF-S2 (Figure 5C); therefore, siTNF-S1 was used for subsequent in vivo experiments with p55⁻/⁻p75⁻/⁻ mice. Compared to treatment with irrelevant siRNA (siIRR), sTNFR1, anti-TNF mAb and siTNF-S1 treatments resulted in significantly increased mortality for HSV-1 infected p55⁻/⁻p75⁻/⁻ mice; p=0.005 (Figure 5D). Mortality for control siIRR treated or untreated p55⁻/⁻p75⁻/⁻ was not different therefore, the siIRR treated mice served as control for the anti-TNF mAb treated mice as well. Infection and treatment of mice with isotype control IgG to serve as a separate control could not be justified, since we and others have previously shown that treatment with normal IgG has no effect on the outcome of HSV infection (9, 67). Procedures for in vivo
neutralization of TNF are summarized in Table 1 and Figure 6 illustrates how these different TNF antagonists interfere with TNF signaling.

**DISCUSSION**

We presented here data that demonstrates an important role for TNF in resistance to mortality following ocular inoculation of HSV-1. Prolonged persistence and higher titers for HSV-1 in the eyes, trigeminal ganglia and brainstems of TNF\(^{-}\) and p55\(^{-}\) mutant mice compared to wild type C57BL/6 mice reveals a role for TNF in control of replication (Figure 1). A protective role for NO produced via induction of iNOS has been demonstrated in several models of HSV-1 infection (2, 44), hence, we suspect that sub-optimal NO production observed for p55\(^{-}\) and TNF\(^{-}\) macrophages contributes to the impaired control of HSV-1 in these mice (Figure 2). These data and other reports of early induction of TNF expression in tissues targeted by HSV-1 are consistent with a protective role for TNF in HSV-1 infection (11, 27, 28, 41, 60). Additionally, intraperitoneal injection of TNF 4 h before, or 8 h after intraperitoneal HSV-1 inoculation of C57BL/6 mice significantly extended their survival compared to untreated C57BL/6 mice (55). Hence, we anticipated and indeed observed, significantly higher mortality (p<0.0002) for TNF null mutant mice (10/18, 56%) compared to wild type C57BL/6 mice (4/49, 8%). Similar mortality rates were reported in previous studies comparing survival of C57BL/6 TNF\(^{-}\) and control C57BL/6 mice challenged with HSV-1 by the corneal route (47, 48).

Most important effects of TNF, including antiviral activity are generally ascribed to signaling via p55 rather than p75 that interacts preferentially with mTNF (4, 71, 73). Finding
that p55\(^{-/-}\) mice were as resistant to HSV-1 ocular challenge as control C57BL/6 mice, \(p > 0.05\). (Figure 1A) suggested that TNF signaling via p75 exerted anti-HSV effects. The antiviral effects of TNF on two poxviruses, vaccinia virus and ectromelia virus, were shown to depend on both p55 and p75 TNF receptors (56). However, we have reported that C57BL/6 and p55\(^{-/-}\)p75\(^{-/-}\) mortality rates were indistinguishable, ranging from 13% to 15% \((p > 0.05)\) in HSV-1 infected mice (43). These results imply that while TNF is required for protection against fatal HSV-1 infection both p55 and p75 receptors are dispensable. Strong support for this conclusion is provided by the nearly identical increase in mortality resulting from treatment of HSV-1 infected C57BL/6 and p55\(^{-/-}\)p75\(^{-/-}\) mice with sTNFR1 or anti-TNF mAb (Figure 5A), both of which are known to neutralize TNF but not LT. A comparable increase in mortality was observed for C57BL/6 mice depleted of macrophages by intraperitoneal injection of liposomes encapsulating a macrophage toxin that is widely used for this purpose (68). This result implicates macrophage produced TNF in protective antiviral responses to HSV-1 consistent with results from studies by others (29, 35, 51). Although TNF contributes to the control of HSV-1 replication, the mechanisms by which TNF protects against fatal HSE is uncertain since HSV-1 titers in CNS tissues were comparable in susceptible TNF\(^{-/-}\) and resistant p55\(^{-/-}\) mice (Figure 1B and 1C).

To mitigate possible confounding effects of reverse signaling through mTNF by sTNFR1 and anti-TNF mAb (34, 49), we utilized siRNA to down regulate TNF in vivo in HSV-1 infected p55\(^{-/-}\)p75\(^{-/-}\) mice and observed an increase in mortality comparable to that obtained with either sTNFR1 or anti-TNF mAb treatment (Figure 5B). It is important to note that siTNF specifically targets TNF and has no cross reactivity with other TNF family member ligands or receptors as illustrated in Figure 6. siTNF specifically down regulated TNF production as
demonstrated by dose dependent down regulation of TNF using three independent target sites in TNF mRNA and by siRNA targeting an irrelevant transcript having no effect (Figure 5B and 5C). Additionally, by in vivo titration we determined a siTNF dose that was highly effective in down regulating TNF at the protein level while avoiding non-specific innate immune responses (5, 19). The most reasonable interpretation of these results is that TNF mediated resistance to fatal HSV-1 infection in mice on the C57BL/6 genetic background is independent of either of known TNF receptors, p55 and p75. The mechanism by which TNF protects against fatal HSE in p55−/−p75−/− mice remains speculative in the absence of formal proof for existence of a novel TNFR. The fact that three mechanistically different approaches, namely treatment with sTNFR1, anti-TNF mAb and siTNF increased mortality to the same extent for HSV-1 infected C57BL/6 and p55−/−p75−/− mice is compelling evidence that only TNF neutralization was involved and argues against reverse signaling via mTNF for sTNFR1 and anti-TNF mAb or neutralization of other TNF ligands. In a related study, the existence of third unknown receptor was invoked to explain the observed resistance of p55−/−p75−/− mice compared TNF−/− mice on C57BL/6 background to a rapidly fatal Leishmaniasis (72). The possibility of developmental defects in secondary lymphoid organs of C57BL/6 TNF−/− influencing the course of disease was excluded in this study using reciprocal bone marrow chimeras.

We show here that resistance in wild type (p55+/+) and p55−/−N13 mice is strictly dependent on TNF signaling as it is impaired by in vivo neutralization of TNF. TNF thus plays a pivotal role in resistance to HSV, which is genetically very complex involving multiple interacting loci (manuscript in preparation). We previously reported that the C57BL/6 allele of the Herpes Resistance Locus, Hrl, linked to p55 on mouse chromosome 6 (c6) confers resistance
to HSV-1 and HSV-2 (43) in mice lacking p55 (p55\(^{-/-}\)N13). Resistance of p55\(^{-/-}\)N13 mice is also
abrogated by in vivo neutralization of TNF (unpublished observation), which indicates a general
requirement for TNF in resistance of mice on the B6 background to HSV-1 infection.

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Figure Legends.

Figure 1. (A), HSV-1 induced mortality in C57BL/6, p55⁻/⁻ and TNF⁻/⁻ mice. Mice were inoculated with 3200 pfu HSV-1 and monitored for mortality and symptoms of encephalitis necessitating euthanasia. Shown is the cumulative survival data from 4 experiments using 18-28 mice from the C57BL/6 (black circles), p55⁻/⁻ (grey circles) and TNF⁺/⁺ strains (grey squares). The TNF⁺ mice are significantly different from the C57BL/6 mice (p<0.0001) whereas the p55⁻/⁻ N13 mice are not (p>0.05). (B), HSV-1 titers in necropsy tissues from C57BL/6, p55⁻/⁻ and TNF⁻/⁻ mice. Tissues from dead mice were collected shortly after death and virus titers were determined. Titers in the indicated tissues is shown for C57BL/6 (black bars), p55⁻/⁻ (grey bars) and TNF⁺/⁺ mice (open bars). Animals were inoculated with 3200 PFU HSV-1 and monitored for mortality; mice with pronounced symptoms of encephalitis were euthanized. Combined data from two experiments resulting in 4-11 deaths per strain are shown as average HSV-1 titer +/- SEM. (C), Persistence of HSV-1 in C57BL/6, p55⁺/⁺ and TNF⁺/⁺ mice after corneal inoculation. The amount of infectious HSV recovered from the infected right eye, the right Tg, brainstem, left Tg) and left eye is shown. HSV-1 titers were determined by plaque assay for tissues collected at the indicated time points. Combined data for 5 experiments with 3-5 mice per strain is shown and data is presented as average HSV titer +/- SEM.

Figure 2. Nitric oxide and TNF production by peritoneal exudate (PE) cells from C57BL/6, p55⁺/⁺ and TNF⁻/⁻ mice. (A), LPS-induced NO production from PE cells of C57BL/6 (black bars), p55⁻/⁻ (grey bars) and TNF⁺/⁺ mice (open bars) in the absence of HSV-1 infection during in vitro culture. (B), LPS-induced NO production from PE cells in the presence of infectious HSV-1
during in vitro culture. (C), TNF production elicited by LPS stimulation in the absence (solid bars) or presence (hatched bars) of infectious HSV-1 in PE cells from C57BL/6 (black bars) and p55⁻/⁻ mice (grey bars). Non-stimulated culture supernatants contained no TNF. All PE cell cultures were pretreated overnight with IFN-γ before use in culture assays. Representative data from three experiments using pooled cells from 5-7 mice are shown. (not done) = insufficient PECs recovered.

**Figure 3. In vivo TNF and macrophage depletion increases mortality in C57BL/6 mice.** (A), C57BL/6 mice were inoculated with 3200 PFU HSV-1, given sTNFR1 on days 0, 2, 4, 6, 8, and 10 and monitored daily for mortality; mice with overt symptoms of encephalitis were euthanized. Mice treated with 10 mg/Kg or 30 mg/Kg sTNFR1 are indicated by squares and triangles, respectively and untreated mice are shown by circles. (B), C57BL/6 (black diamonds) mice were inoculated with 3200 pfu HSV-1 and macrophages were depleted by IP administration of liposome-encapsulated clodronate on days 0, 2, 4, 6, 8, and 10 PI. Mice were monitored for mortality and animals with pronounced symptoms of encephalitis were euthanized. Combined data from 5 experiments using 10-25 mice per strain are shown.

**Figure 4. HSV-specific IgG production in C57BL/6, p55⁻/⁻ and TNF⁻/⁻ mice.** (A), Relative amounts of anti-HSV-1 IgG in sera from C57BL/6 (black circles), p55⁻/⁻ (grey circles), and TNF⁻/⁻ (open circles) mice sacrificed at >day 28 PI are shown. (B), Relative anti-HSV IgG levels in sera after treatment with either 30 mg/Kg sTNFR1 in C57BL/6 (black squares) or clodronate liposomes to deplete macrophages (black triangles); as controls mice were treated with PBS (grey squares). Animals were inoculated with 3200 pfu HSV-1 and serum was collected from mice at
>28 day PI. Absorbance values (450-570 nm; TMB HRP substrate, Pierce) normalized to 1:10 dilution of d28 HSV-positive serum as 100% and HSV-negative serum as 0%. Ranges shown are 1:128 - 1:4096 dilutions of respective serum.

Figure 5. In vivo TNF depletion increases mortality in both C57BL/6 and p55⁺p75⁻ mice. (A), C57BL/6 (squares) and p55⁺p75⁻ mice (circles) inoculated with 3200 PFU HSV-17+ were untreated (black symbols) or treated with sTNFR (blue symbols) on days 0, 2, 4, 6, 8, and 10 PI and monitored for mortality; animals with pronounced symptoms of encephalitis were euthanized. Combined survival data from 6 experiments using a total of 69-121 mice per strain is shown. (B), histogram showing TNF down regulation in LPS stimulated RAW267.4 cells treated with 25 nM siTNF site 1 (red line) compared to siIRR treated, LPS stimulated (solid black line) or non-stimulated RAW cells (dashed black line). (C), dose response for down regulation of TNF by three siRNAs targeting different sites in TNF mRNA; data normalized to RAW cells treated with LPS plus siIRR as control. (D), In vivo neutralization of TNF in p55⁺p75⁻ mice. Mice treated with sTNFR1, 27-mer siTNF (22 µg in 6 doses over 9 days), anti-TNF mAb or siIRR as control and monitored for mortality; animals with pronounced symptoms of encephalitis were euthanized. Combined data from 3 experiments using 10 – 34 mice per group.

Figure 6. Diagram illustrating potential interactions of TNF antagonists with relevant TNF superfamily members. sTNFR1 and anti-TNF mAb can bind soluble or mTNF. PEGylated monomeric sTNFR1 which was used in studies reported here does not bind LT and the anti-TNF mAb binds both sTNF and mTNF but not LT. Thus, the only potential side effects when using sTNFR1 and anti-TNF mAb for in vivo neutralization of TNF are reverse signaling via mTNF.
In contrast, siTNF specifically down regulates TNF but does not interact with other TNF superfamily member ligands or receptors.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Complication in data interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTNFR1</td>
<td>TNF, LT?</td>
<td>Reverse signaling via mTNF, LT binding?</td>
</tr>
<tr>
<td>α-TNF mAb</td>
<td>TNF</td>
<td>Reverse signaling via mTNF</td>
</tr>
<tr>
<td>siTNF</td>
<td>TNF</td>
<td>TLR3 activation at high concentrations*</td>
</tr>
<tr>
<td>clodronate</td>
<td>Macrophages</td>
<td>Depletion of non-macrophage cells</td>
</tr>
</tbody>
</table>

**Table 1.** Effects and complications from in vivo TNF depletion methods.  

*a* The optimized amount of siTNF used in this study did not cause non-specific activation capable of overcoming TNF down-regulation by the siRNA (not shown).

Abbreviations: sTNFR1 = soluble monomeric mouse p55, mTNF = membrane bound TNF (26kDa form), α-TNF mAb = TNF antibody that does not bind LT, siTNF = siRNA targeting TNF, TLR3 = toll-like receptor 3, specific for dsRNA, ?, indicates uncertainty about whether the monomeric sTNFR1 binds LT in vivo.