Effects of Virally Expressed Interleukin-10 on Vaccinia Virus Infection in Mice

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To investigate the *in vivo* role of interleukin-10 (IL-10) in viral infection, we compared infections with a recombinant vaccinia virus (VV) expressing IL-10 (VV-IL10) under control of the VV P7.5 promoter and a control virus (VV- β gal) in normal and severe combined immunodeficient mice. In normal mice, VV-IL10 infection resulted in less natural killer cell activity at 3 days postinfection and less VV-specific cytotoxic T-cell activity at 6 or 7 days postinfection than VV- β gal infection. However, the use of dermal scarification or intraperitoneal, intranasal, or intracerebral inoculation into immunocompetent mice resulted in no difference between VV-IL10 and VV- β gal in visible lesions, mortality, protective immunity to a 100-fold lethal VV challenge, or VV-specific antibody response. In the immunodeficient mice, VV-IL10 infection resulted in greater natural killer cell activity and lower virus replication than VV- β gal infection. These *in vivo* effects were subtler and more complex than had been anticipated. From the VV-IL10 murine model, the Epstein-Barr virus-encoded homolog of human IL-10, BCRF1, may provide a selective advantage by blunting the early human natural killer cell and cytotoxic T-cell responses so that Epstein-Barr virus can establish a well-contained latent infection in B lymphocytes.

Current models of immune responses to foreign pathogens centrally position T helper cells (Th) in balancing antibody and cytotoxic T-cell (CTL) responses (12, 27). Th1 clones secrete interleukin-2 (IL-2) and gamma interferon (IFN- γ), which activate macrophages, promote CTL generation, and enhance delayed-type hypersensitivity (11, 37). Th2 clones secrete IL-4, -5, and -6 and enhance the antibody response (6, 21). Th1 and Th2 clones can also cross-regulate each other by cytokine secretion (29). A newly described Th2-derived cytokine, IL-10, can inhibit IFN- γ secretion from Th1 and natural killer (NK) cells (13, 26). Most IL-10 effects are mediated through monocytes and macrophages (18). IL-10 can also enhance B-lymphocyte and mast cell growth (15, 33, 38).

Epstein-Barr virus (EBV), an intracellular human pathogen, has a close functional and structural homolog (BCRF1) of the murine and human IL-10 genes (17, 40). Human IL-10 and BCRF1 are 90% identical in primary amino acid sequence. The incorporation of the gene for this human cytokine into the EBV genome suggests that the cytokine is important in viral infection.

The purpose of these experiments was to investigate the role of a virus-encoded IL-10 during acute viral infection. In primary human infection with EBV, viral lytic replication initiates in the oropharyngeal epithelium. The virus then infects B lymphocytes, which are the site of latency and from which the virus can reactivate. IFN- γ can specifically inhibit B-cell transformation by EBV *in vitro* and may be important in early infection (16, 24). NK cell and specific antibody responses can be detected within several weeks of primary infection (22, 25). CD8⁺ CTL responses to proteins encoded

by the EBV genome in latently infected B lymphocytes develop more slowly. Their appearance correlates with a fall in the number of latently infected B lymphocytes, which eventually reach a stable low level of 1 in 10⁶ circulating B lymphocytes. Curiously, the EBV IL-10 homolog is only expressed late in lytic EBV infection. Thus, viral IL-10 could be important in blunting or delaying the initial NK cell, CD8⁺ CTL, and IFN- γ responses, enabling EBV to establish a reservoir of latently infected B lymphocytes.

Because direct investigation of the role of the EBV IL-10 homolog in human infection is not possible, and there is no suitable experimental model for primary EBV infection, we chose to investigate the role of the viral IL-10 homolog in acute viral infection by placing the murine IL-10 (mIL-10) gene in the vaccinia virus (VV) genome under the control of a VV promoter and assessing its effects on VV infection in mice. VV infections in mice have been extensively characterized (for a review, see reference 9), and expression of cytokines such as IL-2, IFN- γ , and tumor necrosis factor alpha from recombinant VV has been useful in establishing the role of these cytokines in the murine response to viral infection (14, 20, 23, 32, 35). Cell-mediated immunity (4) and IFN- γ (19, 23) are crucial to the murine defense against VV infection. A cytokine such as IL-10, which might dampen the Th1-mediated responses, might be expected to increase VV virulence by perturbing the balance between the Th1 and Th2 responses and depressing NK cell and CTL functions.

A recombinant VV expressing mIL-10 was made with the pSC11 VV insertion vector (10), which directs expression of an inserted gene from the VV early-late promoter P7.5, providing constitutive expression throughout the viral multiplication cycle. The virus also expresses β -galactosidase activity under the control of the VV late promoter P11. The IL-10 cDNA clone F115 (26) was digested with *Pst*I and *Bam*HI to liberate the IL-10 insert. The fragment was isolated, the ends were repaired, and the fragment was ligated into the unique *Sma*I

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FIG. 1. Expression of IL-10 by VV recombinants. (A) Polyacrylamide gel electrophoresis (15% gel) of 3-h [35 S]methionine-labeled supernatants of TK-143B cells after overnight VV infection. β gal and IL10 refer to infection with VV- β gal and VV-IL10, respectively. Molecular size markers are indicated at the left, and the arrows indicate the three unique bands in the VV-IL10 lane, corresponding to three forms of mIL-10. (B) Table showing ELISA results for IL-10 in the supernatant of VV-IL10- and VV- β gal-infected CV-1 cells at 18 h postinfection and 1 h after removal of the medium at 18 h postinfection.

site of pSC11. A plasmid containing the insert in the correct orientation relative to the P7.5 promoter was isolated and used for transfection into CV-1 cells infected with the WR strain of VV (VV-WR), as described previously (30). This virus was designated VV-IL10. The pSC11 insertion vector inactivates the VV thymidine kinase (*tk*) gene. While the *tk* gene is nonessential for growth in tissue culture, *tk*⁻ VV is attenuated in vivo (8). For this reason, we used *tk*⁻ VV- β gal as a control. VV- β gal is a VV recombinant constructed from the parent pSC11 vector. Both viruses were derived from the same passage of the parental VV-WR strain. In vitro viral infections and titrations were performed as described previously (30).

Figure 1A shows the results of overnight infection with VV- β gal or VV-IL10 followed by labeling for 3 h with [35 S]methionine. The labeled supernatants were mixed with loading buffer and electrophoresed under denaturing conditions. A major band is visible at approximately 20 kDa in the VV-IL10 lane that is absent from the VV- β gal lane. In addition, there are two bands below the 20-kDa band, at 18 and 16 kDa, in the VV-IL10 lane that are not present in the VV- β gal lane. These three bands agree well in both size and relative intensity with the pattern of mIL-10 detected in IL-10 cDNA-transfected COS cell supernatants (26). Figure 1B shows the results of an enzyme-linked immunosorbent assay (ELISA) specific for IL-10 (26) on similar unlabeled VV-infected supernatants. VV- β gal exhibited no IL-10 immunoreactivity, whereas a high level of antigenic IL-10 was observed after overnight infection with VV-IL10. IL-10 secretion continued over a 1-h time period, at 18 h postinfection. The amount of IL-10 in the infected-cell supernatants is 10 to 20% of the amount found in COS cell supernatants after transfection with an expression vector for IL-10 (28). The biological activity of the IL-10 secreted by VV-infected cells cannot be measured directly because VV infection produces a soluble inhibitor of IFN- γ secretion by Th1 cells (25a).

The virulence of VV-IL10 was first compared with the virulence of VV- β gal by intradermal scarification (the least virulent route) in BALB/c mice. At the highest virus dose administered (approximately 2×10^6 PFU), there was no discernible difference in the severity or resolution of infection, and all mice survived (data not shown). Virulence was then compared by intraperitoneal inoculation. At doses of up to 10^7

TABLE 1. Intracerebral LD₅₀ of VV- β gal and VV-IL10 in BALB/c mice^a

Virus dose (PFU)	No. of mice dead/no. in group (% dead)	
	VV- β gal	VV-IL10
10^2	2/5 (40)	0/5 (0)
10^3	7/10 (70)	1/10 (10)
10^4	5/10 (50)	6/10 (60)
10^5	5/5 (100)	4/4 (100)
10^6	5/5 (100)	5/5 (100)

^a Virus was inoculated intracerebrally into the left hemisphere of 4- to 6-week-old BALB/c mice. Deaths that occurred 2 to 14 days postinoculation are included. From these experiments, the LD₅₀ of VV- β gal and VV-IL10 was determined to be 1.77×10^3 and 7.00×10^3 PFU, respectively. The difference was not statistically significant ($P = 0.14$).

PFU per mouse, there was no difference in morbidity, and all mice survived (data not shown). The 50% lethal dose (LD₅₀) of *tk*⁻ VV-WR by intranasal inoculation is in the range of 3×10^6 to 8×10^6 PFU (42). At a dose of 5×10^6 PFU per mouse, there were no differences in the extent or duration of illness, and all mice survived (data not shown). By intracerebral inoculation (8), even *tk*⁻ virus has a measurable LD₅₀. As determined by the moving-angle method (2), the LD₅₀s of VV-IL10 and VV- β gal were 7×10^3 and 2×10^3 PFU per mouse, respectively, and were not statistically different (Table 1). Illness was similar in both groups, with death (measured from 2 to 14 days postinoculation) characteristically preceded by weight loss and paralysis. These experiments indicate that IL-10 does not significantly potentiate VV virulence in BALB/c mice.

The next series of experiments examined whether IL-10 expression interferes with the normal induction of immunity to subsequent challenge with a lethal dose of VV. BALB/c mice were vaccinated with either VV-IL10 or VV- β gal by intraperitoneal inoculation (1×10^6 or 5×10^7 PFU). After 3 weeks to allow recovery (all mice survived primary infection, as expected), the mice were challenged by intracerebral injection of either virus at doses that greatly exceeded the previously determined intracerebral LD₅₀. Mice immunized with as little as 10^6 PFU of VV-IL10 or VV- β gal per mouse were fully protected from challenge with 100 times the intracerebral LD₅₀ of VV- β gal or VV-IL10 (data not shown). Thus, IL-10 does not significantly block the development of host immunity to an otherwise lethal virus challenge, nor does it enable the virus to overcome previously acquired immunity.

After challenge, anti-VV immunoglobulin G (IgG) levels were measured by ELISA (14). Even in mice inoculated and challenged with VV-IL10, there were no differences in anti-VV IgG levels from those in mice which had been vaccinated and challenged with VV- β gal (data not shown). Thus, IL-10 did not skew the immune response toward antibody production.

Since IL-10 has effects on both NK cell and T-cell activity as well as cytokine synthesis in vitro, we investigated whether VV-encoded IL-10 affected NK and T-cell cytotoxic activity in vivo. C57BL/6 mice were inoculated intraorbitally with 10^7 PFU of either VV-IL10 or VV- β gal or else given no VV. At 3 days postinfection, spleen cells were prepared and tested for NK cell activity at various effector-to-target cell ratios against YAC-1 target cells labeled with 51 Cr (43). Table 2 shows the results of cytotoxicity assays at E/T ratios of 100:1, expressed as percent YAC-1 cell lysis or as lytic units per 10^6 spleen cells (as determined by the computer program Von Krough [31]) or per total spleen. In four separate experiments, mice infected with

TABLE 2. Effect of VV- β gal and VV-IL10 infection of C57BL/6 mice on in vivo induction of splenic NK cell-mediated cytotoxicity^a

Expt no.	Virus	% YAC-1 cell lysis ^b	LU/10 ⁶ cells	Total LU/spleen
1	VV- β gal	35 $\pm$ 6	12 $\pm$ 2	784 $\pm$ 82
	VV-IL10	27 $\pm$ 5	5 $\pm$ 1	262 $\pm$ 100
	None	13 $\pm$ 5	2 $\pm$ 2	108 $\pm$ 102
2	VV- β gal	45 $\pm$ 0	9 $\pm$ 3	702 $\pm$ 152
	VV-IL10	29 $\pm$ 5	4 $\pm$ 1	220 $\pm$ 61
	None	17 $\pm$ 2	2 $\pm$ 0.4	113 $\pm$ 14
3	VV- β gal	45 $\pm$ 8	10 $\pm$ 2	1,097 $\pm$ 368
	VV-IL10	34 $\pm$ 2	5 $\pm$ 1	661 $\pm$ 142
	None	10 $\pm$ 1	1 $\pm$ 0.2	98 $\pm$ 35
4	VV- β gal	56 $\pm$ 2	9 $\pm$ 1	421 $\pm$ 115
	VV-IL10	46 $\pm$ 4	3 $\pm$ 0.5	154 $\pm$ 62

^a Mice received 10⁷ PFU of the indicated virus intraorbitally 3 days before analysis. Data are presented as the averages for two (experiment 2), three (experiment 3), or four (experiments 1 and 4) mice per group $\pm$ standard deviations. Lytic units (LU) were calculated on the basis of 10% cytotoxicity in experiments 1, 2, and 3 and 20% cytotoxicity in experiment 4.

^b E/T ratio, 100:1, 4-h ⁵¹Cr release assay.

VV-IL10 had two- to threefold-lower levels of total NK cell-mediated cytotoxicity than mice infected with VV- β gal. VV-IL10-induced NK cell cytotoxicity was still significantly greater than that of uninfected control mouse spleens. At an E/T ratio of 25:1, the VV-IL10-induced NK cell cytotoxicity was still significantly lower than VV- β gal-induced NK cell cytotoxicity, but VV-IL10-induced NK cell cytotoxicity was not significantly greater than that of uninfected controls (data not shown).

Figure 2 shows the NK cell activity after size separation of the splenic cell population by centrifugal elutriation (3). These experiments were performed at an effector-to-target cell ratio of 25:1. Of note is the relatively flat curve for the uninfected mice, indicating a baseline level of NK cell activity in all fractions (resting and activated blast cells). After infection with VV- β gal, there was a pronounced increase in the cytotoxicity of fractions 3 to 6 (activated blast cells), with somewhat less cytotoxicity in fractions 1 and 2 (resting cells). Infection with VV-IL10 results in NK cell activity exclusively in the large blast-size cell fractions (fractions 3 to 6). There was less NK cell activity in the large blast cell fraction than after infection with VV- β gal, and it was not significantly different from the baseline of NK cell activity in the same fractions from uninfected mice. Thus, VV-IL10 infection results in lower NK cell cytotoxic activity overall without selectively preventing NK cell blastogenesis.

CTL activity in C57BL/6 mice was determined 6 or 7 days postinfection with VV-WR-infected MC57G cells as targets. Figure 3 shows that the VV-IL10-infected mice had decreased VV-specific CTL activity across a broad range of E/T ratios. Typically, ⁵¹Cr release of VV-IL10-induced effectors was about 50 to 75% of the level of VV- β gal-induced effectors. However, spleen weights differed significantly between the groups (VV- β gal, 206 $\pm$ 42 mg; VV-IL10, 163 $\pm$ 27 mg; $P < 0.02$, $n = 9$), and there were substantially fewer spleen leukocytes in the VV-IL10-infected mice. When calculated as total lytic units per spleen, VV-IL10 stimulated a CTL response 25 to 50% of that of VV- β gal (Table 3).

To investigate whether these differences in NK and T-cell cytotoxicity were due to differences in viral replication in vivo, virus was measured in infected mice at 1 to 3 days postinfection.

FIG. 2. Killing of YAC-1 cells by elutriated spleen cells from VV- β gal-infected, VV-IL10-infected, and uninfected C57BL/6 mice. Spleen cells from C57BL/6 mice obtained 3 days after infection with VV- β gal ($\blacktriangle$) or VV-IL10 ($\blacksquare$) and from uninfected mice ($\bullet$) were fractionated by centrifugal elutriation, and the individual fractions were used as effector cells against YAC-1 target cells in a 4-h ⁵¹Cr release assay at an E/T of 25:1. Samples were run in a Beckman centrifuge elutriation system at 3,200 rpm, and fractions were eluted at the following flow rates (milliliters per minute): 1, 15; 2, 22; 3, 28; 4, 33; 5, 38; and 6, 46. Fractions 1 and 2 contain small resting cells, whereas fractions 4 to 6 contain blast-sized cells (39). ND, not done.

tion. Virus was not detectable in the spleen (data not shown). Virus titers in the lungs of infected C57BL/6 mice at 1 to 3 days postinfection were low, in the range of 100 to 200 PFU per lung. At 3 days postinfection, there was no significant difference in virus titers in lung tissue; VV- β gal-infected mice had 140 PFU/lung, and VV-IL10-infected mice had 110 PFU/lung. Thus, the decreased NK cell and CTL activity in VV-IL10-infected mice is not due to a lower virus load and consequently to less direct stimulation of NK and T cells.

If VV-IL10 were exerting an effect by rendering virally infected cells less immunogenic rather than suppressing CTL activity, coinfection with both VV-IL10 and VV- β gal would result in the high level of VV-specific CTL induced by VV- β gal. Infection of mice with both viruses resulted in CTL responses that were similar to those after VV-IL10 infection alone (Table 3). These results are most compatible with soluble IL-10 suppressing VV-specific CTL, possibly through an effect on macrophage function.

Mice with severe combined immunodeficiency (SCID) should provide a model for examining the effect of IL-10 on NK cell responses in the absence of adaptive immune processes provided by functional T and B cells (1, 41). Because NK cell responses are typically augmented by IFN in the early stages of viral infection, and because IL-10 possesses the in vitro activity of inhibiting IFN- γ secretion, SCID mice should be a sensitive assay for the influence of IL-10 on NK cell activity and virus clearance. Table 4 shows the NK cell activity of SCID mice infected with either VV-IL10 or VV- β gal at 3 days postinfection. In contrast to the results with normal C57BL/6 mice, SCID mice infected with VV-IL10 had higher levels of NK cell activity at 3 days postinfection than SCID mice infected with VV- β gal. VV titers in the lungs of the infected SCID mice indicate that this difference is not due to a higher virus burden in the VV-IL10-infected mice. In fact, the

FIG. 3. Killing of MC57G target cells by VV-βgal- and VV-IL10-induced VV-specific CTL. MC57G cells (*H-2^b*) were mock infected (open symbols) or infected with VV recombinants (solid symbols) and used as targets for VV-βgal-induced (□, ■) or VV-IL10-induced (○, ●) VV-specific CTL in a 6-h ⁵¹Cr release assay.

higher NK cell activity is associated with slightly lower virus titers in the lungs of VV-IL10-infected mice, suggesting that NK cell activity correlates with the degree of virus clearance.

Given the central importance of NK cells (41), IFN-γ (7, 34), and T cells (4) in the recovery of mice from VV infection and

the *in vitro* activity of IL-10 in inhibiting Th1-mediated enhancement of T-cell cytotoxicity, NK cell activation, and IFN-γ (13), IL-1, IL-6, and tumor necrosis factor alpha secretion (5, 18), we expected that virally expressed mIL-10 would significantly increase the virulence of VV infection in normal and SCID mice. Contrary to this expectation, in immunocompetent mice, the expression of IL-10 by a VV recombinant did not increase the severity of VV infection, although inhibition of NK cell- and virus-specific CTL function was evident. Furthermore, prior infection with VV-IL10 did not alter the antibody response or the protective immune response to challenge with a dose of 100 times the LD₅₀ of VV-βgal. The lack of a dramatic effect of IL-10 *in vivo* is probably due to the increased complexity of IL-10's activity *in vivo* and to counteracting modulatory responses.

The paradoxical effects of virally expressed mIL-10 in increasing NK cell activity and decreasing virus replication in SCID mice were particularly surprising. These results indicate that the presence of mature T or B cells or the effects of their cytokines can alter the effects of IL-10 on NK cell function *in vivo*. Other evidence that IL-10 can have NK cell stimulatory effects has recently arisen in studies of the effects of EBV protein BCRF1 on the activation of HLA-unrestricted NK cells in cultures of human peripheral blood mononuclear cells incubated with latently infected B lymphocytes (36).

The finding that mIL10 expressed from the VV genome affects an immunocompetent animal's initial NK cell and CTL response to VV infection highlights aspects of IL-10's functions which may have direct relevance to BCRF1 effects in EBV infection in humans. EBV initiates infection in the oropharyngeal epithelium and then spreads to B lymphocytes. An immediate NK cell response and a rapidly evolving CTL response ensue, directed against viral proteins expressed in latently infected B cells (39). During acute primary infection, EBV must establish latent infection in B cells, since reactivation of lytic infection in B cells is necessary for reseeding of the oropharynx and spread to new nonimmune hosts. In establish-

TABLE 3. Effect of VV-βgal and VV-IL10 infection on the generation of VV-specific CTL^a

Expt no.	Virus	Dose (PFU)	Total spleen cells (10 ⁷)	% Lysis of VV-WR-infected MC57G cells ^b	LU/spleen ^c
1	VV-βgal	10 ⁷	23	59	595
	VV-IL10	10 ⁷	13	39	154
2	VV-βgal	10 ⁷	12 ± 2	51 ± 14	1,293 ± 202
	VV-IL10	10 ⁷	8 ± 1	26 ± 10	334 ± 171
3	VV-βgal	10 ⁷	11 ± 1	83 ± 10	765 ± 59
	VV-IL10	10 ⁷	9 ± 2	62 ± 5	357 ± 41
	Both	5 × 10 ⁶ each	8 ± 2	42 ± 7	265 ± 128
4	VV-βgal	10 ⁷	15 ± 1	55 ± 2	834 ± 47
	VV-IL10	10 ⁷	11 ± 1	49 ± 4	482 ± 44
	Both	5 × 10 ⁶ each	11 ± 2	54 ± 3	596 ± 195
	Both	10 ⁷ each	13 ± 1	52 ± 8	604 ± 157
5	VV-βgal	10 ⁷	13 ± 2	75 ± 5	1,626 ± 381
	VV-IL10	10 ⁷	6 ± 1	69 ± 2	766 ± 12
	Both	5 × 10 ⁶ each	7 ± 2	63 ± 6	760 ± 154
	Both	10 ⁷ each	5 ± 1	55 ± 9	529 ± 282

^a C57BL/6 mice received the indicated virus at the indicated dose intraorbitally (experiments 2 and 5) or 7 (experiments 1, 3, and 4) days before analysis. For experiment 1 only spleens were processed together and pooled after individual weights were determined. Results represent the means ± standard deviation for two (experiments 1 and 3), three (experiments 4 and 5), or four (experiment 2) mice per group.

^b 100:1 E/T ratio, 6-h assay.

^c The number of lytic units (LU) per spleen was calculated on the basis of 10% (experiment 2) or 20% (experiments 1, 3, 4, and 5) cytotoxicity of VV-WR-infected MC57G cells.

TABLE 4. Splenic NK cell activity and virus titers in lungs of SCID mice infected with VV- β GAL or VV-IL10^a

Expt no.	No. of mice	Day postinfection	% YAC-1 cell lysis ^b		PFU/lung	
			VV- β gal	VV-IL10	VV- β gal	VV-IL10
1	4	3	15 $\pm$ 3	29 $\pm$ 9 ^c	640 $\pm$ 158	245 $\pm$ 134 ^d
2	3	3	21	61	2,653 $\pm$ 2,084	<207 $\pm$ 155 ^e
3	3	3	7	37	2,920 $\pm$ 1,287	1,180 $\pm$ 580
4	3	3	8	24	ND ^f	ND

^a Mice received 10⁷ PFU of the indicated virus intraocularly 3 days before analysis. Results are given as means $\pm$ standard deviation except for YAC-1 lysis in experiments 2 through 4, for which spleens were pooled on day 3 postinfection.

^b E/T ratio, 100:1; 4-h assay.

^c $P < 0.05$ versus VV- β gal.

^d $P < 0.01$ versus VV- β gal.

^e $P < 0.1$ versus VV- β gal (below detection limit [20 PFU] in one mouse).

^f ND, not determined.

ing a persistent latent infection in B lymphocytes, EBV encounters the need to expand the number of latently infected B cells without inducing uncontrolled proliferation. Our data and the recently published data on BCRF1 effects in inhibiting the reactivation of NK cell and CTL reactivity in human peripheral blood mononuclear cell cultures (36) are consistent with the proposal that EBV has selectively incorporated BCRF1 in order to blunt the initial NK cell and CTL responses without affecting long-term immunity, which is essential to both viral and host viability. In blunting the immune response in a manner that does not impair the ability to eventually clear an acute viral infection, the IL-10 activity of BCRF1 may also affect the manifestations of acute infectious mononucleosis which are due to secondary effects of the immune response.

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