Cowpox virus induces interleukin-10 both in vitro and in vivo

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Cowpox virus infection induces interleukin-10 (IL-10) production from mouse bone marrow-derived dendritic cells (BMDCs) or cells of the mouse macrophage line (RAW264.7) at about 1800 pg/ml, whereas infections with vaccinia virus (strains WR or MVA) induced much less IL-10. Similarly, in vivo, IL-10 levels in bronchoalveolar lavage fluids of mice infected with cowpox virus were significantly higher than those after vaccinia virus infection. However, after intranasal cowpox virus infection, although dendritic and T-cell accumulations in the lungs of IL-10 deficient mice were greater than those in wild-type mice, weight-loss and viral burdens were not significantly different. IL-10 deficient mice were more susceptible than wild-type mice to re-infection with cowpox virus even though titters of neutralizing antibodies and virus-specific CD8 T cells were similar between IL-10 deficient and wild-type mice. Greater bronchopneumonia in IL-10 deficient mice than wild-type mice suggests that IL-10 contributes to the suppression of immunopathology in the lungs.

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Introduction

IL-10 was first described as a CSIF (cytokine synthesis inhibitory factor), a cytokine produced by T-helper 2 (Th2) cells that inhibited the production of cytokines by Th1 cells (Fiorentino et al., 1989). It is now known to be produced by cells of numerous types, including B cells (Burdin et al., 1997; O’Garra et al., 2004), macrophages, dendritic cells (Boonstra et al., 2006), mast cells (Ishizuka et al., 1999), and eosinophils (Nakajima et al., 1996), as well as various T cells including CD8 and CD4 T cells (reviewed by Hawrylowicz and O’Garra, 2005; O’Garra et al., 2008; O’Garra and Vieira, 2007). The primary role of IL-10 is to act as a key regulator of immunity to infection (reviewed by Couper et al., 2008). IL-10 is an immunosuppressive cytokine that inhibits multiple macrophage and DC functions, including: their synthesis of proinflammatory cytokines; expression of costimulatory molecules and MHC class II proteins; antigen presentation; and DC trafficking (reviewed by Couper et al., 2008; Grutz, 2005; O’Garra et al., 2008). In a normal course of events, IL-10 can act to curtail inflammatory and adaptive immune responses that otherwise might be damaging to the host. Indeed, IL-10 is one of the principal active agents of regulatory T cells (Li and Flavell, 2008; Roncarolo et al., 2006) and regulatory B cells (Mauri and Ehrenstein, 2008).

In its role as a key regulatory cytokine, IL-10 is a crucial factor in the outcome of several viral diseases (reviewed by Blackburn and Wherry, 2007; Filippi and von Herrath, 2008). A number of viruses including lymphocytic choriomeningitis virus (Brooks et al., 2008, 2010), hepatitis C virus (Brady et al., 2003; Saito et al., 2008), hepatitis B virus (Hyodo et al., 2004), murine gammaherpesvirus 68 (Siegel et al., 2008), and HIV (Contreras et al., 2004; Gee et al., 2006, 2007; Granelli-Piperno et al., 2004; Gupta et al., 2008) induce elevated levels of IL-10 production during infection which correlate with the impairment of T-cell responses and the failure to control viral replication. Herpes simplex virus can stimulate infected T cells to selectively synthesize IL-10, and elevated levels of IL-10 have also been found in T cells of mice during acute infections with Sin Nombre virus (Schountz et al., 2007; Sloan and Jerome, 2007).

Underscoring the importance of IL-10 in viral infections, several herpes- and poxviruses, including Epstein–Barr virus (Hsu et al., 1990; MacNeil et al., 1990), human cytomegalovirus (Jenkins et al., 2008; Kotenko et al., 2000; Lockridge et al., 2000), orf virus (Fleming et al., 2000, 1997), lumpy skin disease virus (Tulman et al., 2001), and Yaba-like disease virus (Bartlett et al., 2004), encode viral proteins that are homologues of IL-10 or the IL-10-like family members IL-19, IL-20,
and IL-24. The properties of these viral IL-10s are typically distinct from those of the host IL-10, and the biological roles of each viral IL-10 are not completely understood. Nonetheless these viral proteins are thought to mimic actions of IL-10 to promote viral replication and contribute to the pathogenic properties of each virus.

Interestingly, although the viruses of the orthopoxvirus genus have acquired numerous homologues of host genes (reviewed by Hughes et al., 2010), including several genes encoding proteins that inhibit or modify host immune responses, none of these viruses is known to encode a viral homologue of IL-10. The orthopoxviruses may have failed to acquire genes encoding IL-10 homologues because they fail to derive benefit from such homologues. In this regard, available information has been equivocal. On the one hand, vaccinia virus-vector expression of mouse IL-10 in immunocompetent C57BL/6 mice did result in both the suppression of natural killer cell activity at 3 days post-infection, and reduced (25–50%) virus-specific CTL activity at 6–7 days post-infection in comparison to infection with control vaccinia virus. On the other hand, infections of BALB/c mice, by either dermal scarification, intraperitoneal, intranasal, or intracerebral inoculation, with recombinant vaccinia viruses expressing mouse IL-10 did not differ significantly from infections with wild-type vaccinia viruses in any of the following: visible lesions; viral load in the lungs; morbidity; mortality; protective immunity to a 100-fold lethal challenge with vaccinia virus; or vaccinia virus-specific antibody response (Kurilla et al., 1993).

An alternative explanation for the orthopoxviruses’ failure to encode homologues of IL-10 is that some of these viruses may have acquired the capacity to use the host-encoded IL-10 to their advantage. The first evidence of this was provided by Slezak et al. (2000) who showed that vaccinia virus infection of human monocytes resulted in a modest induction of IL-10 (46 pg/ml) by 24 hr post-infection. Subsequently, Maloney et al. (2005) showed that at least one orthopoxvirus protein might affect IL-10 expression under certain circumstances, in that the vaccinia virus protein A52R can strongly enhance lipopolysaccharide-induced IL-10 production in cultured RAW264.7 cells. Liu et al. (2005) found that vaccinia virus infection of primary human keratinocytes resulted in increased expression of immunoregulatory cytokines including IL-10 (30 pg/ml). However, the viral induction of IL-10 was cell-type specific, insofar as there was no induction of IL-10 by vaccinia virus infection of dermal microvascular endothelial cells, or X52 cells, a line of cells established from mouse epidermis-derived dendritic cells (Deng et al., 2006). Intriguingly, studies of the effects of cowpox virus infection of BALB/c mice (Erwin, Hutt, and Lyons, personal communication), as well as studies of Knorr et al. (2006) and Smee et al. (2008) with vaccinia and cowpox viruses, showed that while both viruses could induce elevated levels of IL-10 in serum and lung tissue, cowpox virus induced IL-10 accumulations could be up to 40-fold greater than those induced by vaccinia virus.

Collectively, these results suggested that cowpox virus, which possesses many more accessory genes than vaccinia virus, might have a special capacity to induce IL-10 as part of its strategy to suppress host immune responses. The objectives of this study were to determine the effects of cowpox virus infection on host IL-10, and to determine the effects of IL-10 on cowpox virus replication in vivo.

**Results**

**Cowpox virus induces secretion of IL-10 from mouse bone marrow derived dendritic cells**

To determine if cowpox virus can induce the production of IL-10 in host cells, cultures of mouse bone marrow-derived dendritic cells (BMDC) were infected with cowpox virus, and cytometric bead arrays were used to measure the accumulation of IL-10 in the cell culture medium. As shown in Fig. 1A, cowpox virus-induced levels of IL-10 (~1760 pg/ml) by 24 hr after infection. Control cultures of BMDCs were treated with a mixture of Pam3CSK4 and ODN 1826, (an unmethylated oligonucleotide with CpG motifs), here collectively referred to as Pam/CpG, to stimulate Toll-like receptors (TLRs) 2 and 9 respectively. Pam/CpG treatment of the BMDCs produced a modest but measurable induction of IL-10, consistent with that of previous studies (Samarasinghe et al., 2006).

To determine whether infection with other orthopoxviruses would similarly induce the production of IL-10 in host cells, additional cultures of mouse BMDCs were infected with vaccinia virus strain WR (VV-WR), or the highly attenuated strain of vaccinia virus, modified vaccinia Ankara (MVA), and the production of IL-10 in the cell culture media was measured by cytometric bead array 24 hr after infection. As shown in Fig. 1A, only cowpox virus, and neither VV-WR nor MVA was able to induce the production of IL-10 by BMDCs. To determine whether active infection was required for IL-10 induction, parallel cultures of BMDCs were treated with either UV- or heat-inactivated viruses. The inactivated viruses did not induce detectable IL-10.

![Fig. 1. Cowpox virus induces higher amounts of IL-10 in cultured cells than vaccinia virus WR or MVA.](image-url)

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production by BMDCs, suggesting that either processes during viral entry into the cell, or some products of viral replication, but not simply inactivated virus particles, are required to stimulate IL-10 induction. IL-10 induction was stimulated by cowpox virus infection in the presence or absence of cytosine arabinoside, an inhibitor of viral DNA replication, showing that events during the early phase of virus replication are sufficient to induce the observed IL-10 accumulation (Fig. 1B).

Cowpox virus induces IL-10 in mouse macrophage-derived RAW264.7 cells

To determine whether cowpox virus-specific induction of IL-10 could be reproduced in cells other than primary BMDCs, cells of a mouse macrophage cell line (RAW264.7 cells) were infected with cowpox, VV-WR, MVA, or mock infected with media alone. A parallel culture of cells was treated with LPS as a positive control for IL-10 induction. RAW264.7 cells were used both because they are derived from mouse macrophages, which together with dendritic cells are an important source of cytokines early after infection in the mouse (reviewed by Lohmann-Matthes et al., 1994; Peters-Golden, 2004), and because RAW264.7 cells are known to produce IL-10 in response to LPS treatment (Hasko et al., 1996). The levels of IL-10 in the culture media were determined 6 hr after infection. As shown in Fig. 1C, RAW264.7 cells infected with cowpox virus produced high amounts of IL-10 (~1800 pg/ml), whereas cells infected with either VV-WR or MVA produced much lower levels (~80 and 100 pg/ml respectively), consistent with earlier determinations of IL-10 induction by vaccinia viruses in cultured cells (Liu et al., 2005; Slezak et al., 2000). As expected, control cultures treated with LPS also produced high levels of IL-10 (~2000 pg/ml), whereas IL-10 accumulation above background levels was not detected in the culture media from untreated RAW264.7 cells.

In summary, the preceding results showed that cowpox virus induced significant levels of IL-10 production from primary BMDC and macrophage-derived RAW264.7 cells, while VV-WR and MVA did not. The induction of IL-10 by cowpox virus required viral replication, but neither viral DNA replication nor viral late replication, which suggests that either viral entry, or products of early viral replication are sufficient to trigger IL-10 induction.

Cowpox virus induces more IL-10 in vivo than vaccinia virus

To determine whether cowpox virus induced more IL-10 in vivo than the less virulent vaccinia virus, the bronchoalveolar lavage fluid (BAL) was collected from infected mice and assayed for IL-10. For this purpose, cohorts of C57BL/6 mice (n = 10 per group) were infected intranasally with 10^4 PFU of CPXV, VV, or MVA, or mock infected with PBS. BAL fluid was collected 6 days after infection. The levels of IL-10 in the BAL fluids were determined by cytometric bead array (Bio-Rad). Bars represent the SEM. For infections with CPXV, VV and MVA, n = 10; for PBS, n = 5. Values of p were calculated by two-tailed Student’s t test: CPXV versus VV-WR p = 0.0189; CPXV versus MVA p = 0.0006. Dashed line indicates the minimum level of detection of IL-10.

**Fig. 2.** Cowpox virus induces higher amounts of IL-10 in the lungs of infected mice than vaccinia viruses WR or MVA. (A) C57BL/6NCrl mice (8–10 week old female mice) were infected intranasally with 10^4 PFU of CPXV, VV, or MVA, or mock infected with PBS. BAL fluid was collected 6 days after infection. The levels of IL-10 in the BAL fluids were determined by cytometric bead array (Bio-Rad). Bars represent the SEM. For infections with CPXV, VV and MVA, n = 10; for PBS, n = 5. Values of p were calculated by two-tailed Student’s t test: CPXV versus VV-WR p = 0.0189; CPXV versus MVA p = 0.0006. Dashed line indicates the minimum level of detection of IL-10. (B) Viral load in the lungs of mice infected with similar, sublethal doses of CPXV, VV or MVA. C57BL/6NCrl mice (8–10 week old female mice) were infected intranasally with 10^4 PFU of virus. For all groups, n = 3. Mice were euthanized 6 days after infection, and the titers in the lungs were determined by plaque assay (CPXV and VV-WR) or immunostaining (MVA). Differences in the means among groups were tested by two-tailed Student’s t test indicating no significant difference (p = 0.72) in lung titers of CPXV and VV-WR-infected mice.

IL-10 suppresses DC and T-cell accumulation in the lungs of mice intranasally infected with sublethal doses of cowpox virus

Although neither weight loss nor viral load following sublethal infection with cowpox virus was affected by the absence of IL-10, examination of the inflammatory response in the lungs of infected mice revealed marked differences between wild-type and IL-10 deficient mice. In the late stages of infection (days 14–21), there were greater numbers of dendritic cells CD4, and CD8 T cells in the lungs of IL-10 deficient mice as compared with wild-type mice (Figs. 4A–C). In addition, histological analyses of lungs of mice at 14 days after infection showed that wild-type and IL-10 deficient mice (Figs. 4D and E) had predominantly mononuclear cellular infiltrates that were consistent with the increased cellularity determined by flow cytometry. In both wild-type and IL-10 deficient mice, the infiltrates were greatest around the subsegmental pulmonary arteries and associated bronchioles, becoming more diffuse in the surrounding tissue. In many cases, the lesions were larger in the lungs of IL-10 deficient mice than in those of wild-type mice suggesting a greater degree of pulmonary inflammation in the absence of IL-10.

These results indicate that both the magnitude and the duration of the inflammatory responses to viral infection are increased in the effects of infection of wild-type control animals. As shown in Fig. 3A, after intranasal infection with a sublethal dose of virus (10^5 PFU/mouse), the course of disease in IL-10 deficient mice was very similar to the course of disease in wild-type mice, with all animals surviving virus challenge, and no statistically significant difference in the weight losses between the groups. Consistent with this result, additional cohorts of wild-type and IL-10 deficient mice (n = 5 mice per group per timepoint) challenged with cowpox virus contained similar viral loads in the lungs (Fig. 3B). Taken together, these results showed that IL-10 deficient mice were not significantly more sensitive or resistant than wild-type mice to primary challenge with a sublethal dose of cowpox virus. Similarly, IL-10 deficient mice were not significantly different from wild-type mice in their responses to infection with cowpox virus at a 10-fold higher dose, which resulted in 20% weight loss within 5 days of infection (Fig. 3C), at which time the mice were euthanized.

Weight loss and viral burdens are not significantly different in wild-type and IL-10 deficient mice after sublethal cowpox virus infection

To begin to determine whether IL-10 induction contributed to the pathogenicity of cowpox virus, the effects of sub-lethal cowpox virus infections of mice genetically deficient in IL-10 were compared to the
lungs of IL-10 deficient mice as compared to response in wild-type mice.

**IL-10 deficient mice are more susceptible than wild-type mice to re-infection with cowpox virus**

To determine whether wild-type and IL-10 deficient mice were similarly protected from re-challenge with a lethal dose of cowpox virus, groups of mice were infected intranasally with cowpox virus (10^4 PFU per mouse), and then challenged with a lethal dose (10^6 PFU per mouse) 10 weeks after the primary infection. As expected, all wild-type mice were protected from re-challenge, with mice exhibiting no weight loss or other signs of morbidity (Fig. 5A). The IL-10 deficient mice were also protected from re-challenge, but unlike wild-type mice they exhibited a transient weight loss of up to about 8% (Fig. 5A) although all IL-10 deficient mice exhibited a transient weight loss of up to about 10% in wild-type mice, and CPXV-infected IL-10 deficient mice, n = 9. Mice were weighed daily, and the mean percentage of the initial weight for each day is shown. Bars indicate SEM. Mice were euthanized if they lost more than 20% of their initial weight or became unresponsive. (B) Mice were infected as described in panel A. At the indicated times post-infection, 5 mice per group were euthanized. Lungs were harvested, and the viral titers per lung were determined. The dashed line indicates level of detection of the plaque assay. There were no statistically significant differences (as determined by two-tailed Student’s t test) between virus titers in lungs of wild-type versus IL-10 deficient mice at any time post-infection. Infectious virus was not detected (ND) at 21 or 28 days post-infection. (C) As per Panel A, except mice were infected intranasally with 10^5 PFU of CPXV.

**Fig. 3.** Comparison of CPXV-induced weight loss after primary intranasal infection of wild-type and IL-10 deficient mice. (A) C57BL/6J (wild-type) or B6.129P2-Ii10tm1Cgn/J (IL-10−/−) mice (female, 8–10 week old mice) were infected intranasally with 10^6 PFU of CPXV, or mock-infected with PBS. For mock-infected wild-type mice, and mock-infected IL-10−/− mice, n = 5; for CPXV-infected wild-type mice, and CPXV-infected IL-10−/− mice, n = 9. Mice were weighed daily, and the mean percentage of the initial weight for each day is shown. Bars indicate SEM. Mice were euthanized if they lost more than 20% of their initial weight or became unresponsive. (B) Mice were infected as described in panel A. At the indicated times post-infection, 5 mice per group were euthanized. Lungs were harvested, and the viral titers per lung were determined. The dashed line indicates level of detection of the plaque assay. There were no statistically significant differences (as determined by two-tailed Student’s t test) between virus titers in lungs of wild-type versus IL-10 deficient mice at any time post-infection. Infectious virus was not detected (ND) at 21 or 28 days post-infection. (C) As per Panel A, except mice were infected intranasally with 10^5 PFU of CPXV.

One of the major correlates of protection against poxvirus infection is the development of virus neutralizing antibodies (Panchanathan et al., 2006, 2010). To begin to determine the basis for the enhanced susceptibility of IL-10 deficient mice to rechallenge, the production of serum antibody against cowpox virus in wild-type and IL-10 deficient mice was compared. As shown in Fig. 5B, sera collected at multiple timepoints after primary infection (from the same cohort of animals shown in Figs. 3–4) were assayed by ELISA for binding activity against the cowpox virus B5 protein. The cowpox virus B5 protein is expressed on the outer membrane of the extracellular enveloped virion (EV), and antibodies against B5 have been shown to limit dissemination of poxviruses in vivo (Benhnia et al., 2009; Galmiche et al., 1999; Law and Smith, 2001). As shown in Fig. 5B, anti-B5 titers of wild-type and IL-10 deficient mice were similar at all timepoints analyzed. Titers in IL-10 deficient mice were slightly lower than titers in wild-type mice, but because the antibody levels present in both wild-type and IL-10 deficient mice were at or above the levels that are predictive of protection (Barefoot et al., 2008) the difference is unlikely to be biologically significant. Similarly, analyses of the same sera for neutralizing activities against cowpox virus showed that sera from both groups of mice neutralized live cowpox virus equally well (Fig. 5C). Taken together, these results suggest that protective anti-cowpox virus humoral responses were intact in wild-type and IL-10 deficient mice, and that the susceptibility of IL-10 deficient mice to rechallenge cannot be attributed to failure of the humoral immune response in those animals.

To determine whether cellular responses were intact in IL-10 deficient mice, the production of cowpox virus-specific CD8 T cells in wild-type and IL-10 deficient mice was analyzed. Using cells from the same cohort of mice as in Figs. 3–4, an MHC Class I tetramer was used to identify Kb-restricted CD8 T cells recognizing the immunodominant epitope (TSYKFESV) of the cowpox virus B8 protein. As shown in Fig. 5D, wild-type and IL-10 deficient mice were similarly protected from re-challenge with a lethal dose of cowpox virus (10^6 PFU per mouse), and then challenged with a lethal dose (10^6 PFU per mouse) 10 weeks after the primary infection. As expected, all wild-type mice were protected from re-challenge, with mice exhibiting no weight loss or other signs of morbidity (Fig. 5A). The IL-10 deficient mice were also protected from re-challenge, but unlike wild-type mice they exhibited a transient weight loss of up to about 8%, but all IL-10 deficient mice had to be euthanized on the 7th day after challenge (Fig. 5A). Although all IL-10−/− mice exhibited a transient weight loss of up to about 10% (Fig. 5A) although all IL-10 deficient mice did survive. At a 10-fold higher challenge dose (10^7 PFU per mouse) all wild-type mice were protected from re-challenge, with mice exhibiting transient weight loss of up to about 8%, but all IL-10 deficient mice lost weight rapidly, and had to be euthanized on the 7th day after challenge (Fig. 5A). These results suggested that IL-10 was required for optimal protection against re-infection, especially at higher rechallenge doses.

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IL-10 deficient mice were more susceptible than wild-type mice to rechallenge.

To gain greater insight into the mechanisms contributing to the increased susceptibility of immunized IL-10 deficient mice versus immunized wild-type mice to challenge with cowpox virus, we repeated the challenge experiment, and in addition took samples to assay for potential differences in adaptive immune responses, lung pathology, or virus titers between wild-type and IL-10 deficient mice after secondary challenge.

As shown in Fig. 6A, when mice were primed with 10^3 PFU of cowpox virus, and rechallenged 10 weeks later with a lethal dose (10^6 PFU) of cowpox virus, there was a significant difference in weight loss and survival between wild-type and IL-10 deficient animals. As in the first study, wild-type mice were immune to secondary challenge, and all animals survived. The majority of the IL-10 deficient mice however succumbed to disease by the seventh day after secondary challenge. All naive mice (wild-type and IL-10 deficient mice) rapidly succumbed to infection with a lethal dose (10^6 PFU) of CPXV (Fig. 6B). One possibility that could explain the difference in resistance to secondary challenge was that adaptive immune responses capable of clearing the virus (neutralizing antibody and CD8 T cells) might be deficient in the IL-10 deficient mice. Consistent with this idea, the post-challenge antibody titers (Fig. 6C), and the expansion of CD8 T cells (Fig. 6D) in the IL-10 deficient mice were lower than those in wild-type mice. Accordingly, viral replication may not have been as well controlled in the IL-10 deficient animals as in wild-type mice. Consistent with this possibility, at 3 and 6 days after rechallenge, viral loads in the lungs of infected IL-10 deficient mice were slightly higher than those of wild-type mice (Fig. 6E). Perhaps more importantly, while viral loads of wild-type mice declined from day 3 to day 6, those of IL-10 deficient animals increased.

An additional possibility was that a vigorous inflammatory response may have contributed to pulmonary pathology in the IL-10 deficient mice. To test this possibility, pathology in the lungs of wild-type and IL-10 deficient mice was scored according to four criteria as shown in Fig. 7). In naive mice challenged with 10^6 PFU of cowpox virus, both wild-type and IL-10 deficient mice showed a similar pattern of pathology, with extensive fibrinohemorrhagic necrosis around the pulmonary arteries and their segmental branches. Notably, there was no evidence of lymphocyte infiltration in the naive mice challenged with CPXV, which is consistent with previous reports describing a deficient primary response to poxviruses. In mice...
rechallenged with cowpox virus, pathology was significantly different, with the most dominant finding in both wild-type and IL-10 deficient mice being a marked perivascular lymphocytic infiltrate. Both wild-type and IL-10 deficient mice also demonstrated marked bronchiolitis and bronchopneumonia during secondary challenge. Importantly however, IL-10 deficient mice had a much higher degree of bronchopneumonia than wild-type animals at both timepoints tested (Fig. 7).

Discussion

The results of this study show that cowpox virus, which unlike a number of viruses, such as orb virus, lacks the capacity to encode a viral homologue of IL-10, can induce host cell IL-10 both in vitro and in vivo. In addition, both in vitro and in vivo, at similar doses or at similar viral loads, cowpox virus has greater capacity than vaccinia virus to induce IL-10, suggesting that the induction of IL-10 is not simply a non-specific host response to orthopoxviral infection, but that it may reflect yet one more way in which cowpox virus controls immune responses.

A cowpox virus-specific mechanism of induction of IL-10 would be in accordance with the results of previous studies suggesting that cowpox virus could induce greater levels of IL-10 than vaccinia virus in vivo (Knorr et al., 2006; Smee et al., 2008), as well as the much greater array of accessory genes present in cowpox virus genome of 225-kb (Genbank AF482758) as compared to that of the 195-kb genome of vaccinia virus WR (GenbankAY243312).

The effects of IL-10 on orthopoxvirus infections, using vaccinia virus as the model virus, have been the subject of two earlier studies. Kurilla et al. (1993) examined the effects of viral-vectored expression of mouse IL-10 on vaccinia virus infection in mice. They showed that the effects of this viral expression of IL-10 were subtle, with infections by a variety of routes (including dermal scarification, intraperitoneal, intranasal, and intracerebral routes) resulting in little difference between control virus and IL-10-expressing VV as judged by visible lesions, mortality, antibody response, or protection against subsequent challenge with a 100-fold lethal dose of VV. In immunocompetent mice, they noted that the recombinant vaccinia virus expressing IL-10 induced less natural killer (NK) cell and VV-specific cytotoxic T-cell activities than control virus. Consistent with these studies van Den Broek et al. (2000) showed that after intraperitoneal infection, IL-10 deficient mice cleared vaccinia virus more rapidly than wild-type mice. Together, these two studies suggested that IL-10 could have the effect of suppressing host defenses against vaccinia virus.

In this study, after intranasal infections with cowpox virus, we detected no significant differences between IL-10 deficient mice and wild-type mice with respect to any of the following: weight loss; clinical signs; temperature; viral load in the lungs; antibody or cellular immune responses to viral proteins. Poxviruses are cleared from the host by the
combined action of neutralizing antibody and cellular responses involving cytotoxic T lymphocytes, and NK cells (Chaudhri et al., 2006, 2009; Panchanathan et al., 2006; Xu et al., 2004). Presumably, in the vaccinia model, cellular responses were augmented in the absence of IL-10, because IL-10 has potent immunosuppressive activity. In our cowpox virus model, there was no apparent increase in the activity of cellular antiviral responses in IL-10 deficient mice. This result could mean that the effects of IL-10 produced during infection with cowpox virus infection are less evident than those on vaccinia virus because cowpox virus encodes more immunomodulatory proteins than vaccinia virus. Specifically, cowpox virus can interfere with both NK and T cell functions in ways that vaccinia virus cannot. For example, cowpox encodes a high affinity antagonist of the NKG2D, an activating receptor of NK lymphocytes as well as some CD8 TCR-αβ T cells, splenic TCR-γδ T cells, and murine macrophages (Campbell et al., 2007; Obiedy and Sharland, 2009), whereas vaccinia virus does not. Also, cowpox virus encodes two proteins (CPXV12 and CPXV203) that abrogate MHC class I expression to evade antiviral CD8 T cells (Alzhanova et al., 2009; Byun et al., 2007, 2009), whereas vaccinia virus does not. Also, cowpox virus encodes two proteins (CPXV12 and CPXV203) that abrogate MHC class I expression to evade antiviral CD8 T cells (Alzhanova et al., 2009; Campbell et al., 2007; Sharland, 2009), whereas vaccinia virus does not. Also, cowpox virus encodes two proteins (CPXV12 and CPXV203) that abrogate MHC class I expression to evade antiviral CD8 T cells (Alzhanova et al., 2009; Byun et al., 2007, 2009), whereas vaccinia virus does not. Therefore, even though cowpox virus may be able to induce more IL-10 than vaccinia virus, it may nonetheless be much more competent than vaccinia virus at suppressing immune responses even in the absence of IL-10.

Surprisingly, IL-10 deficient mice immunized with cowpox virus, and then challenged with lethal doses of cowpox virus, were not protected as well as wild-type mice, even though the titers of
antibodies against the B5 protein, titers of neutralizing antibodies, and assays of CD8 T cells recognizing the immunodominant epitope (TSYKFESV) of the cowpox virus B8 protein, failed to show any significant differences in these parameters between IL-10 deficient mice and wild-type mice (Fig. 5). However, upon re-infection, the antibody and CD8+ T-cell responses were lower in IL-10 deficient mice than wild-type mice, which may have contributed to increased viral loads in the IL-10 deficient mice. It is also possible that after challenge, the immunized IL-10 deficient mice may mount a more robust cytokine response than wild-type mice, leading to an exacerbation of immunopathological responses, including pronounced weight loss, even in the presence of what would normally be expected to be protective antibody titers. Previously, IL-10 has been shown to play a role in suppressing immunopathological responses to *Toxoplasma gondii* (Gazzinelli et al., 1996) and *Trypanosoma cruzi* (Hunter et al., 1997).

The results of this study comparing cowpox virus infections in wild-type and IL-10 deficient mice confirm that IL-10 has an important role in reducing immunopathological effects during primary and secondary viral infections. Further, they suggest that a diminished capacity to produce IL-10 may exacerbate reactions to cowpox virus infections, reinfections, or challenges post-immunization. The ability of cowpox virus to induce IL-10 suggests that this property is advantageous to the virus, but additional studies will be needed to determine how this property contributes to viral replication in the natural environment.

Materials and methods

**Viruses and cells**

Cowpox virus, strain Brighton red, and vaccinia viruses, strains Western Reserve (WR) and MVA, were prepared by standard methods (Carroll and Moss, 1997). All viruses were purified by velocity gradient centrifugation as previously described (Joklik, 1962). All virus stocks were tested by culture on chocolate agar, and by PCR screening, to confirm the absence of contamination with bacteria, including mycoplasma. Viruses were quantified by plaque assay on human 143B cells, BSC-1 cells, or BHK cells as previously described (Lynch et al., 2009). Bone marrow cells were harvested from 8 to 16-week old female C57BL/6NCrl mice (from Charles River Laboratories), and cultured into BMDCs as described (Inaba et al., 1992; Lee et al., 2005).

**Reagents**

Lyophilized recombinant mouse IL-4 and GM-CSF (R&D Systems, Minneapolis, MN) were suspended in sterile, distilled water at 10 pg/ml. Trioxsalen (4′-Aminomethyl-Dihydrochloride) was diluted in sterile, distilled water to 1 mg/ml, and filter sterilized. Lyophilized LPS from *Escherichia coli* 0111:B4 (InvivoGen, San Diego, CA), a TLR4 agonist was reconstituted at 5 mg/ml with sterile endotoxin-free water, for use at final concentration of 1 µg/ml. Lyophilized Pam3CSK4 (InvivoGen), a TLR2/TLR1 agonist, was reconstituted at 1 mg/ml with sterile endotoxin-free water, for use at final concentration of 1 µg/ml. Lyophilized ODN 1826 (InvivoGen), an unmethylated oligonucleotide with CpG motifs that stimulates mouse TLR9, was reconstituted at 500 µM in sterile endotoxin-free water, for use at final concentration of 5 µM. Lyophilized recombinant mouse IL-10 (provided with OptEIA ELISA set, BD Biosciences) was reconstituted with 1 ml sterile, distilled water to 175 ng/ml. SuperBlock consisted of PBS, pH 7.4 containing 4% Bovine serum albumin, 0.05% Tween-20 and 0.05% sodium azide.

**Inoculation of mice**

Eight to 10-week-old female C57BL/6j mice or IL-10 deficient mice (B6.129P2-Il10<sup>tm1Cgn</sup>/J) were obtained from Jackson Laboratories. Mice were housed in microisolator cages in a biosafety level 2-equipped animal facility, with mice housed for at least 1 week before experiments were initiated. For intranasal challenge with viruses, mice were lightly anesthetized with isoflurane using a vaporizer, and each mouse was administered the indicated amount of virus in 30 µl total volume. The Institutional Animal Care and Use Committee of Duke University approved all animal experiments. After challenge mice were monitored daily for weight loss and change in body temperature (Physitemp rodent thermometer, Physitemp Inc., Clifton, NJ).
Measurement of IL-10

Murine IL-10 (in cell culture media, serum or the bronchoalveolar lavage fluid of animals) was quantified using an OptEIA ELISA set (BD Biosciences), or Bio-Plex assay (Bio-Rad Laboratories), according to the manufacturer’s instructions. All samples were assayed in duplicate. Numbers reported are in pg/ml as determined via linear regression using a standard curve.

Preparation of organs for titration of infectious virus

Mice were euthanized via anesthesia overdose. Immediately after death, the left atrium was nicked and lungs were perfused with sterile PBS through the right ventricle. Lungs were aseptically removed, weighed, and frozen. To obtain suspensions for titration, lungs were thawed, and homogenized using a tissue homogenizer in 3× their volume (0.1 ml PBS for every 100 g weight). Suspensions were sonicated, centrifuged to remove solids, aliquoted, and stored at −80 °C. For titration, samples were thawed, serially diluted, and assayed on semiconfluent BSC-1 cells (CPXV and VV-WR) or BHK cells (MVA). Plaques were visualized after 48 h by staining with crystal violet.

Bronchoalveolar lavage and lung parenchymal cell isolation

BAL fluid and lung cells were collected as previously described (Lin et al., 2008). Briefly, mice were euthanized by isoflurane inhalation. For BAL collection, the trachea was cannulated with an 18-gauge angiocath connected to a 1 ml syringe, and the lungs flushed with PBS until a total of 2 ml was collected. BAL fluid was centrifuged and the supernatant frozen immediately at −80 °C for subsequent assays. To obtain leukocytes, lungs were perfused with 3 ml of PBS. Lungs were minced and incubated for 30–40 min at 37 °C with 5 ml HBSS containing 5% FBS, 10 mM HEPES, 1 mg/ml collagenase (crude, type 1A) and 0.2 mg/ml DNase I (from bovine pancreas, Sigma). The collagenase digestion was stopped with 1 ml cold 120 mM EDTA. Cells were dissociated through a 70 μm mesh strainer and centrifuged at 220×g for 20 min over an 18% Nycodenz cushion. Low-density cells were collected, diluted in HBSS with 5% FBS, 10 mM EDTA and 10 mM HEPES (HBSS-5), centrifuged, and suspended in red blood-cell lysing media (Sigma) for 5 min. Cells were washed, and resuspended with HBSS-5, then passed through a 35 μm mesh strainer to remove DNA aggregates. Cells were centrifuged, and resuspended in HBSS-5, then counted and stained for flow cytometry.

Flow cytometry

All antibodies were used at the staining saturation point. Cell surface staining was performed as previously described (Lin et al., 2008). Lung cells were washed twice with flow buffer (HBSS supplemented with 5% FBS and 10 mM EDTA), then resuspended in flow buffer containing 5% normal mouse serum, 5% normal rat serum (Jackson ImmunoResearch), and 1% rat IgG2a, anti-CD16/CD32 (BD Biosciences). The cells were incubated on ice for at least 10 min, then antibodies were added, and the cells incubated for at least 20 min. Cells were washed twice with flow buffer. Cells were resuspended in flow buffer, and phenotyped on a FASCS Aria or LSR II flow cytometer (BD Biosciences). Unstained cells and cells stained with each antibody were used to compensate the data. The data were analyzed using Flowjo software (Tree Star). Cells were gated on FSC-A/FSC-H to removed doublets, and FSC-A/SSC-A to remove debris (data not shown). The following antibodies were used to stain cells: rat IgG2a, anti-mouse CD8α-APC and Armenian hamster IgG anti-mouse CD11c-PE-Cy5.5 (eBioscience), rat IgG2a, anti-mouse CD8α-APC and Armenian hamster IgG anti-mouse CD11c-PE-Cy5.5 (eBioscience).

ELISA for binding antibody

The ectodomain (amino acids 1–279) of the B5 protein of CPXV, was expressed, purified, and used to assay for binding antibody as described (Barefoot et al., 2008). Briefly, 96-well Immulon II ELISA plates (Fisher) were coated with purified B5 protein (200 ng per well) in bicarbonate buffer (0.1 M NaHCO₃) overnight at 4 °C. Wells were washed, blocked with Superblock (15% normal goat serum, 0.04% whey in PBS-Tween), and incubated with diluted sera. Antibody was detected with alkaline-phosphatase conjugated goat anti-mouse Ig, and antibody reactivity was detected by adding pNPP substrate (4-nitrophenyl phosphate Sigma N2640) at 1 mg/ml as directed by the manufacturer, incubating for 45 min, and reading Abs at 405 nm. Serum Ig endpoint titers are reported as the reciprocal of the highest dilution that gave absorbance greater than 2-fold over background, where background is the absorbance for pre-immune serum at the same dilution. Sera from the animals were assayed individually, and compared to individual pre-immune titers. Buffer alone was included as a negative control, and serum with a known endpoint dilution of B5 was used as a positive control for the assay.

Plaque reduction neutralization assays were conducted as described (Law and Smith, 2001) to measure serum neutralizing activities against cowpox virus mature virions.

Tetramer assay

Splenocytes were obtained by disrupting spleens between the frosted ends of two microscope slides. Red blood-cells were removed using red blood-cell lysing buffer (Sigma). Cells were washed and resuspended for staining. Briefly, 5×10⁶ cells were added to the bottom of a V-bottom plate, and blocked with unconjugated streptavidin (Molecular Probes), and FcBlock (Pharmingen) for 15 min at room temperature. Cells were then labeled with FITC-conjugated anti-CD62L, APC-conjugated anti-CD8, and PE-conjugated tetramer. The tetramer was an MHC class I Kb tetramer recognizing the immunodominant Kb-restricted B8 epitope of vaccinia virus (NTSFKFESV-C) as described (Tschark et al., 2005). The tetramer was obtained from the NIH Tetramer Facility.

Histopathologic scoring

Lungs were harvested as described above and were submitted to the Duke Pathology Research Laboratory for tissue processing, embedding, sectioning, and staining with hematoxylin and eosin per standard diagnostic protocols. Six levels of tissue sections were taken from each lung. Each histopathologic endpoint was scored on a scale of 0–4 using standard criteria as follows. All slides were coded and scored “blinded.” Periartrial fibronohemorrhagic degeneration was seen only in the naive mice (0 = no degeneration; 1 = trace degeneration; 2 = mild degeneration; 3 = moderate degeneration; 4 = severe degeneration). Perivascular lymphocytic infiltrates were seen only in rechallenged mice (0 = no infiltrates; 1 = trace infiltrates; 2 = mild infiltrates; 3 = moderate infiltrates; 4 = marked infiltrates). Bronchiolitis was seen in all animals (0 = no bronchiolitis; 1 = mild respiratory epithelial edema/hypertrophy; 2 = moderate bronchiolitis with neutrophilic infiltrates; 3 = severe bronchiolitis with neutrophilic infiltrates and focal breakdown of the bronchial wall; 4 = severe necrotizing bronchiolitis with obliteration of the bronchial wall). Bronchopneumonia was seen in all animals (0 = no bronchopneumonia; 1 = mild focal bronchopneumonia; 2 = moderate bronchopneumonia with clustering and focal confluence across two bronchioles and associated lobular units; 3 = severe bronchopneumonia with confluence across three or more bronchioles and...
associated lobular units; 4 = severe bronchopneumonia with regional sublobar or lobar involvement.

Statistical analyses

Numerical data analyses were performed using Prism Software (GraphPad Software). Data are presented as the mean ± SEM or points representing individual animals with mean as indicated in the figure legends.

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