Herpes Simplex Virus Type 2 (HSV-2)-Induced Mortality following Genital Infection is Blocked by Anti-TNF-α Antibody in CXCL10 Deficient (CXCL10−/−) Mice

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Abstract

The role of TNF-α was evaluated in CXCL10 deficient (CXCL10\textsuperscript{−/−}) mice which succumb to genital HSV-2 infection and possess elevated levels of virus and TNF-α but not other cytokines in the central nervous system (CNS) and vaginal tissue within the first seven days following virus exposure. Anti-TNF-α but not control antibody treatment offset the elevated mortality rate of CXCL10\textsuperscript{−/−} mice despite increased CNS viral titers. In addition, TNF-α neutralization suppressed recruitment of leukocyte subpopulations into the CNS associated with reduced CCL2 and CXCL9 expression. Collectively, the results implicate TNF-α as the principal mediator of mortality in response to genital HSV-2 infection.
Herpes simplex virus type 2 (HSV-2) is one of the most common causes of genital ulcer disease in humans that can result in fatal CNS infection (4, 14, 30, 49). Nearly six to eight hundred thousand cases are reported annually such that as many as forty to sixty million individuals are infected with HSV-2 in the United States (50). During replication in the vaginal epithelium cells, the virus enters sensory nerve endings and by retrograde transport, traffics to sacral ganglia where it establishes a latent infection in resident neurons (8, 19). Following reactivation, the virus can traffic by anterograde transport and cause recurrent infection at the original portal of entry as well as adjacent sites (42). In the immunocompromised patient as well as newborns, the infection can be quite severe ultimately resulting in death (20, 22, 46). Experimental evidence suggests elevated levels of TNF-α may be a key factor in neuropathogenesis following viral infection (16, 48).

In response to CNS virus infection, TNF-α is produced by astrocytes, microglia, neurons, and infiltrating hematopoetic cells (34, 52) acting through two structurally-related cell surface receptors TNFR1 (p55) and TNFR2 (p75) constitutively expressed on neurons and glial cells (35, 48). TNF-α regulates leukocyte trafficking by inducing a number of factors including cell adhesion molecules (ICAM-1, VCAM-1), selectins (E- and P-selectins), and chemokine expression (17, 18, 31, 47). In addition, it regulates NK cell differentiation (24), a cell population critical in the control of genital HSV-2 infection (44). Relative to acute HSV-1 infection, TNF-α suppresses virus replication and dissemination into the CNS by means which appear to be independent of either TNFR1 or TNFR2 (25, 41). At the cellular level, TNF-α synergizes with IFN-γ in the induction of nitric oxide (36), a molecule that also has potent anti-HSV action (6, 29). Although TNF-
α has many positive anti-viral attributes as noted above, it can also be detrimental to brain function possibly through the induction of high mobility group box 1 protein expression (11, 21, 38). Recently, it was found CXCL10⁻/⁻ mice are highly susceptible to genital HSV-2 infection based on increased mortality and virus titer associated with elevated TNF-α (45) but not IL-6 (data not shown). It was hypothesized the increase in HSV-2-mediated mortality of CXCL10⁻/⁻ mice was due to excessive expression of TNF-α as opposed to an increase in virus titer within the CNS. In order to test this hypothesis, anti-TNF-α antibody was administered to HSV-2-infected CXCL10⁻/⁻ mice. Sixty percent of isotype, control antibody (Ab)-treated CXCL10⁻/⁻ mice succumbed to infection on day 7 post infection which was significantly (p<.01) higher in comparison to anti-TNF-α Ab-treated CXCL10⁻/⁻ or non-treated, WT mice (Fig. 1a). To determine whether survival lasted throughout the acute infection, mice were monitored for fourteen days post infection. In this case, late mortality of anti-TNF-α Ab treated CXCL10⁻/⁻ mice was observed (Fig. 1b). The mortality of anti-TNF-α Ab treated CXCL10⁻/⁻ mice was delayed to day 12 post infection compared to isotype, control Ab-treated CXCL10⁻/⁻ mice in which all mice succumbed by day 8 post infection (Fig. 1b). Similarly, to CXCL10⁻/⁻ mice, HSV-2 WT mice benefitted by anti-TNF-α Ab treatment in comparison to isotype control Ab-treated WT mice (Fig. 1b). To determine what impact the administration of anti-TNF-α Ab had on virus replication, anti-TNF-α Ab-treated CXCL10⁻/⁻ mice were compared to isotypic control Ab-treated and non-treated WT mice measuring virus levels recovered in the vaginal tissue, spinal cord, and brain stem. The administration of anti-TNF-α Ab did not modify the amount of infectious virus recovered in the vaginal tissue, spinal cord, or brain stem of CXCL10⁻/⁻ mice in
comparison to isotypic control Ab-treated, HSV-2-infected CXCL10−/− mice (Fig. 1C). In fact, a trend for an increase in virus disseminated to all tissues surveyed was observed consistent with the notion that TNF-α Ab suppresses HSV replication (25, 41). Both treated groups of CXCL10−/− mice possessed significantly more infectious virus compared to WT mice.

Adhesion molecules (e.g., selectins, ICAM-1, and VCAM-1) which facilitate trafficking of leukocytes into inflamed tissue and cause leakiness of endothelial lining are induced or up-regulated by TNF-α (1, 5). Since neutrophils, macrophages, and T cells are thought to contribute to immune surveillance of HSV-2 (32, 33, 36) as well as serve as a source of inflammatory mediators that can harm the nervous system, it was reasoned the application of anti-TNF-α Ab to HSV-2-infected mice would prevent or suppress the extravasation of leukocytes into the CNS. In support of this idea, significantly reduced neuropathology evident by a reduced level of infiltrating cells, degeneration of tissue, and loss of neurons was observed in the brain stem and spinal cord of anti-TNF-α Ab-treated CXCL10−/− mice compared to isotype Ab-treated CXCL10−/− mice (Fig. 2). Consistent with this observation, there was a significant reduction in the absolute number of CD45 hi leukocytes, activated macrophages (defined as F4/80+Gr1+), and neutrophils (F4/80−Gr1+) residing in the CNS of anti-TNF-α Ab-treated mice compared to isotype control Ab-treated CXCL10−/− or non-treated WT mice on day 7 p.i. (Fig.3a-c). Similarly, the absolute number of CD4+ and CD8+ T cells was also significantly reduced in the CNS of anti-TNF-α Ab-treated, HSV-2-infected CXCL10−/− mice (Fig. 3d-e). No significant difference was found in the number of leukocytes residing in the CNS of WT versus isotype control Ab-treated CXCL10−/− mice except for
CD8+ T cells which were significantly reduced in the brain stem of the HSV-2-infected CXCL10−/− isotype control Ab-treated group (Fig. 3b). These results are consistent with other findings that show abrogation of TNF-α or TNFR1 expression significantly reduces leukocyte infiltration (13, 43).

TNF-α can induce CCL2 expression and mobilize monocytes/macrophages and T cells through the G protein-coupled, seven transmembrane domain chemokine receptor, CCR2 (2, 3, 26, 27). Likewise, neutrophils are highly responsive to another TNF-α-induced chemokine, CXCL1 (23, 40), and CXCL9 and CXCL10 are potent chemoattractant molecules for T cells (12). Since neutrophils and macrophages along with T cells were reduced in the brain stem and spinal cord of HSV-2-infected, CXCL10−/− mice treated with anti-TNF-α antibody, expression of CCL2, CXCL1, and CXCL9 was assessed in the CNS of the infected mice as a means to identify a possible scenario responsible for the dramatic change in leukocyte influx. CCL2 expression in the spinal cord and brain stem of anti-TNF-α Ab-treated, HSV-2-infected CXCL10−/− mice was reduced in comparison to the isotypic control Ab-treated, HSV-2-infected animals similar to the level found in infected WT mice (Fig. 4a-b). There was no significant difference in the expression of CXCL1 in the brain stem or spinal cord comparing the anti-TNF-α Ab-treated group to either the isotypic control or WT mice (data not shown). CXCL9 levels were also reduced in the anti-TNF-α Ab-treated, HSV-2-infected mice in comparison to HSV-2-infected WT mice (Fig. 4c-d).
Of the chemokines induced by TNF-α, CXCL9 operates thru CXCR3 and has been found to be instrumental in chemotaxis of CD8⁺ T cells to the CNS following genital HSV-2 infection (45) whereas CCL2 selectively attracts immune cells including macrophages, monocytes, PMNs and T cells that bear the receptor CCR2 (39). The association of CCL2 and CCR2 in neuropathogenesis has also been demonstrated in other primate and rodent models of neuronal injury or virus infection (10, 28, 51).

We also find strong expression of CCL2 in both spinal cord and brain stem of HSV-2-infected CXCL10⁻/⁻ mice associated with an increase in TNF-α (45). It is tempting to speculate a delay in mortality of HSV-2 infected CXCL10⁻/⁻ mice treated with anti-TNF-α Ab is due to a reduction in infiltrating leukocytes as a result of reduced CCL2 expression including macrophages that secrete a variety of soluble factors that can result in neuropathology (including TNF-α and iNOS) consistent with a recent observation in an experimental autoimmune encephalomyelitis model (7).

Therefore, the absence of CXCL10 appears to be overcompensated by the ensuing immune response with TNF-α having a lead role in the neuropathology as opposed to a direct effect of virus within the CNS.

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**Figure legends**

**FIG. 1.** Anti-TNF-α treatment offsets elevated mortality rate of CXCL10−/− mice. (A) Wild type (WT) and CXCL10−/− mice (8) (n=18 mice/group) were rendered susceptible to genital HSV-2 using DepoProvera (36) and infected with HSV-2 (2000 pfu/vagina). On day 5 post infection, 100 µg of anti-mouse TNF-α or isotypic control Ab was
administered retro-orbitally into HSV-2-infected CXCL10−/− mice. Mice were monitored and recorded for survival up to day 7 post infection. The results are shown as mean ± SEM of six experiments. **(B) Survival study was repeated including WT mice with anti-mouse TNF-α or isotypic control Ab treatment, and mice were monitored and recorded for survival up to day 14 post infection. The results are shown as mean ± SEM of three experiments (n=8 mice/group).** (C) Mice were exsanguinated and vaginal tissue, spinal cords, and brain stems were removed, processed, and assayed for viral titer by standard plaque assay (14). The viral titer is expressed as mean log pfu ± SEM summarized from three experiments (n=9 mice/group). **p<.01, *p<.05 comparing WT to anti-TNF-α Ab-treated CXCL10−/− mice or WT to isotypic control Ab-treated CXCL10−/− mice as determined by ANOVA and Tukey’s post hoc T-test.**

**FIG. 2. Reduced inflammatory pathology and infiltrates in CNS of anti-TNF-α treated CXCL10−/− mice.** Following DepoProvera treatment, CXCL10−/− mice (n=3/group) were infected with HSV-2 (2000 pfu/vagina). On day 5 post infection, 100 µg of anti-mouse TNF-α or isotypic control Ab was administered retro-orbitally into HSV-2-infected CXCL10−/− mice. On day 7 post infection, mice were exsanguinated and the brain stem and spinal cord were removed from each mouse, and processed for histological analysis following H & E staining. Tissues from uninfected CXCL10−/− mice were used as control. Magnification is 400x.

**FIG. 3. Macrophage, neutrophil and T cell infiltration into the CNS.** DepoProvera-treated wild type (WT) and CXCL10−/− mice (n=9/group) were infected with HSV-2 (2000
pfu/vagina). On day 5 post infection, 100 µg of anti-mouse TNF-α or isotypic control antibody was administered intravenously (retro-orbitally) into HSV-2-infected CXCL10-/- mice. On day 7 post infection, mice were exsanguinated and the brain stem and spinal cord were removed from each mouse, processed, and analyzed for (A) total infiltrating leukocytes (i.e., CD45^hi), (B) activated macrophages (i.e., F4/80^+Gr1^+), (C) neutrophil, (D) CD3^+CD4^+ T cell or CD3^+CD8^+ T cell (spinal cord) and (E) CD3^+CD4^+ T cell or CD3^+CD8^+ T cell (brain stem) content by flow cytometry (44). Bars represent the mean ± SEM from three experiments. **p<.01, *p<.05 comparing the anti-TNF-α Ab-treated CXCL10-/- mice to the other two groups as determined by ANOVA and Tukey’s post hoc T-test.

FIG. 4. CCL2 and CXCL9 levels in the CNS of mice. DepoProvera-treated wild type (WT) and CXCL10-/- mice (n=12/group) were infected with HSV-2 (2000 pfu/vagina). On day 5 post infection, 100 µg of anti-mouse TNF-α or isotypic control antibody was administered intravenously (retro-orbitally) into HSV-2-infected CXCL10-/- mice. On day 7 post infection, mice were exsanguinated and the spinal cord and brain stem were removed from each mouse, processed, and analyzed for (A) CCL2 (spinal cord), (B) CCL2 (brain stem), (C) CXCL9 (spinal cord), and (D) CXCL9 (brain stem) content by ELISA. Samples were run in duplicate and analyzed along with known standard provided for each analyte by the manufacturer with the unknown amount extrapolated from the standard curve generated for each assay. The bars represent the mean ± SEM of the indicated chemokine from three experiments. *p<.05 comparing the isotypic control Ab-treated CXCL10-/- mice to the other two groups in (A) or comparing the anti-TNF-α
Ab-treated CXCL10−/− mice to the WT mice in (B) as determined by ANOVA and Tukey’s post hoc T-test.
Figure a shows the percent cumulative survival over days post infection for different groups: WT, CXCL10−/− + Iso, and CXCL10−/− + anti-TNFα.

Figure b displays similar data with a longer time frame: WT + Iso, WT + anti-TNFα, CXCL10−/− + Iso, and CXCL10−/− + anti-TNFα.

Figure c presents bar graphs comparing HSV-2 log pfu/tissue levels in the Vagina, Spinal Cord, and Brain Stem for WT, CXCL10−/− + Iso, and CXCL10−/− + anti-TNFα groups.