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## *Induced IL-10 Splice Altering Approach to Antiviral Drug Discovery*

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# Induced IL-10 Splice Altering Approach to Antiviral Drug Discovery

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Ebola virus causes an acute hemorrhagic fever lethal in primates and rodents. The contribution of host immune factors to pathogenesis has yet to be determined and may reveal efficacious targets for potential treatment. In this study, we show that the interleukin (IL)-10 signaling pathway modulates Ebola pathogenesis. IL-10$^{-/-}$ mice and wild-type mice receiving antisense targeting IL-10 signaling via disrupting expression through aberrant splice altering were resistant to ebola virus infection. IL-10$^{-/-}$ mice exhibited reduced viral titers, pathology, and levels of IL-2, IL-6, keratinocyte-derived chemokine (KC), and macrophage inflammatory protein-1 α and increased interferon (IFN)-γ relative to infected wild-type mice. Furthermore, antibody depletion studies in IL-10$^{-/-}$ mice suggest a requirement for natural killer cells and IFN-γ for protection. Together, these data demonstrate that resistance to ebola infection is regulated by IL-10 and can be targeted in a prophylactic manner to protect against lethal hemorrhagic virus challenge.

## Introduction

EBOLA VIRUS (EBOV), a member of the filovirus family, causes hemorrhagic fever with lethality rates ranging from 20% to 88% in humans. Disease is characterized by high viral titers, rapid viral propagation, hemorrhage, clotting abnormalities, lymphocyte death, and liver dysfunction (reviewed in Hoenen et al., 2006). There is currently no approved vaccine or treatment other than supportive care (Burnett et al., 2005). Due to extreme risk, research is limited to biosafety level (BSL)-4 containment facilities and thus little known about the immunological aspects of the disease. Furthermore, most human infections occur in remote areas that lack modern scientific equipment, making field research difficult. Recent increases in EBOV outbreak frequency coupled with the potential for weaponization underscores the importance of a clearer understanding of the host pathogen interaction in this disease.

Very few studies in humans have focused on serum cytokine responses in survivors versus non-survivors after EBOV infection. Survivors of EBOV-Zaire (ZEBOV) infection transiently produced increased levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, interferon (IFN)-γ, and macrophage inflammatory protein 1 (MIP-1) α and β, fol-lowed by increased plasma levels of IL-6R, TNF-R, and IL-1RA (Baize et al., 2002). However, non-survivors generated high levels of IL-10, TNF-R, and IL-1RA, and moderate amounts of IL-6 and TNF-α prior to succumbing to the disease (Villinger et al., 1999; Baize et al., 2002). Survivors of EBOV-Sudan infection did not exhibit elevated TNF-α or IFN-γ but did have higher IFN-γ levels with fatal cases showing increased IL-10, IL-6, IL-8, and MIP-1α plasma levels (Hutchinson and Rollin, 2007). There have also been reports of asymptomatic EBOV infection in humans, which resulted in low-level viral replication and were associated with early proinflammatory cytokine levels (IL-1β, IL-6, TNF-α, MIP-1α and β, and MCP) followed by "dampening" of the inflammatory response with IL-1RA, TNFRI and II, and IL-10 expression (Leroy et al., 2001).

Together, these findings suggest that host factors including those that regulate cytokine expression profiles influence the outcome of survivable or harmful immune responses to EBOV. However, these fail to elucidate the types of responses needed for survival, since background levels and time of infection cannot be determined in these kinds of studies.

Interleukin-10 (IL-10) is a key modulator of immune responses (reviewed in Couper et al., 2008; Mosser and Zhang,

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2008). Production of IL-10 can downregulate inflammatory cytokine production, functionally suppress natural killer (NK) cell activity, and inhibit dendritic cell (DC) antigen presentation and co-stimulatory activity, thus dampening Th1 responses vital to combating virus infection. Previous studies have shown that inhibition of IL-10 can ameliorate pathogenesis in a wide range of infections, including bacterial, nematode, and fungal as well as chronic viral infections (Ejrnaes et al., 2006; Couper et al., 2008). However, little work has been done to elucidate a role for IL-10 in acute viral infections in general, or specifically what might be observed in a lethal challenge model upon inhibiting its signaling capacity. Although there is speculation for IL-10 involvement in EBOV pathogenesis inferred from other studies where inappropriate expression abolishes antiviral cytotoxic T lymphocyte activity, no direct evidence has been shown (reviewed in Mohamadzadeh, 2009). Here, we show that reduction of IL-10 signaling early in the infection process significantly improves survival in mice lethally challenged with ZEBOV.

## Materials and Methods

### Mice and virus

B10.129P2 (B6)-IL10Cgn/J, IFN-$\gamma^{-/-}$ and control mice were obtained from JAX mice. C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center. Antisense treatment was given as a 50-$\mu$g dose of the indicated PPMO (peptide-conjugated derivative of the phosphorodiamidate morpholino oligomer) compound at both 24 and 4 hours before or 24 hours after intraperitoneal injection with 1,000 PFU of mouse-adapted EBOV under maximum containment in a BSL-4 laboratory at the U.S. Army Medical Research Institute of Infectious Diseases. Animals were observed for illness at least twice daily for at least 28 days. Research was conducted under an institutional animal care and use committees–approved protocol in compliance with the Animal Welfare Act, Public Health Service policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 2011.

### Antisense compounds

PPMO compounds were designed as sequences complementary to GenBank accession number and named for their target and RNA position either targeting the translational initiation site (AUG), a splice acceptor (SA) or splice donor (SD) site of a specific exon (Table 1). All PPMOs were synthesized and purified by Sarepta Therapeutics, Inc., as previously described (Summerton and Weller, 1997).

### Reverse transcription-polymerase chain reaction

RNA was prepared via Qiagen RNeasy mini-prep kits according to the manufacturer's recommendations. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on an iCycler instrument (BioRad) using Invitrogen's One-Step qRT-PCR kit according to the manufacturer's recommendations using primers flanking exon 4 of IL-10 forward 5′-AAGCCTTATCGGAAATGATCC-3′ and reverse 5′-TTCATGGCCTTGTAGACACC-3′. Amplicons were resolved on a 0.8% agarose ethidium bromide–stained gel.

### Cell culture, cytokine assays, and cell depletion

Murine splenocytes and bone marrow derived dendritic cells (DCs) were isolated and cultured as previously described (Marshall et al., 2007). Cytokine levels from culture supernatant or plasma were measured by ELISA or using Luminex murine 20-plex cytokine kit from Luminex Corporation. Depletion of immune cell subsets was done by injecting mice with 300 mg of antibody on days $-3$, $-1$, $+3$, and $+8$ after infection (IFN-$\gamma$ injections were on days $-3$, $-1$, $+1$, $+3$, and $+6$). Clones used were XMG1.2 (IFN-$\gamma$), GK1.5 (CD4), 53-6.7 (CD8a), and PK136 (NK1.1). Statistical comparisons were made using the students *t*-test and Prism software.

TABLE 1. INCREASED SURVIVAL IN MICE WITH REDUCED INTERLEUKIN-10 EXPRESSION

| Gene target | Percentage survival (survivors/total)[a] | PMO name (sequence)[b] |
|---|---|---|
| IL-10 | 72.2% (36/50) | IL104SA4 (GGAGAAATCGATGCTGAAGAA) |
| IL-10 | 30% (6/20) | IL10AUG (GCAGTGCTGAGCCAGGCATG) |
| IL-10 | 15% (3/20) | IL10SD3 (ACAGCGCCTCAGCCGCATCC) |
| IL-10 receptor alpha | 55% (11/20) | MuIL10RA-SA6 (TCAGGTTGGTCACAGTGAAAT) |
| IL-10 receptor alpha | 50% (10/20) | MuIL10RA-SA2 (AAGIGCTTGGCAGTTCTGTCC) |
| IL-10 receptor alpha | 43.3% (13/30) | IL10Ra-AUG (GCAAACGCGACAACATGGTG) |
| Influenza nucleoprotein | 10% (2/20) | Influenza-NP (AGCAAAAGCAGIGTAGATAATC) |
| None (naïve mice)[c] | 12% (6/50) | N/A |
| IL-10 knockout[d] | 70% (7/10) | N/A |
| None (wild-type)[d] | 30% (3/10) | N/A |

[a]The number of mice surviving following challenge with 1000 PFU of mouse-adapted ebola virus.
[b]All phosphorodiamidate morpholino oligomers (PMOs) were tagged on the 5′ end with the arginine-rich peptide tag P007. Indicated PMOs were administered via intraperitoneal (i.p.) injection to male or female C57BL/6 mice at 24 and 4 hours prior to i.p. challenge (50 $\mu$g/ dose).
[c]Untreated naïve control C57BL/6 mice from Charles River-Frederick were included in all experiments using PMO treatment.
[d]Interleukin (IL)-10 knockout or control C57BL/10 mice were purchased from Jackson Laboratories.

## Results and Discussion

Antisense phosphorodiamidate morpholino oligomers (PMOs) have shown potent sequence-specific antiviral *in vitro* and *in vivo* efficacy against numerous RNA viruses by selective targeting of viral gene products (reviewed in Stein, 2008). Among these is the antisense targeting of EBOV VP35 protein expression, which provided significant protection against lethal challenge. Important to the studies shown here is the capability of a PPMO to deliver antisense into murine leukocytes, both *in vitro* and *in vivo* (Marshall et al., 2007; Mourich et al., 2009). The ability to target protein expression *in vivo* with PPMOs make these particularly useful agents for determining the role of different host immune factors in the pathogenesis of EBOV.

Among the group of PPMO-targeted host molecules providing significant protection was IL-10 and its cognate receptor alpha chain (Table 1). IL-10 is a cytokine produced by DCs, monocytes-macrophages, B cells, and various subsets of CD4+ and CD8+ T cells (Mosser and Zhang, 2008). Moreover, DCs have been shown to be a cell that provides early and sustained foci of EBOV infection and are likely the cell types producing IL-10 early in the infection cycle. Therefore, we sought to confirm the antisense activity of IL-10 PPMO by altered mRNA splicing and protein inhibition in these cells. Treatment of lipopolysaccharide-stimulated DCs or a-CD3-stimulated T cells (data not shown) with the PPMO targeting the exon 4 splice acceptor of IL-10 pre-mRNA (IL-10 SA4) resulted in the production of an amplicon having the predicted size of IL-10 mRNA missing the portion encoded by exon 4 as determined by gel electrophoresis (Fig. 1). The sequences were verified after isolation and cloning. The splice altering activity was found to be dose- and time-dependent as was the effect on IL-10 protein production measured in the culture supernatants taken from PPMO-treated DCs (Fig. 1). *In vivo* splice altering activity was confirmed in splenocytes isolated from PPMO-treated mice following a-CD3 or Zymosin injection.

The influence of IL-10 on Th1 responses in chronic viral infections, including inhibition of NK cell and macrophage activity as well as an ability to ameliorate immunopathology has been well established (Couper et al., 2008). However, it is not clear how IL-10 affects the pathology of acute virus infection including EBOV (Mohamadzadeh, 2009). Previous reports in human EBOV outbreaks have reported a positive correlation between survival and reduced IL-10 serum levels (Leroy et al., 2001; Hutchinson and Rollin, 2007). However, it is not known whether reducing IL-10 expression or signaling capability could lead to increased EBOV survival.

To test if disruption of the IL-10 signaling pathway could diminish EBOV lethality, we utilized PPMO to target expression of IL-10 or the cognate receptor alpha chain IL-10RA in the C57BL/6 mouse model of EBOV infection. As shown in Table 1, IL-10 (SA4) and IL-10RA PPMO treatment significantly improved mouse survival (up to 72%) against EBOV challenge compared to controls, including irrelevant PPMO targeting influenza nucleoprotein or a PPMO targeting IL-10 sequence (E3SD) that does not influence IL-10 mRNA splicing or protein expression, demonstrating that the protection is not due to off-target or nonspecific effects by PPMOs. This is the first direct evidence that targeting IL-10 can enhance resistance to EBOV lethality. To confirm the role of IL-10 in EBOV pathogenesis, we infected IL-10 knockout (IL-10$^{-/-}$) or control wild-type
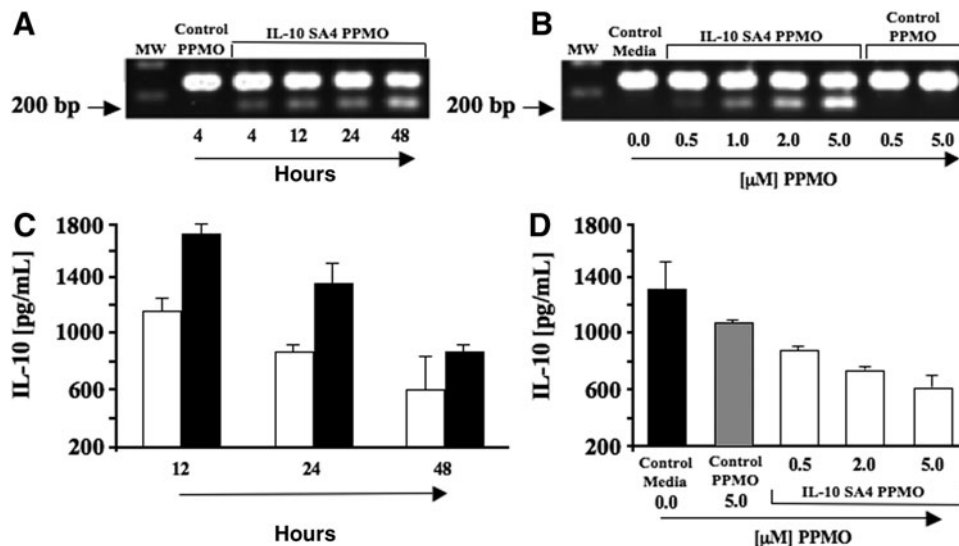


**FIG. 1.** Confirmation of interleukin (IL)-10 SA4 peptide-conjugated derivative of the phosphorodiamidate morpholino oligomer (PPMO) activity exhibits a dose- and time-dependent effect on mRNA and protein expression in dendritic cells (DCs). **(A)** RT-PCR of mRNA isolated from 12-, 24-, and 48-hour cultured DCs after treatment with either control or IL-10 SA4 PPMO [5 μM]. Lane 1, molecular weight (MW) marker. Lane 2, control PPMO at 4 hours. Lanes 3, 4, 5, 6, IL-10 SA4 PPMO treatment for 4, 12, 24, and 48 hours, respectively. **(B)** Dose range effect at 48 hours post treatment. Lane 1, molecular weight marker. Lane 2, untreated cells. Lanes 3, 4, 5, are 0.5, 1, 2, and 5 μM IL-10 SA4 PPMO, respectively. Lanes 6, 7, 5.0 and 0.5 μM control PPMO, respectively. **(C)** ELISA detection of IL-10 in culture media over 12, 24, and 48 hours with 5 μM treatment with either control IL-10 E3SD (black bars) or IL-10 SA4 (white bars) PPMO. **(D)** Response to dose range from 0.5–5.0 μM of IL-10 SA4 PPMO for 48 hours. RT-PCR, reverse transcription-polymerase chain reaction.

(WT) C57BL/10 mice. As shown in Table 1, IL-10$^{-/-}$ mice were partially protected against EBOV compared with WT mice. Additionally, we have shown that IL-10 modulation can also afford significant protection in a mouse model for Marburg virus infection (data not shown), indicating that filoviruses likely utilize IL-10 signaling as a common immune modulating mechanism to establish infection and pathology.

To analyze mechanisms of protection in IL-10$^{-/-}$ mice, serial sampling experiments were performed. IL-10$^{-/-}$ or WT mice were euthanized at periods after infection to measure viral titer. Interestingly, viremia was only slightly reduced in serum and spleen in IL-10$^{-/-}$ mice relative to WT on day 7 (Fig. 2A). This was surprising, since it is often thought that viral load correlates with severity of disease. However, since IL-10$^{-/-}$ mice are only partially protected against EBOV infection, this

may be explained by a minor decrease in replication. One hallmark of EBOV infection is severe lymphocyte apoptosis (Baize et al., 1999; Bradfute et al., 2007). Histopathological analysis revealed a decrease in lymphocyte apoptosis in the spleens of IL-10$^{-/-}$ mice, compared to WT mice correlating with reduced terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling of apoptotic cells and cell debris (Fig. 2B). Although the role of lymphocyte apoptosis in EBOV infection is unclear (Bradfute et al., 2008), it is interesting to note that IL-10$^{-/-}$ mice exhibit less splenocyte apoptosis than WT. In addition, we observed fewer apoptotic cells and cell debris in the liver of IL-10$^{-/-}$ mice as opposed to WT (Fig. 2B). Since liver damage is a hallmark of EBOV infection, apoptosis of cells associated with the liver (i.e., Kupffer cells, fibroblastic reticular cells, endothelial cells, and



**FIG. 2.** Viral replication and pathology in IL-10 $(-/-)$ knockout (KO) mice. **(A)** Viral replication in serum and spleen late in ebola virus (EBOV) infection. $n = 4$ for days 3 and 7; $n = 2$ for day 0. $*p = 0.058$; $\ddagger p = 0.062$. Black and white bars indicate wild-type (WT) mice and IL-10 $-/-$ mice, respectively. **(B)** TUNEL staining of liver and spleen on day 7 of lymphocytes and hepatocytes in IL-10 $(-/-)$ mice relative to WT (*arrows* indicate positive staining).
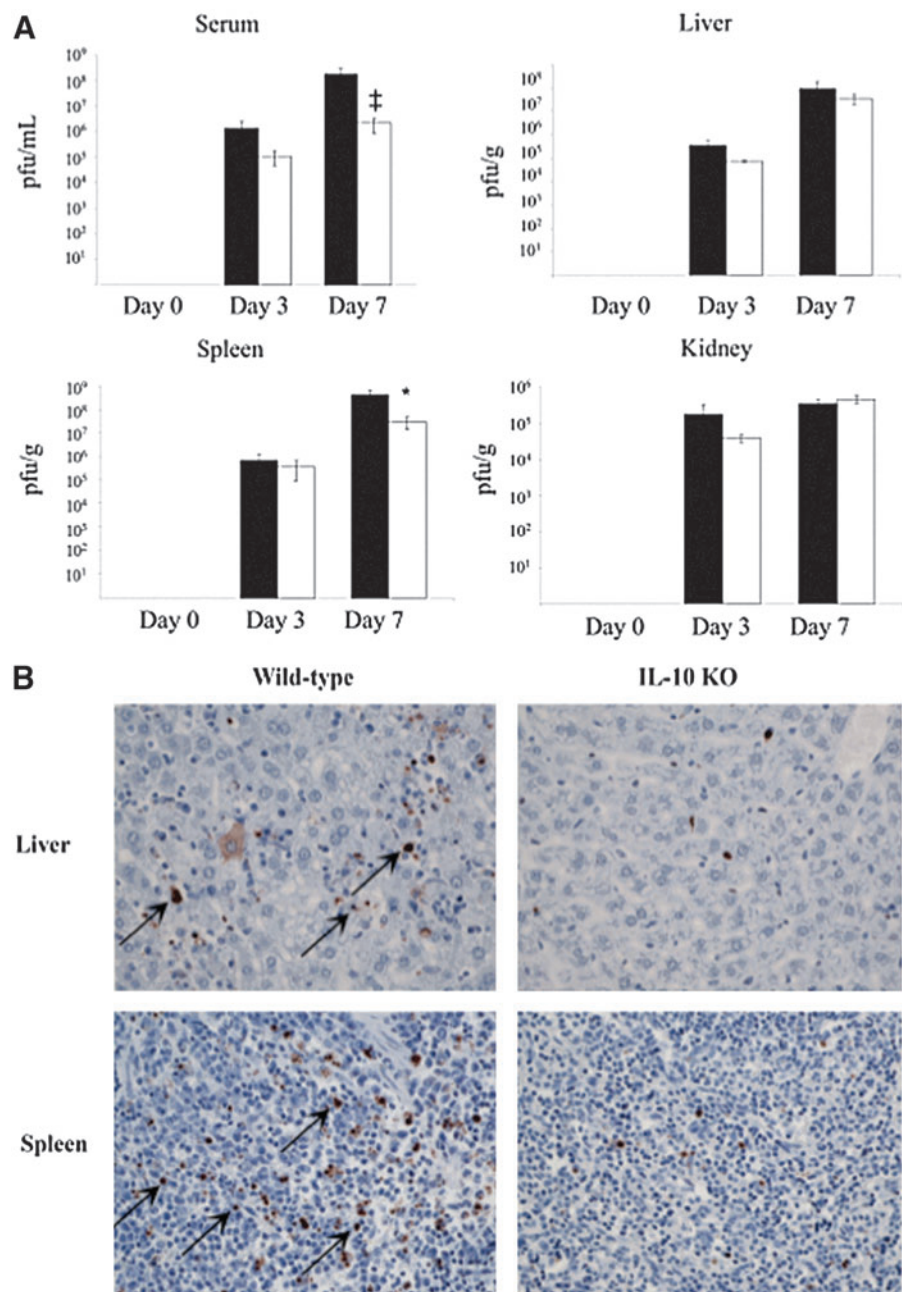
hepatocytes) may play a role in EBOV-induced liver dysfunction.

To determine cytokine expression profiles in WT versus IL-10$^{-/-}$ mice following EBOV infection, serum cytokines were assessed on days 0, 3, 6, and 7 post-infection. Data from days 6 and 7 were analyzed together as a single group. IL-10 was produced in WT, but not IL-10$^{-/-}$ mice at both days 3 and 6/7 (data not shown), similar to human data suggesting increased IL-10 levels correlate with fatal outcome. In day 6/7 serum samples, several cytokines exhibited increased expression in WT but at lower levels in IL-10$^{-/-}$ mice, including fibroblast growth factor (FGF)-basic, IL-1b, IL-2, IL-6, IL-13, keratinocyte-derived chemokine (KC), and macrophage/monocyte chemoattractant protein-1 (MCP-1) (Fig. 3). It is important to note that timing of various cytokine expression profiles can result in very different responses to infection. For example, in human EBOV infection, production of proinflammatory cytokines early after EBOV infection correlate with survival, but production of the same cytokines later in infection is also found in lethal infection (Baize et al., 1999; Leroy et al., 2001; Hutchinson and Rollin, 2007). Similarly, data presented here suggest that suppression of proinflammatory cytokines late in infection in IL-10$^{-/-}$ mice correlates with protection against EBOV infection.

To determine what cell types provide protection against EBOV infection in IL-10$^{-/-}$ mice, different populations of lymphocytes were depleted in IL-10$^{-/-}$ mice using antibody treatment. As shown in Fig. 4A, CD4$^{+}$ cells were not required for protection in IL-10$^{-/-}$ mice. However, NK cells were absolutely required, as NK-depleted IL-10$^{-/-}$ mice succumbed to EBOV infection with the same kinetics as WT mice. IL-10$^{-/-}$ mice receiving IFN-γ neutralizing antibody also completely succumbed to EBOV infection, whereas mice receiving isotype control had increased survival (24% average) relative to WT mice (0% survival). These data are consistent with a model that reduced IL-10 leads to protection mediated by NK cell-derived IFN-γ, resulting in control of EBOV infection.

It has been inferred from other models (Mohamadzadeh, 2009) as well as supported by these data that lethal EBOV infection may induce a T helper type 2 cell (Th2)-skewed immune response that is ineffective against EBOV disease. To test this, we infected either WT or IFN-γ$^{-/-}$ mice with EBOV. As shown in Fig. 4B, mice lacking IFN-γ succumbed to EBOV
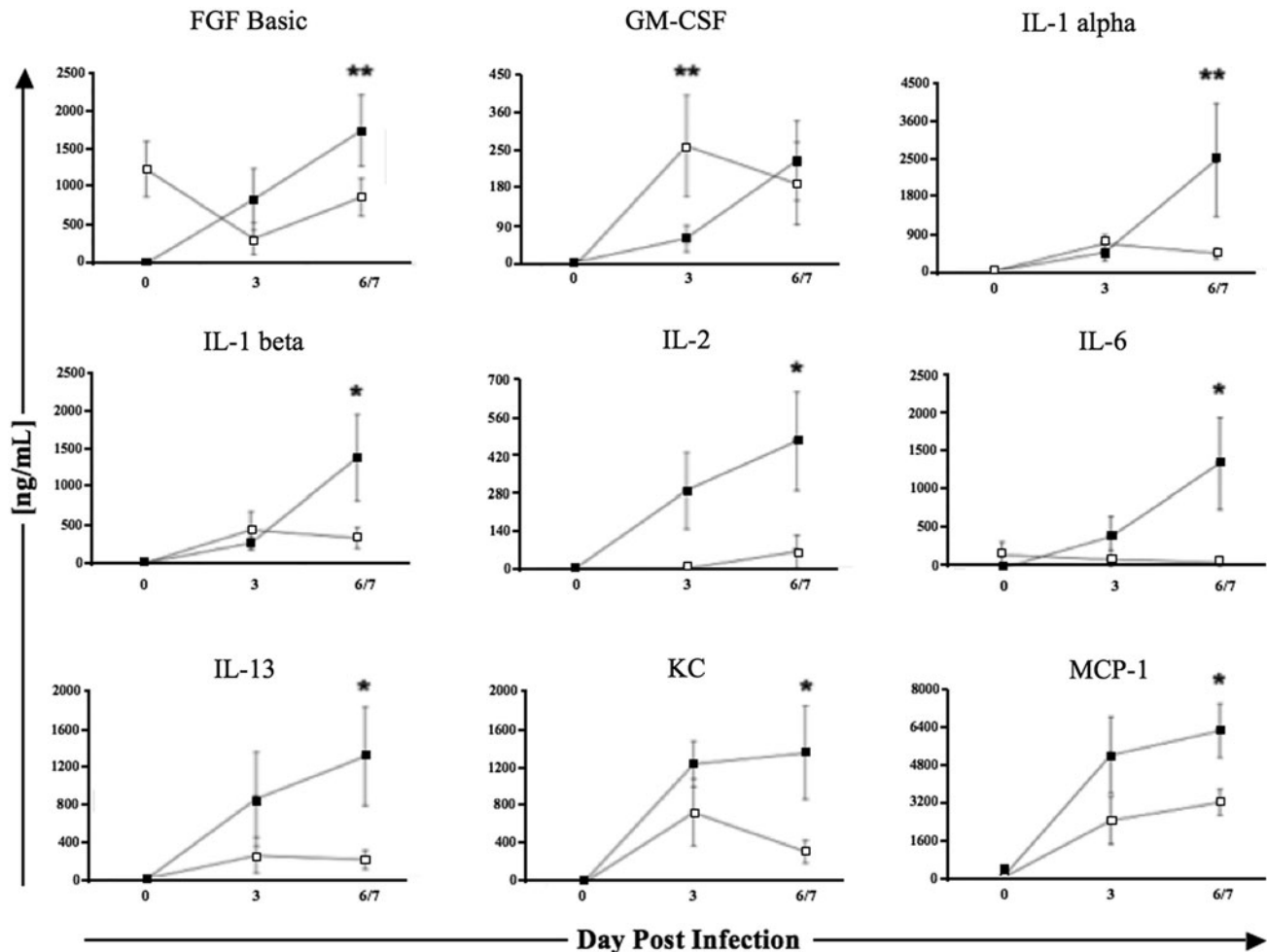


**FIG. 3.** Decrease in proinflammatory cytokine expression late in EBOV infection in IL-10 KO mice. Serum cytokine levels were determined by multiplex bead array analysis using the Luminex system. *Black closed square* symbols indicating WT mice compared with *white open squares* show that IL-10($-/-$) mice have decreased proinflammatory cytokine levels late in EBOV infection. $n = 2$ for day 0; $n = 7$ for day 3; and $n = 10$ for day 6/7. *$p < 0.05$, **$p < 0.06$. FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemotactic protein-1.
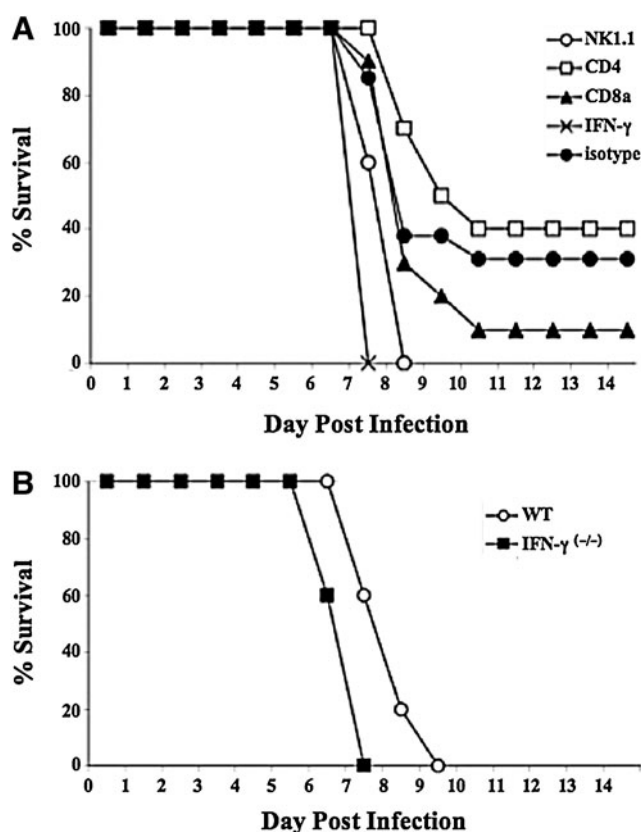
**FIG. 4.** Natural killer (NK) cells and interferon-gamma (IFN-γ) are necessary to IL-10$^{-/-}$ mice protection against EBOV. **(A)** Survival against EBOV challenge of IL-10 (−/−) mice injected with depleting antibody to CD4, CD8a, NK1.1, or IFN-γ neutralizing antibody. $n=10$ for NK1.1, CD4, CD8a, IFN-γ; $n=5$ for isotypes ($n=13$ combined), $n=15$ for WT. **(B)** WT compared with IFN-g (−/−) KO mice infected with EBOV; $n=10$.

infection more quickly than WT mice. The outcome of a T helper cell response is influenced by several factors including IL-10 expression by developing Th2 cells, which can be inhibitory to subsequent Th1 differentiation. T-bet is a Th1-specific transcription factor and promotes Th1 development while inhibiting Th2. Using T-bet$^{-/-}$ mice in the EBOV lethal infection model we observed that this also resulted in marked increase an in susceptibility compared to wild type strain.

Here, we show that diminished IL-10 signaling improves survival to EBOV infection. NK cells and IFN-γ are required for protection in IL-10$^{-/-}$ mice, confirming the findings that they play important roles in successful vaccination against EBOV (Warfield et al., 2004; Warfield et al., 2005). Furthermore, this protection is associated with decreased expression of several inflammatory cytokines late in infection, as well as a small decrease in viral titers. IL-10 is known to suppress Th1 responses, through inhibition of IFN-γ. Indeed, neutralization of IFN-γ caused IL-10$^{-/-}$ mice, and IFN-γ$^{-/-}$ mice to succumb more quickly to EBOV infection than WT. Together, these data suggest that the Th1/Th2 balance plays an important role in survival to EBOV infection, and skewing to a Th2 response is detrimental. It is interesting to note that in human EBOV infection, the correlation with IL-10 expression and survival is not absolute; many survivors pro-

duced levels of IL-10 similar to patients who succumbed to infection (Hutchinson and Rollin, 2007). Similarly, we show here that in IL-10$^{-/-}$ mice or antisense treated mice targeting IL-10 signaling are only partially protected against EBOV infection. This shows the importance and relevance of the mouse model in dissecting mechanisms of immune protection for EBOV infection. We report for the first time that directly diminishing IL-10 production or signaling improves survival to EBOV infection; validating that this is a potential host target that appears to directly modulate the outcome of EBOV infection and could be targeted with antibody (to IL-10RA) (Ejrnaes et al., 2006) or antisense (to IL-10 or receptor) as potential treatment regimen.

Many pathogens have evolved to evade, alter, or diminish immune responses, usually not to a lethal level for the host, yet sufficient to allow replication and spread to new hosts. Chronic viruses tend to influence virus-specific adaptive responses allowing for long-lived infection, while acute viruses spread quickly and efficiently induce innate and cellular responses they are cleared rapidly. However, accidental hosts can become infected and subsequent complex interactions with a "foreign" immune system can result in unrestrained highly destructive replication and or immune-induced pathology. Understanding which host factors contribute to increased virulence or lethality of hemorrhagic viruses could provide a pathway to produce broadly applicable therapeutics lessening the risk of death from other known or newly emerging acute viral diseases.

### Author Disclosure Statement

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