IL-18 Expression Results in a Recombinant Vaccinia Virus That Is Highly Attenuated and Immunogenic

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Interferon- γ (IFN- γ) is an attenuating factor for vaccinia virus (VACV), decreasing its virulence *in vivo* by more than a million fold. It is also a highly effective adjuvant when administered at the time of immunization with protein antigens. However, recombinant VACV (rVACV) vaccines expressing IFN- γ do not induce enhanced immune responses. It is possible that the IFN- γ expressed by rVACVs induces both an antiviral state and increased immunological clearance, thus resulting in decreased levels of antigen expression due to reduced viral replication and spread. We conjectured that delaying expression of IFN- γ would result in enhanced production of antigens by rVACVs thus resulting in increased immune responses to foreign antigens. Interleukin (IL)-18, also known as IFN- γ inducing factor, is a cytokine that induces T and NK cells to produce IFN- γ . In this study, we demonstrated that an rVACV expressing bioactive murine IL-18 replicated to low but detectable levels *in vivo*, unlike an rVACV expressing IFN- γ . Moreover, the rVACV expressing IL-18 was significantly attenuated in both immunocompromised and immunocompetent mice. This attenuation was dependent on IFN- γ , as IL-18 expression failed to attenuate VACV in IFN- γ knock-out mice. Cytotoxic T-cell (CTL) and anamnestic antibody responses were slightly increased in animals vaccinated with the rVACV expressing IL-18. Thus, induction of IFN- γ because of IL-18 expression resulted in an rVACV that replicated to low but detectable levels *in vivo*, yet elicited slightly better CTL and anamnestic humoral immune responses.

Introduction

VACCINIA VIRUS (VACV) has many characteristics desirable for the development of live recombinant vaccines, combining the advantages of subunit vaccines with those of attenuated live vaccines by inducing both humoral and cell-mediated immune responses to VACV and expressed foreign antigens (Moss 1996). Although recombinant VACVs (rVACVs) have been very effective as vaccines for diseases such as rabies and rinderpest (Kieny and others 1984; Yilma and others 1988; Giavedoni and others 1991; Pastoret and Brochier 1996; Verardi and others 2002), they have not been as successful for some other diseases such as vesicular stomatitis and acquired immunodeficiency syndrome (Mackett and others 1985; Giavedoni and others 1993; Ahmad and others 1994). In addition, the extensive use of VACV as a smallpox vaccine has uncovered adverse side effects in a small percentage of normal vaccinees, and more severe complications in immunodeficient individuals (Fulginiti and others 2003). For these reasons, it is imperative to increase both the safety and the efficacy of rVACV vaccines.

One strategy our laboratory has pursued to enhance the safety and efficacy of rVACV vaccines is the co-expression of cytokines. Interferon- γ (IFN- γ) is a cytokine with potent immunoregulatory, antineoplastic, and antiviral properties. Expression of IFN- γ by rVACVs attenuates the virus by at least a million-fold (Kohonen-Corish and others 1990; Giavedoni and others 1992; Legrand and others 2005). In addition, IFN- γ enhances the primary and secondary immune responses of mice and cattle immunized with a mixture of the cytokine and the vesicular stomatitis virus (VSV) glycoprotein (VSV-G) (Anderson and others 1988). However, when IFN- γ is expressed by rVACVs, no enhancement of immune responses to VACV or other co-expressed heterologous antigens is observed (Verardi and others 2001; Legrand and others 2005). This phenomenon may be explained by the fact that the expressed IFN- γ is focused at sites of viral replication, thereby inducing an antiviral state in uninfected neighboring cells and limiting viral spread (Ramsay and others 1993). Limited replication, and increased clearance, could restrict antigen expression and presentation, resulting in the observed lack of adjuvant activity.

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We looked for means to delay expression of IFN- γ to allow low levels of viral replication and high levels of antigen expression. Interleukin (IL)-18, also known as IFN- γ inducing factor, is a proinflammatory cytokine that belongs to the IL-1 family of ligands (Okamura and others 1995; Ushio and others 1996). IL-18 is synthesized as an inactive 24 kDa precursor (pro-IL-18) that undergoes proteolytic cleavage by caspase-1, also known as IL-1 β -converting enzyme (ICE), to generate a mature bioactive 18 kDa molecule (Gu and others 1997; Gram and others 2012). Macrophages and dendritic cells are the primary sources for active IL-18, which in turn induces IFN- γ production from T, B, and NK cells (Dinarelli 1999; Gracie and others 2003; Reddy 2004). Thus, we predicted that rVACVs expressing IL-18 would be able to replicate to low levels and induce enhanced immune responses before the expressed IFN- γ accelerated viral clearance.

Previously, we showed that the insertional inactivation of VACV serine protease inhibitor (serpin) homolog genes attenuates rVACVs without compromising the effectiveness of the immune response (Legrand and others 2004). The B13R gene of VACV encodes a serpin that inhibits ICE (Kettle and others 1997) from processing pro-IL-1 β and pro-IL-18 to their bioactive forms. Thus, we used the B13R gene loci for the insertion of the IL-18 gene and insertional inactivation of the B13R gene to ensure expression of mature bioactive IL-18 by VACV.

We tested the safety and efficacy of an rVACV expressing the VSV-G protein as a model antigen in a mouse model. This allowed us to evaluate humoral and cell-mediated immune responses to a heterologous protein and to VACV itself (Legrand and others 2004, 2005). We were able to demonstrate that IL-18 expression results in a highly attenuated rVACV that replicates to low but detectable levels *in vivo*, that such attenuation is dependent on IFN- γ induction, and that despite the higher levels of replication, vector immunity was only slightly increased.

Materials and Methods

Cells and viruses

African green monkey kidney (BS-C-1 and BS-C-40), murine L929, hamster BHK-21, and human A549 and HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO₂. The rVACVs v50 (Mackett and others 1985), v50 Δ B13R (Legrand and others 2004), and v50 Δ B13RM γ (Legrand and others 2005), all derived from the Western Reserve strain of VACV, have been described elsewhere. rVACVs were propagated in HeLa S3 cells and titered in BS-C-1 cells. The New Jersey serotype of VSV (Yilma and others 1985) was propagated and titered in BHK-21 cells.

Animals

Nude BALB/cBy mice (CBYJ.Cg-Foxn1^{nu}/J), normal BALB/c mice, and BALB/c IFN- γ knockout mice [C.129S7(B6)-Ifng^{tm1Ts}/J] were purchased from the Jackson Laboratory (Bar Harbor, ME). C.B-17 SCID mice (C.B-Igh-1^b/IcrTac-Prkdc^{scid}) were purchased from Taconic (Germantown, NY). Normal (BALB/c $\times$ C57BL/6) F₁ hybrid mice (CB6F₁/Hsd) were purchased from Harlan Sprague Dawley (Indianapolis, IN). All animals were maintained according to National Institutes of Health (NIH) guidelines and animal

care protocols approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Construction of the VACV transfer vector

The murine IL-18 gene was cloned from mouse spleen cDNA (Clontech, Mountain View, CA) with primers 5'-TCAAACCGGCCGAATCACTTCCTC-3' and 5'-ACAAACCGGCCGCACCTAACTTTG-3', designed according to GenBank IL-18 sequence (accession No. D49949). The resulting 631 bp polymerase chain reaction fragment was then cloned into pCRBlunt (Invitrogen, Carlsbad, CA) and sequenced. The cloned IL-18 gene (flanked by *Eag* I sites, shown underlined on the primers) was digested with *Eag* I, resulting in a 614 bp fragment, and subcloned into the unique *Not* I site of p2B13Rgpt (Legrand and others 2004), originating the VACV transfer vector p2B13RgptIL-18.

Generation of rVACV

v50 Δ B13RIL-18 was generated by homologous recombination using cationic liposome-mediated transfection of BS-C-1 cells infected with v50 (Mackett and others 1985) at 0.05 plaque-forming units (PFU) per cell. Recombinant *gpt*-positive VACVs were plaque purified on BS-C-40 cells from transfection supernatants, using *gpt*-selection medium (Falkner and Moss 1988). The expression of the *lacZ* gene by the final plaque-purified rVACV was tested by cytochemical staining of infected cell monolayers as previously described (MacGregor and others 1991). The overall genomic structure of v50 Δ B13RIL-18 was determined by *Hind*III restriction analysis of VACV DNA, which was purified by a small-scale method employing micrococcal nuclease (Lai and Chu 1991).

IL-18 detection by immunoblotting

BS-C-1 cell monolayers in 6-well plates were infected with the rVACVs a multiplicity of infection (MOI) of 10 and incubated for 24 h in a final volume of 2 mL DMEM with 2.5% FBS. Cells were harvested by centrifugation, and supernatants were collected and concentrated using Centriprep concentrators (Millipore, Billerica, MA). Sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 15% gel. As a control, 1 μ g of recombinant murine IL-18 (MBL International, Woburn, MA) was used. Samples were then transferred onto an Immobilon-P membrane (Millipore). IL-18 protein was detected after incubation with an anti-IL-18 antibody (R&D Systems, Minneapolis, MN) and a horseradish peroxidase-conjugated goat anti-mouse IgG monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Binding was detected by chemiluminescence using the ECL plus system (GE Healthcare, Piscataway, NJ).

Murine IL-18 detection by ELISA and bioassay

BS-C-1 cell monolayers in 6-well plates were infected with the rVACVs at a MOI of 0.5 and incubated for 6, 12, 24, 48, and 72 h in a final volume of 2 mL DMEM with 2.5% FBS. Supernatants were then harvested, passed through 0.2- μ m filters, and serially diluted in DMEM with 5% FBS. IL-18 expression was determined with a murine IL-18 enzyme-linked immunosorbent assay (ELISA) kit (MBL International). The expression of biologically active murine IL-18 was detected based on the IL-18-induced stimulation of IFN- γ

expression by spleen cells (Yoshimoto and others 2001). Briefly, BALB/c mice were inoculated with 400 µg of anti-asialo GM1 antibody (Wako Pure Chemical Industries, Osaka, Japan) 3 days before being used as a source of splenic T cells. T cells were then stimulated with murine IL-12 (R&D Systems) at a final concentration of 20 ng/mL. Next, 100 µL of stimulated T cells and 100 µL of serially diluted IL-18 sample supernatants or a recombinant murine IL-18 control (MBL International) were seeded in 96-well plates and incubated at 37°C for 72 h. Supernatants were then harvested by centrifugation at 150g for 10 min and serially diluted in DMEM with 5% FBS. IL-18-induced IFN-γ production was assessed with the murine IFN-γ Quantikine ELISA kit (R&D Systems).

Viral growth kinetics

Triplicate monolayers of BS-C-1, A549, and L929 cells were infected at a MOI of 0.01 for 1 h in 12-well plates and *in vitro* virus replication was determined as previously described (Verardi and others 2001).

Virulence studies in immunodeficient mice

Survival was measured in groups of 10 immunodeficient BALB/cBy nude mice (7–8-week-old males) and C.B-17 SCID mice (6-week-old females) challenged intraperitoneally (i.p.) with 10⁷ PFU of rVACV, in a final volume of 250 µL of sterile phosphate-buffered saline (PBS). Animals were examined twice daily.

Clearance studies in immunodeficient and immunocompetent mice

Groups of BALB/cBy nude mice and CB6F₁ normal mice (7–8-week-old females) were inoculated i.p. with 10⁷ PFU of rVACV. Ovaries were removed, weighed, homogenized, and resuspended in DMEM at 10% wt/vol. Next, ovaries were lysed by freeze-thawing and trypsinization. Viral titers were determined by plaque assay on BS-C-1 cell monolayers.

Virulence studies in IFN-γ knockout mice

Groups of 10 normal BALB/c mice or BALB/c IFN-γ knockout mice (7–8-week-old females) were given 5 × 10⁷ PFU of rVACV intranasally (i.n.) in a final volume of 10 µL of sterile PBS, under light anesthesia. All animals were examined and weighed daily.

Humoral immune response studies

Groups of 11 CB6F₁ mice (6–7-week-old females), under light anesthesia, were immunized intramuscularly with 10⁵ PFU of rVACV in a final volume of 50 µL of sterile PBS. Animals were boosted i.p. 4 weeks post-vaccination with 2 × 10⁵ PFU of VSV in a volume of 100 µL. Mice were bled at 0, 2, 4, and 6 weeks post-infection. Serum samples were pooled for each group. Antibody titers to VSV and VACV were determined by ELISA as previously described (Legrand and others 2004).

T helper cell proliferation studies

Groups of 6 CB6F₁ (H-2^{b/d}) mice (7-week-old females) were immunized i.p. with 10⁷ PFU of rVACV in a final volume of 250 µL of sterile PBS. Splenocytes were harvested

10 days post-vaccination. Baculovirus-expressed VSV-G (0.5 µg/mL) in complete RPMI-1640 medium supplemented with 50 mM 2-β-mercaptoethanol was added to 1 × 10⁵ splenocytes in flat bottom 96-well plates. The mitogen Con A at 2 µg/mL served as a positive control whereas uninfected naïve mouse splenocytes served as a negative control. Splenocytes were incubated for 4 days at 37°C. On the fourth day, [³H] thymidine was added [0.5 µCi per well (1 Ci = 37 GBq)], and cells were incubated 18 h at 37°C. Cells were then harvested to determine incorporation of radioactivity. Data analysis was based on counts per minute (cpm) in triplicates and expressed as a stimulation index. The stimulation index was calculated as (cpm in the presence of the antigen)/(cpm in the control culture).

Cytotoxic T-cell (CTL) studies

The same splenocytes harvested for the proliferation studies above were used for Cytotoxic T-cell (CTL) studies. For primary immune responses, effector splenocytes (5 × 10⁴–5 × 10⁵ cells) in complete RPMI-1640 medium supplemented with 50 mM 2-β-mercaptoethanol were stimulated with ultraviolet (UV)-treated VSV-infected target splenocytes (1 × 10⁴) for 4 h in U-bottom 96-well plates. For secondary CTL responses, effector splenocytes (5 × 10⁴–5 × 10⁵ cells) were stimulated with UV-treated/γ-irradiated (3,000 Gy)-VSV-infected target splenocytes (1 × 10⁴ cells) as well as a sub-optimal level of IL-2 (1 ng/mL) for 5 days at 37°C. Cytotoxicity was measured with the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). For both primary and secondary immune responses, infected L929 cells (H-2^k) served as a negative target control. Percent specific cytolysis was calculated as (OD Experimental–Effector Spontaneous–Target Spontaneous)/(OD Target Maximum–Target Spontaneous) × 100, where OD stands for optical density.

Data analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA).

Results

v50ΔB13RIL-18 expresses functional murine IL-18

To evaluate the effects of IL-18 expression by VACV, we cloned the IL-18 gene from murine splenic cDNA and constructed a rVACV expressing murine IL-18 (v50ΔB13RIL-18) at the B13R gene site that was stable and free of the parental strain v50 (Mackett and others 1985) (data not shown). The v50 parental strain of VACV expresses VSV-G at its thymidine kinase (TK) site (Mackett and others 1985). We used a previously developed rVACV with a deletion of the B13R gene, v50ΔB13R (Legrand and others 2004), and an rVACV that in addition expressed IFN-γ, v50ΔB13RMγ (Legrand and others 2005), as control viruses. IL-18 production by v50ΔB13RIL-18, under control of a synthetic early/late VACV promoter (Chakrabarti and others 1997), was assessed by immunoblotting (Fig. 1A). The expressed IL-18 protein is 18 kDa, consistent with the size of the mature biologically active IL-18 protein. No inactive pro-IL-18 (24 kDa) was detected by immunoblotting. Production of IL-18 was also measured at 6, 12, 24, 48, and 72 h post-infection by a direct quantitative ELISA (Fig. 1B). At a MOI of 0.5, IL-18

FIG. 1. Cells infected with v50ΔB13RIL-18 secrete functional murine interleukin-18 (IL-18) that does not affect viral growth kinetics *in vitro*. **(A)** Supernatants of BS-C-1 cells infected at a multiplicity of infection (MOI) of 10 were concentrated and IL-18 expression was detected by immunoblotting. A protein of about 18 kDa that corresponds to the mature and biologically active IL-18 was expressed only by v50ΔB13RIL-18. Recombinant murine IL-18 was used as a positive control. **(B, C)** BS-C-1 cells were infected at an MOI of 0.5 and at the indicated time points, supernatants were harvested and IL-18 expression was determined by enzyme-linked immunosorbent assay (ELISA) **(B)** and by an IL-18 bioassay that measures the stimulation of interferon- γ (IFN- γ) expression by spleen cells **(C)**. The data shown represent the mean of each sample assayed in duplicate. **(D)** BS-C-1 cells were infected at a MOI of 0.01 and at the indicated time points, intracellular viral fractions were collected and titered on BS-C-1 cells. The data shown represent the mean viral yields from triplicate samples assayed in duplicate. Error bars indicate the standard error of the mean (SEM).

production by v50ΔB13RIL-18 was first detected at 48 h post-infection. Finally, the activity of rVACV-expressed IL-18 was tested in a bioassay that measures the induction of IFN- γ (Fig. 1C). The IL-18 expressed by v50ΔB13RIL-18 was able to successfully induce the secretion of IFN- γ starting at 48 h post-infection.

IL-18 expression has no effect on VACV replication in vitro

The *in vitro* growth kinetics of v50ΔB13IL-18 was essentially identical to that of v50ΔB13RMγ and the control rVACV, v50ΔB13R (Fig. 1D). This was true for replication in African green monkey BS-C-1 cells, murine L929 cells, and human A549 cells, and for both intracellular and extracellular virus fractions (only data for intracellular virus fractions of BS-C-1 cells are shown). Moreover, the plaque phenotypes of the rVACVs and their parental strains were indistinguishable in cell culture (data not shown).

IL-18 expression greatly attenuates VACV virulence in immunodeficient mice

T cell-deficient BALB/c nude mice were inoculated i.p. with 10^7 PFU of rVACV. v50ΔB13RIL-18 and v50ΔB13RMγ-inoculated mice survived significantly longer than mice inoculated with v50ΔB13R ($P < 0.005$ and $P < 0.0001$, respectively, log-rank test). The median survival time for v50ΔB13R was 46.5 days, and for v50ΔB13RIL-18 was > 120 days. Mice inoculated with v50ΔB13RMγ did not suffer any mortality during the 120 days of observation, and mice receiving v50ΔB13RIL-18 had a survival rate of 60% 120 days post-infection ($P < 0.05$ when compared with v50ΔB13RMγ) (Fig. 2A). Similarly, T and B cell-deficient C.B-17 SCID mice inoculated i.p. with 10^7 PFU of v50ΔB13RIL-18 and v50ΔB13RMγ (Fig. 2B) survived significantly longer than mice inoculated with v50ΔB13R ($P < 0.0001$, log-rank test). SCID mice were more sensitive to VACV infection than nude mice. The median survival time for animals receiving v50ΔB13R or v50ΔB13RIL-18 was 29.5 or 97.5 days, respectively. The rVACV expressing IFN- γ did not cause mortality in the SCID mice ($P < 0.05$ when compared with v50ΔB13RIL-18). For each of the experiments, control immunodeficient

mice inoculated with sterile PBS ($n = 4$) remained healthy throughout the observation period of 120 days.

Co-expression of IL-18 increases VACV clearance in immunodeficient and immunocompetent mice

Female athymic immunodeficient mice were inoculated i.p. with 10^7 PFU of rVACV and viral titers in ovaries were

FIG. 2. IL-18 expression by vaccinia virus (VACV) significantly increases survival of immunodeficient mice. Groups of 10 athymic BALB/cBy nude mice **(A)** or C.B-17 SCID mice **(B)** were intraperitoneally inoculated with 10^7 plaque-forming units of recombinant VACV. Statistical analyses (log-rank test) of v50ΔB13RIL-18 versus v50ΔB13R or v50ΔB13RMγ yielded the P values shown. Control immunodeficient mice inoculated with sterile phosphate-buffered saline ($n = 4$) and all mice inoculated with v50ΔB13RMγ remained healthy throughout the observation period of 120 days.

determined at 3, 6, and 12 days post-infection (Fig. 3A). High viral titers of the control v50ΔB13R were detected in the ovaries, peaking 6 days post-infection, whereas v50ΔB13RMγ viral titers were undetectable at any time point tested ($P < 0.05$, Mann-Whitney test). Low levels of v50ΔB13RIL-18 viral replication were observed at 3 days post-infection in the ovaries of infected nude mice (difference non-significant when compared to v50ΔB13R), and even lower titers were detected 6 and 12 days post-infection ($P < 0.05$ when compared with v50ΔB13R), but the level of replication was significantly higher than v50ΔB13RMγ ($P < 0.05$).

Groups of female immunocompetent CB6F₁ hybrid mice were also inoculated i.p. with 10^7 PFU of rVACV. Three days post-inoculation, the mice were sacrificed and ovaries removed for virus titration. Once again, reduced viral levels of v50ΔB13RIL-18 were detected compared with the control virus v50ΔB13R ($P < 0.05$, Mann-Whitney test), and viral replication was not observed in the ovaries of mice inoculated with rVACV co-expressing IFN-γ ($P < 0.05$ when compared with v50ΔB13RIL-18) (Fig. 3B).

Attenuation provided by IL-18 is dependent on IFN-γ induction

To test the importance of IFN-γ induction on the ability of IL-18 to attenuate VACV, a virulence study was conducted using IFN-γ knockout (IFN-γ^{-/-}) mice. Normal and IFN-γ^{-/-} BALB/c mice were inoculated i.n. with 5×10^7 PFU of rVACV; no mortality was observed with this dose. Normal mice inoculated with v50ΔB13RMγ or v50ΔB13RIL-18 had no weight loss, whereas mice inoculated with the same dose of v50ΔB13R had marked weight loss (Fig. 4A). The difference was statistically significant by analysis of variance (ANOVA) followed by Tukey's post-test from days 5 through 9 post-infection ($P < 0.05$), and highly significant at days 5 through 7 ($P < 0.001$). In the IFN-γ knockout mice, however, v50ΔB13RIL-18-infected animals displayed weight loss similar to

v50ΔB13R-inoculated animals, while IFN-γ expression by the rVACV completely abolished weight loss. The difference, compared with v50ΔB13RMγ-inoculated animals, was significant from days 4 through 12 post-infection ($P < 0.05$) and highly significant at days 5 through 7 ($P < 0.001$) (Fig. 4B). This strongly suggests that IFN-γ is essential for the attenuating activity of IL-18 on VACV.

IL-18 expression results in higher secondary humoral responses to VSV-G

Groups of 11 CB6F₁ normal mice were intramuscularly inoculated with 10^5 PFU of rVACV and serum was collected 0, 2, 4, and 6 weeks post-infection. Antibody titers to VACV and VSV in pooled serum samples were determined by ELISA. Although there was a significant increase in attenuation as a result of IL-18 co-expression, no statistical differences were observed in the humoral immune responses to either homologous (VACV) or heterologous (VSV-G) antigens in mice inoculated with the respective rVACVs (Fig. 5). Animals were boosted i.p. 4 weeks post-vaccination with VSV and secondary responses to VSV-G were observed, with the group initially vaccinated with v50ΔB13RIL-18 showing a 4-fold increase in VSV-G antibody titer over the other groups.

VACV co-expressing IL-18 induce potent cell-mediated immune responses

Groups of 6 CB6F₁ normal mice were inoculated i.p. with 10^7 PFU of rVACV and relative T-helper cell proliferative responses to soluble VSV-G protein were measured by the incorporation of [³H] thymidine (Fig. 6A). The i.p. route is routinely used to measure VACV-mediated cellular immune responses (Legrand and others 2004, 2005). T-helper responses, calculated as a stimulation index, did not differ among v50ΔB13R, v50ΔB13RMγ, or v50ΔB13RIL-18-inoculated groups. The stimulation index for all of the groups was strong,

FIG. 3. Co-expression of IL-18 increases VACV clearance in immunodeficient and immunocompetent mice. Groups of 3 female BALB/cBy nude (immunodeficient) mice (**A**) or 5 female CB6F₁ immunocompetent mice (**B**) were intraperitoneally inoculated with 10^7 plaque-forming units (PFU) of recombinant VACV (rVACV). At the indicated days post-infection, the mice were sacrificed, their ovaries removed, and viral titers were determined (as PFU/organ). Bars indicate mean titers per group. Virus was undetectable in the ovaries of all mice inoculated with v50ΔB13RMγ. Statistical analyses (Mann-Whitney test) comparing the 3 rVACVs yielded P values < 0.05 (except for v50ΔB13R versus v50ΔB13RIL-18 at day 3 in panel **A**, where the P value was > 0.05).

FIG. 4. Attenuation provided by IL-18 is dependent on IFN- γ induction. Normal (IFN- $\gamma^{+/+}$) and IFN- γ knock-out (IFN- $\gamma^{-/-}$) BALB/c mice were inoculated intranasally with 5×10^7 PFU of rVACV and weighed daily. **(A)** Normal mice inoculated with v50 Δ B13RM γ and v50 Δ B13RIL-18 did not show any weight loss, whereas mice inoculated with the same dose of v50 Δ B13R had marked weight loss. **(B)** IFN- γ knock-out mice inoculated with v50 Δ B13RIL-18 displayed weight loss similar to v50 Δ B13R-inoculated animals. Statistical analyses (analysis of variance [ANOVA] followed by Tukey's post-test) of v50 Δ B13RM γ or v50 Δ B13RIL-18 versus v50 Δ B13R **(A)**, and v50 Δ B13R or v50 Δ B13RIL-18 versus v50 Δ B13RM γ **(B)**, yielded the *P* values shown.

approximately 6 times higher than that of naive controls. Finally, specific CTL responses were determined. All rVACVs induced potent primary and secondary CTL responses. However, both primary (Fig. 6B) and secondary (Fig. 6C) CTL responses induced by v50 Δ B13RIL-18 were higher than those induced by v50 Δ B13R and v50 Δ B13RM γ , reaching as high as 44% and 55%, respectively (Fig. 6). Infected L929 cells (H-2^k) served as negative target controls. Lysis values for negative control targets was <5% (data not shown).

Discussion

VACV is frequently used as an effective recombinant vaccine vector due to its ability to induce both humoral and

cell-mediated immune responses, and ease in vector construction, ability to incorporate large amounts of foreign DNA, and a wide host range (Moss 1996). Previously, we demonstrated that TK⁻ rVACVs with a deletion in the B13R or B22R gene and expressing IFN- γ have no detectable viral replication *in vivo* yet elicit immune responses similar in magnitude to those induced by rVACVs without the cytokine gene (Legrand and others 2005). Thus, it is conceivable that rVACVs that replicate to a low level before viral clearance might result in increased heterologous antigen expression and enhanced immune responses compared with the highly attenuated rVACV expressing IFN- γ . This has been demonstrated in other vaccine viral vectors, for example, replication-competent recombinant adenoviruses elicit higher levels of humoral and cell-mediated immune responses than nonreplicating vaccines (Peng and others 2005). Thus, we constructed an rVACV that expresses the cytokine IL-18, which then induces IFN- γ expression by the host.

IL-18 has been successfully used as an immune modulator to elicit augmented immune responses. Administered either as a protein or via plasmid DNA, IL-18 magnified the immune responses against tumor cells (Heuer and others 1999; Yamanaka and others 1999; Hara and others 2000; Nagai and others 2000), pathogens (Kremer and others 1999; Ohkusu and others 2000; Li and others 2013), and subunit antigens (Eberl and others 2000; Degen and others 2005; Shi and others 2007). IL-18 has been shown to have anti-VACV activity through the activation of innate and adaptive immune responses (Tanaka-Kataoka and others 1999; Gherardi and others 2003), and having antiviral effects on herpes simplex virus type-1 (Fujioka and others 1999). Moreover, IL-18 has been demonstrated to be an important modulator in the innate control of genital herpes simplex virus type-2 (Harandi and others 2001) and encephalomyocarditis virus-induced acute myocarditis (Kanda and others 2000). Studies have also demonstrated that some haplotypes of IL-18 and the IL-1 complex are associated with increased risk of fever after smallpox vaccination in humans (Stanley and others 2007), and that single-nucleotide polymorphisms/haplotypes in the IL-18 and IL-18 receptor genes are associated with variations in humoral immune responses to the smallpox vaccine in humans (Haralambieva and others 2011).

To study the ability of IL-18 to enhance immune responses by delayed expression of IFN- γ , the IL-18 gene was cloned from murine splenic cDNA and used to construct an rVACV expressing the mature bioactive form of IL-18 (Fig. 1A–C). To enhance the production of bioactive IL-18, a VACV gene (B13R) that encodes a serpin that inhibits ICE from processing pro-IL-18 to its mature bioactive form (Dobbelstein and Shenk 1996) was deleted. Fortunately, deletion of the VACV genes C12L (encoding an IL-18-binding protein that inhibits mouse IL-18) (Symons and others 2002; Falivene and others 2012) and F1L (which inhibits inflammasome/ICE activation) (Gerlic and others 2013) were not necessary for expression of bioactive IL-18 by our rVACV. Our previous studies showed that the deletion of the B13R gene has no effect on virus replication *in vitro*, whereas *in vivo*, it reduced rVACV virulence and pathogenicity in both immunocompetent and immunodeficient mice (Legrand and others 2004).

As we previously showed with IFN- γ , IL-18 expression had no effect on viral replication *in vitro* (Fig. 1D). These studies also demonstrated that IFN- γ expression induced by IL-18 commenced between 24 and 48 h post-infection (Fig.

FIG. 5. IL-18 expression elicits strong humoral responses to rVACV. Groups of 11 CB6F₁ normal mice were intramuscularly inoculated with 10^5 PFU of the rVACV. Animals were boosted intraperitoneally 4 weeks post-vaccination with vesicular stomatitis virus (VSV). Antibody titers to VACV (**A**) and VSV (**B**) were determined by ELISA from pooled samples assayed in duplicate.

1B), compared to 3–6 h post-infection with the IFN- γ -expressing VACV (data not shown), as predicted. On the other hand, expression of IL-18 greatly increased attenuation of VACV *in vivo* as measured by median survival rates and clearance studies in immunodeficient mice (Figs. 2 and 3A), similar to a previous study by Gherardi and others (2003). As expected, this attenuation was not as marked (60% survival and limited clearance) as that achieved with the rVACV co-expressing IFN- γ (100% survival and complete clearance). This may be due to the very low number of mature T cells that express the IL-18 receptor observed in immunodeficient nude and SCID mice and thus their ability to induce the production of IFN- γ was constrained, decreasing viral clearance and survival. Normal immunocompetent mice also had detectable replication of the IL-18-expressing VACV, but

not the IFN- γ -expressing VACV (Fig. 3B), suggesting that the attenuation of the virus *in vivo* was highly dependent on early and relatively high concentrations of IFN- γ for both normal and immunodeficient mice. The postponement of IFN- γ production by the IL-18-expressing VACV *in vitro* almost certainly occurred *in vivo* as well, therefore delaying viral clearance (Fig. 3), and probably allowing for greater homologous and heterologous antigen expression. To further investigate the role of IFN- γ in the attenuating activity of IL-18, we compared the virulence of the rVACVs in mice without a functional IFN- γ gene. Normal (IFN- $\gamma^{+/+}$) mice did not have any weight loss when inoculated i.n. with v50ΔB13RM γ or v50ΔB13RIL-18 (Fig. 4A). On the other hand, IFN- γ knockout (IFN- $\gamma^{-/-}$) mice displayed marked weight loss when inoculated with v50ΔB13RIL-18 or the

FIG. 6. VACV co-expressing IL-18 elicits strong cell-mediated immune responses. Groups of 6 CB6F₁ normal mice were intraperitoneally with 10^7 PFU of rVACV and splenocytes were harvested 10 days post-vaccination. (**A**) For the T-helper responses, harvested splenocytes were stimulated with 0.5 μ g/mL of VSV glycoprotein, incubated for 4 days, and pulsed with [3 H] thymidine. Data analysis was based on cpm in triplicates and expressed as a stimulation index. (**B**) For primary cytotoxic T-cell (CTL) studies, effector splenocytes were stimulated with haplotype-matched (H-2^{b/d}) ultraviolet (UV)-treated VSV-infected target splenocytes. (**C**) For secondary CTL studies, effector splenocytes were re-stimulated with the VSV-infected target splenocytes. Infected L929 cells (H-2^b) served as negative target controls. Lysis values for negative control targets was <5% (data not shown). Specific cytotoxicity values at the indicated effector:target cell ratios represent the means of triplicate experiments assayed in duplicate. Error bars represent SEM.

control strain v50ΔB13R, but not when inoculated with v50ΔB13RMγ (Fig. 4B). These results suggest that induction of IFN-γ is probably the most important function of IL-18 for VACV attenuation, and contrasts with the previous suggestion that VACV clearance by cytokines such as IL-12 and IL-18 may not be related to the production of IFN-γ (Gherardi and others 2003).

Despite being highly attenuated, the rVACV expressing IL-18 induced potent immune responses. Previous reports indicated IL-18 co-administration elevates cell-mediated immune responses but not humoral responses (Billaut-Mulot and others 2001; Kim and others 2001). Both the primary and secondary CTL responses induced by v50ΔB13RIL-18 were higher than those induced by the 2 other rVACVs (Fig. 6), although these differences were small as measured by the CTL assay. A previous report indicated that CD8⁺ T cell responses were slightly enhanced in BALB/c mice as well (Gherardi and others 2003). Similar to published results from other groups using IL-18 to enhance immune responses to DNA vaccines, there were no statistical differences in the primary humoral immune responses to either homologous (VACV) or heterologous (VSV-G) antigens in mice inoculated with the respective rVACVs. However, secondary antibody responses to VSV-G after VSV boost were elevated 4-fold in v50ΔB13RIL-18-vaccinated animals (Fig. 5).

In conclusion, we demonstrated that IL-18 can be used as an effective attenuating agent for VACV, increasing the safety of rVACV vectors. This attenuation appeared to be primarily dependent on IFN-γ induction. The delayed, natural expression of IFN-γ resulted in an rVACV that replicated to low but detectable levels *in vivo* and elicited slightly better CTL and anamnestic humoral immune responses. Therefore, IL-18 co-expression by rVACVs provides another method for increasing recombinant vaccine safety and efficacy.

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Author Disclosure Statement

No competing financial interests exist.

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