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Preferential Replication of Vaccinia Virus in the Ovaries is Independent of Immune Regulation Through IL-10 and TGF-β

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Abstract

Vaccinia virus (VACV) exhibits a strong tropism for ovarian tissue and can cause ovary pathology and sterility. Why VACV preferentially accumulates in this organ is not known. Here we show that multiple immune cell populations infiltrated the ovaries following VACV infection, including virus-specific CD8 T cells making both IFN-γ and TNF. This was also accompanied by the induction of interleukin (IL)-10 and TGF-β, suggesting that VACV may exploit the ovarian environment for immune evasion via induction of these suppressive cytokines. To test this we used several strategies, including neutralizing these cytokines, and exogenous targeting of the T-cell response, to determine if this inhibited virus replication in the ovaries. We found that the VACV-specific CD8 T-cell immunity and the clearance of virus were not enhanced in the ovaries of infected mice in which IL-10 receptor (IL-10R) was blocked with antagonist antibody. VACV replication was also only moderately affected in the ovaries of infected IL-10 knockout mice. Similarly, blockade of TGF-β with antagonist antibody demonstrated no effect on CD8 T-cell immunity or VACV replication. Lastly, an agonist antibody targeting the tumor necrosis factor receptor superfamily member OX40 (TNFRSF4) enhanced the number of VACV-specific CD8 T cells producing IFN-γ in lymphoid tissue, but had no effect on CD8 T-cell infiltration of the ovaries or on the viral load. Collectively, the results indicate that preferential replication of VACV in the ovaries may not be dependent on immune suppressive mechanisms in this tissue.

Introduction

Vaccinia virus (VACV) is a large DNA orthopoxvirus similar to variola, monkeypox, buffalopox, and cowpox. VACV represents a useful tool for understanding how immunity to viruses is regulated, as well as what factors might determine how to create strong vaccines. Smallpox, long considered to be the most deadly and persistent human pathogenic disease, was eradicated by 1977 through vaccination with live VACV (1), and attenuated vaccinia virus vectors have since been developed as vaccine vehicles for multiple infectious diseases (2–4). In addition to this, VACV also has many attributes that make it an attractive vector for tumor-directed gene therapy and oncolytic virotherapy (5,6).

It has been known for many years that VACV exhibits a strong tropism for ovarian tissue and can cause ovary pathology and sterility (7–9). Immunohistochemistry studies have revealed viral infection in ovarian follicles and in adjacent ovarian stromal tissues (10). Although the virus can be found in peripheral tissues, it accumulates preferentially in the ovaries and persists in this site longer than other tissues. Similarly, systemic delivery of wild-type and tumor-selective VACV has shown that the highest level of infectious virus is isolated from the tumor and ovary, with little to no viral recovery from other normal organs when a tumor-selective VACV was used (10–12). Thus, although the use of VACV-based vectors to develop vaccine vehicles for infectious disease as well as for cancer immunotherapy is promising, the effectiveness may be associated with VACV-mediated damage to the ovaries and loss of reproductive function. Interestingly, although VACV has been studied for decades, the reason why it preferentially accumulates in the ovaries is unclear. Furthermore, the majority of animal studies of immunity to VACV measure virus replication in the ovaries, with the idea that clearance of virus from this organ is a reflection of systemic immunity. However, it is not clear whether the immune response in the ovaries is equivalently regulated compared to peripheral sites or in secondary lymphoid organs. Understanding how VACV preferentially replicates in the ovaries may assist the design of safer vaccination and immunotherapeutic strategies, as well as aid basic studies of immunity to this virus.

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Interestingly, the propensity for VACV to accumulate in the ovaries is not unlike that of cytomegalovirus (CMV) to replicate at high levels in the salivary glands, representing a site of transmission to susceptible hosts. Recently we found that persistent mouse CMV (MCMV) replication in the salivary glands is accompanied by the appearance of IL-10-expressing CD4 T cells specifically within this organ, but not elsewhere. This suggested that the induction of this regulatory cytokine might represent a means by which the virus persisted in this location. In line with this, blockade of IL-10R signaling, or increasing the ratio of protective CD4 T cells expressing IFN-γ in the salivary glands, strongly reduced the persistent replication of MCMV in this organ (13). These data suggested that MCMV takes an active role in the exploitation of this mucosal tissue for immune evasion. Similarly to MCMV, VACV possesses a wide range of immune evasion strategies it uses in order to survive. Although ovarian immunity generated during VACV infection has yet to be studied, recent data have suggested that there are correlations between ovarian immune cells and infection or inflammation (14,15). We therefore sought to investigate whether VACV may employ the ovaries as a target for immune evasion through the induction of an organ-specific immune-suppressive environment.

Here, we show that multiple immune cell populations infiltrated the ovaries following VACV infection, accompanied by increased levels of regulatory cytokines, including IL-10 and TGF-β. However, in virus-infected mice in which the IL-10R was blocked, VACV-specific CD8 T-cell immunity, and the clearance of virus, was not enhanced in the ovaries. VACV replication in the ovaries of infected IL-10 knockout mice was largely unaffected. Similarly, blockade of TGF-β with antagonist antibody had no effect in regulating the virus-specific T-cell response in the ovary or in controlling VACV replication. Furthermore, an agonist antibody targeting the tumor necrosis factor receptor superfamily member OX40 (TNFRSF4) increased the number of VACV-specific CD8 T cells producing IFN-γ in the periphery, but had no effect on CD8 T-cell accumulation in the ovaries, or viral load in the ovaries. Thus, preferential replication of VACV in the ovaries is largely independent of the regulatory influences of TGF-β and IL-10, and is not impacted by enhancing peripheral CD8 T-cell responses.

Materials and Methods

Mice

Eight- to 12-wk-old female C57BL/6 and IL-$10^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The studies reported here conformed to the Animal Welfare Act and the National Institutes of Health (NIH) guidelines for the care and use of animals in biomedical research. All experiments were conducted following the guidelines of the La Jolla Institute for Allergy and Immunology's Institutional Animal Care and Use Committee.

Virus infection and antibody treatment

The VACV Western Reserve strain was purchased from the American Type Culture Collection (Manassas, VA), grown in HeLa cells, and tittered on VeroE6 cells (47). Mice were infected with 2×10^5 PFU VACV by the intraperitoneal route, and in some experiments was injected IP at the time of infection with 250 μg of either rat IgG or anti–IL-10Rα (clone 1B1.3a), purified by protein A affinity chromatography. In other experiments, mice were injected IP on day 1 with 100 μg of either rat IgG or anti-OX40 (clone OX86) purified by protein A affinity chromatography; or were injected IP on days -3, -2, -1, and 0 of infection with 100 μg of either rat IgG or anti-TGF-β (clone 1D11), purified by protein A affinity chromatography.

RT-PCR and real-time PCR

Ovaries from VACV-infected mice were flash-frozen in liquid nitrogen before being homogenized with a Tissue Master (OMNI International, Kennesaw, GA). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). An aliquot of total RNA was reverse-transcribed to cDNA by using SuperScript III (Invitrogen). Data are presented as normalized to ribosomal protein housekeeping gene L32. The primers used were: IFN-γ FWD: AAC GCT ACA CAC TGC ATC TTG G REV: GCC GTG GCA GTA ACA GCC; TGF-β FWD: TGA CGT CAC TGG AGT TGT ACG G REV: GGT TCA TGT CAT GGA TGG TGC; IL-10 FWD: GGG AAG ACA ATA ACT GCA CCC A REV: CAG TCC GCA GCT CTA GGA GC; and Foxp3 FWD: GGC CCT TCT CCA GGA CAG A REV: GCT GAT CAT GGC TGG GTT GT.

Peptides and tetramers

Vaccinia virus peptide epitope B8R (20–27; TSYKFESV) was used as described previously (48,49). MHC/peptide tetramers for the epitope B8R/H-2Kb, conjugated to allophycocyanin, were obtained from the NIH Tetramer Core facility (Emory University, Atlanta, GA).

Immunofluorescence labeling

Ovary cells and splenocytes after lysing RBCs from infected mice were resuspended in RPMI-1640 medium (Gibco, Carlsbad, CA), supplemented with 10% FCS (Omega Scientific, Tarzana, CA), 1% L-glutamine (Invitrogen), 100 μg/mL streptomycin, 100 U/mL penicillin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Then 1–2×10^6 cells were plated in round-bottomed 96-well microtiter plates in 200 μL with medium or VACV peptide at 1 μg/mL for 1 h at 37°C. GolgiPlug (BD Biosciences, San Jose, CA) was then added to the cultures according to the manufacturer's instructions, and the incubation continued for 6–9 h. Cells were stained with anti-CD8 (PerCP; 53-6.7) and CD62L (PE; MEL-14), followed by fixation with Cytofix-Cytoperm (BD Biosciences) for 20 min at 4°C. Fixed cells were subjected to intracellular cytokine staining in BD Perm/Wash buffer for 30 min at 4°C. Anti-TNF (FITC; MP6-XT22) and IFN-γ (APC; XMG1.2) were obtained from eBioscience (San Diego, CA) and used at a 1:100 dilution. Samples were analyzed for their proportion of cytoplasmic cytokines after gating on $CD8^+CD62L^{low}$ T cells with a FACSCalibur flow cytometer using CellQuest (BD Biosciences) and FlowJo software (Tree Star, San Carlos, CA).

Measurement of serum VACV-specific IgG titers

Serum was obtained after centrifugation of blood samples collected with a heparinized capillary pipette from the

retro-orbital plexus. All samples were stored at −20°C until analyzed for Ab activity. The level of specific Abs against VACV in serum was quantitated with an ELISA as previously described (47,50).

VACV-titer assay

Tissues from individual mice were homogenized and sonicated for 1 min, with a pause every half minute, using an ultrasonic cleaner (model 1210; Branson Ultrasonics, Danbury, CT). Serial dilutions were made and the virus titers were then determined by plaque assay on confluent VeroE6 cells.

Statistical analysis

Statistical significance was determined by a two-tailed Student's *t*-test. Unless otherwise indicated, data represent the means ± SEM. Significance was set at $p < 0.05$.

Results

VACV preferentially replicates in the ovaries

To directly compare accumulation of VACV in the ovaries versus other visceral organs, naive B6 mice were inoculated IP with 2×10^5 PFU of VACV of the Western Reserve strain, and viral titers were determined in the ovaries as well as in the spleen, brain, lungs, kidneys, heart, and livers at different time points post-infection. VACV was found in most organs within 24 h, and similar titers were found in the ovaries, spleen, kidney, and liver at this time. However, by 48 h, virus titers in the ovaries were at least 100 times higher than in all other organs examined (Fig. 1). VACV replication in the ovaries continued through day 7 of infection, while virus levels were either maintained or decreased elsewhere (Fig. 1). At day 15, the virus was not detected in most organs, but was still present in the ovaries, albeit at strongly reduced levels (Fig. 1). At day 20, the virus was cleared from the ovaries (data not shown). As the ovary was the smallest organ assayed, the amount of virus per gram of tissue was significantly higher than in the other organs.

VACV infection in the ovaries induces immune cell infiltration and promotes both regulatory and inflammatory cytokine production

To evaluate if preferential replication of VACV in the ovaries is associated with organ-specific immune regulation, we first determined whether immune cells could infiltrate into the ovaries after VACV infection. A low percentage of hematopoietic cells, including T and B cells, were found in the ovaries in a naïve mouse. Seven days following VACV infection, a striking amount and diversity of hematopoietic cells, including CD8 T cells, NK cells, macrophages, granulocytes, and B cells, had infiltrated the ovaries (Fig. 2a). The total number of each infiltrated population was also significantly higher in the ovaries from VACV-infected mice, which had enlarged in size and showed apparent pathology compared to naïve ovaries (Fig. 2b). There was a predominance of CD8 T cells that entered the ovary, whereas the CD4 T-cell number was not increased in infected ovaries (Fig. 2b). Although the average number of $CD11c^+$ dendritic cells (DCs) was increased in infected ovaries, it was not statistically significant compared to naive ovaries (Fig. 2b). The migration of immune cells into ovarian tissue following VACV infection was likely associated with virus activity, as the kinetics of CD8 T-cell infiltration was tightly connected with the kinetics of VACV replication within the ovaries. The number of infiltrating CD8 T cells peaked at day 7, and dropped to a naive level around day 15 post-infection (data not shown), corresponding to when the virus titer in the ovaries was decreasing. This suggested a potential role for ovarian immune cells, and specifically CD8 T cells, in modulating virus accumulation or clearance in this organ.

We previously found that during infection with MCMV, induction of IL-10 in the salivary glands allowed this virus to replicate for extended times in this location (13). Levels of another suppressive cytokine, TGF-β, also increase during many viral infections, such as with lymphocytic choriomeningitis virus (LCMV), human cytomegalovirus (HCMV), and human respiratory syncytial virus (RSV) (16–18). Overexpression of TGF-β following myeloablative conditioning post–bone marrow transplantation resulted inimpaired effector T-cell responses to murine gammaherpesvirus (MHV) infection (19), and blocking TGF-β during RSV infection resulted in the rescue of RSV-induced cell-cycle arrest, and a significant reduction in viral protein expression (18). We therefore asked whether VACV might preferentially replicate in the ovaries because of comparable immune suppression mechanisms. Gene expression of IL-10 was significantly upregulated by days 2–3 of infection, and peaked in the ovaries at day 7 (Fig. 3a). Similarly, TGF-β was also induced within the same time frame in the ovaries (Fig. 3b). Elevated IL-10 and TGF-β were not, however, accompanied by an influx of conventional regulatory T cells (Treg), as Foxp3, the key transcription factor, was not upregulated (Fig. 3c). In addition to regulatory cytokines, a strong

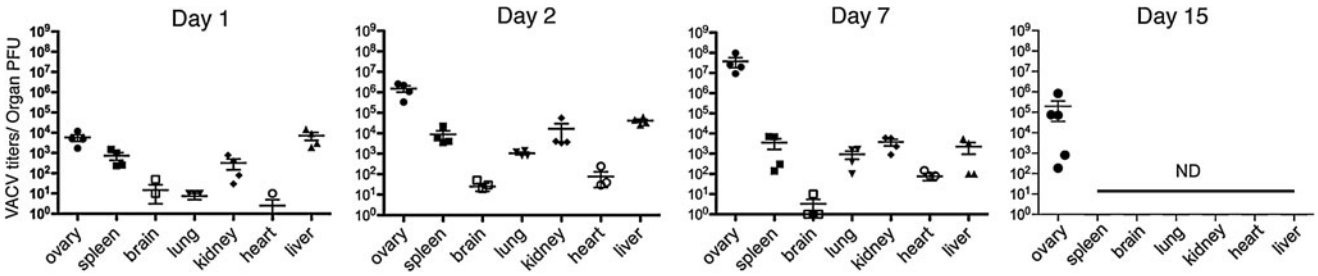


FIG. 1. VACV preferentially replicates in the ovaries. WT mice were infected IP with VACV-Western Reserve (2×10^5 PFU/mouse). On days 1, 2, 7, and 15 post-infection, VACV titers were determined in the indicated organs as described in the materials and methods section. Data are representative of three separate experiments.

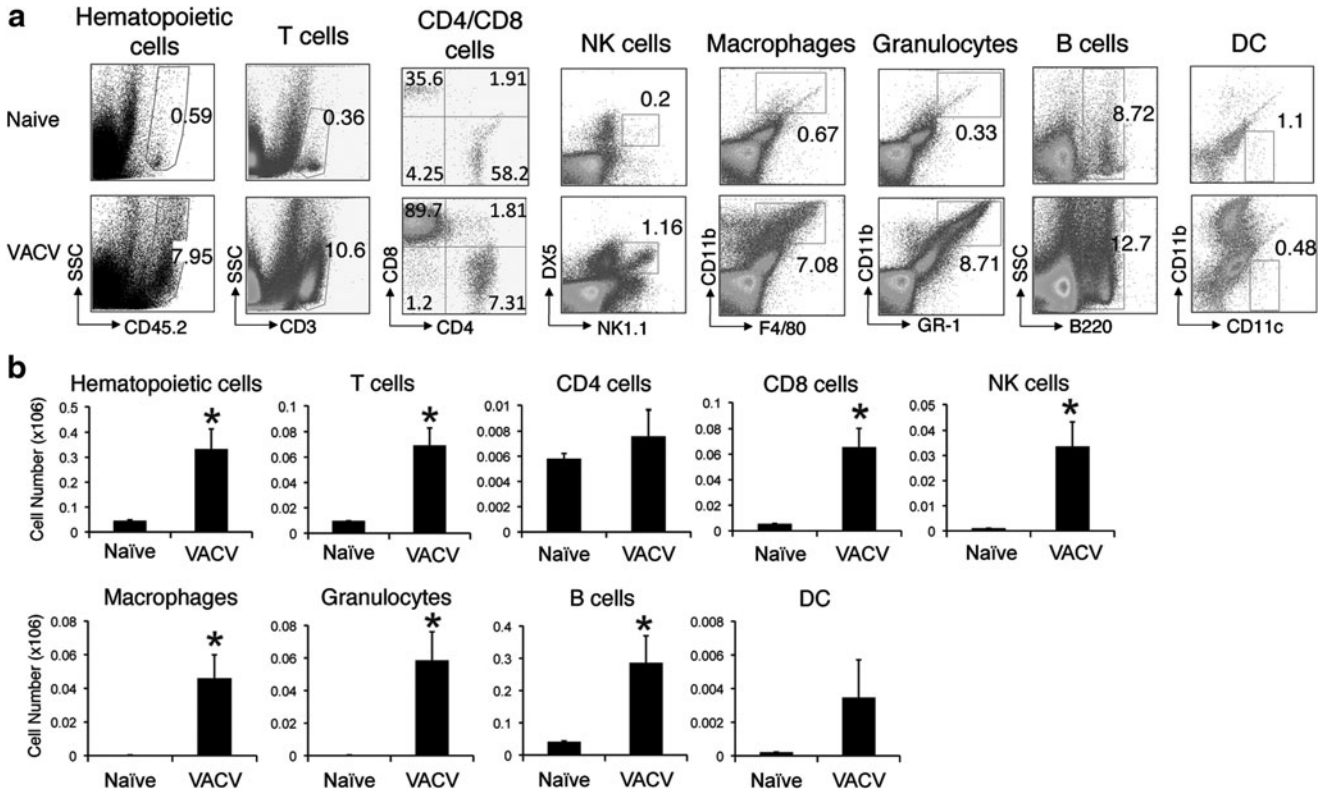


FIG. 2. Immune cells infiltrate the ovaries after VACV infection. WT mice were infected IP with VACV-Western Reserve (2×10^5 PFU/mouse). On day 7 post-infection, the ovaries were removed and cell suspensions were stained for CD45.2, CD3, CD4/CD8, NK1.1/DX5, F4/80/CD11b, GR-1/CD11b, B220, or CD11c/CD11b to identify populations of hematopoietic cells, T cells, $CD4^+/CD8^+$ cells, NK cells, macrophages, granulocytes, B cells, and $CD11c^+$ DC. CD4/CD8 T cells were gated on $CD45.2^+CD3^+$ cells, and the other cell populations were gated on total live cells. (**a**) Percentages of cells in gated populations are indicated, compared to those in ovaries from naïve mice. (**b**) Total numbers of each subset. Data are representative of two separate experiments.

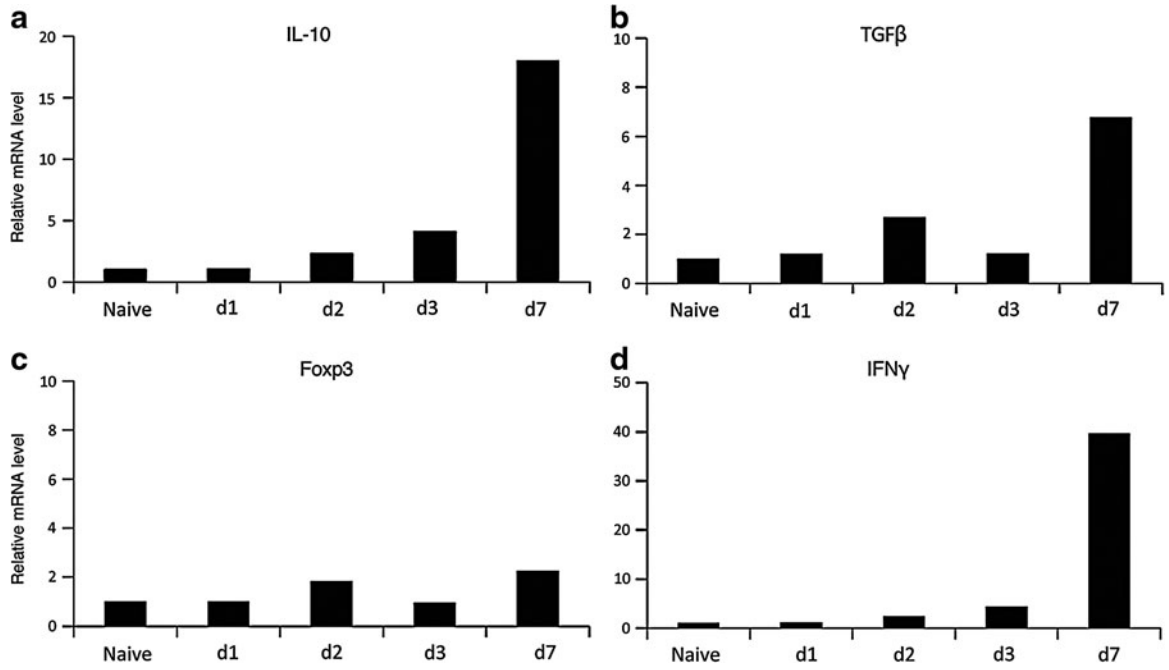


FIG. 3. VACV induces both regulatory and inflammatory cytokines in the ovaries. Quantitative real-time PCR analysis of IL-10 (**a**), TGF-β (**b**), Foxp3 (**c**), and IFN-γ (**d**) mRNA expression in the ovaries of naïve mice, or mice infected with VACV, for the indicated times. Data are representative of two separate experiments.

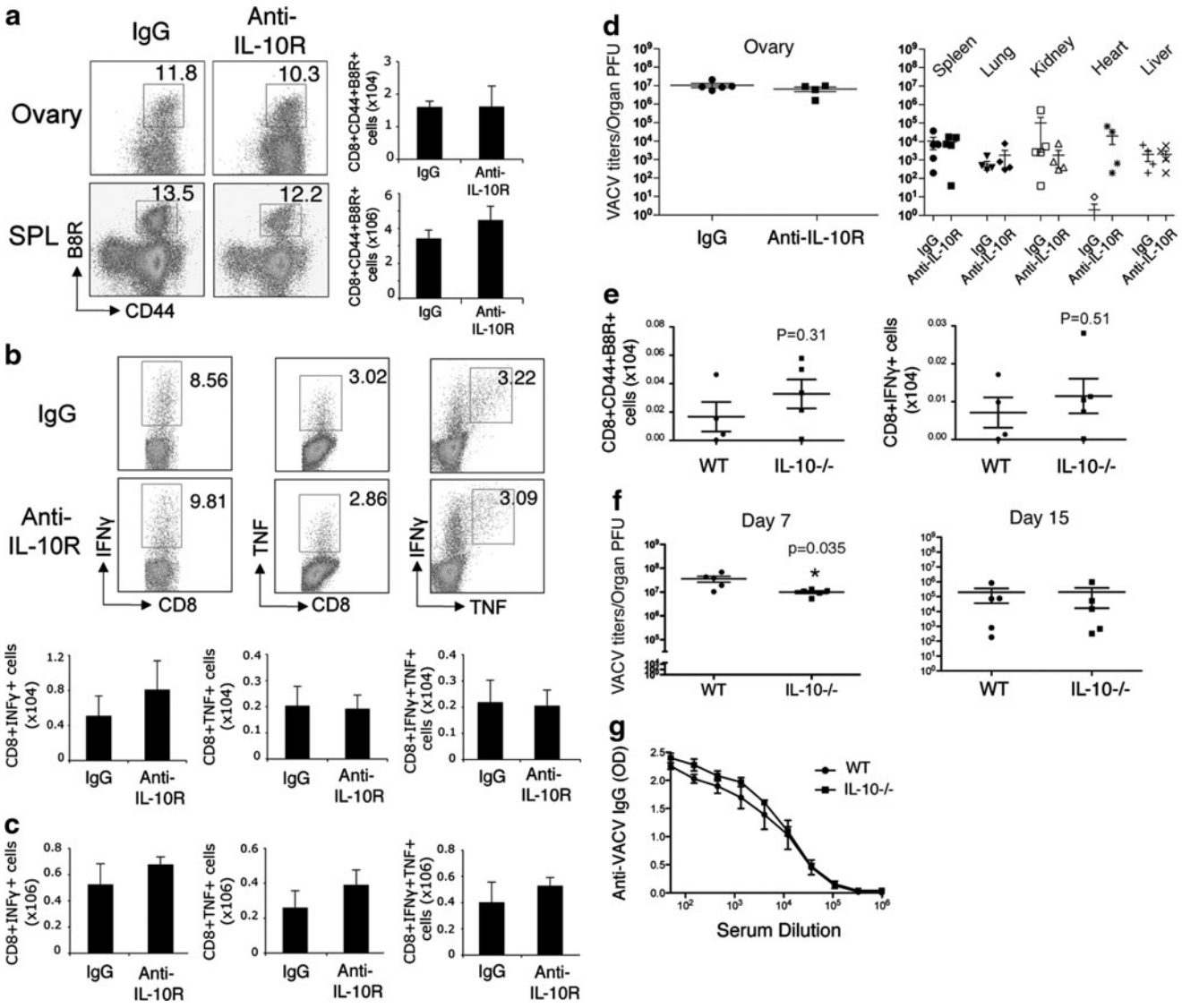


FIG. 4. IL-10 plays a limited role in regulating VACV replication in the ovaries. WT mice were infected IP with VACV-Western Reserve (2×10^5 PFU/mouse), and treated with 250 μg of rat IgG or anti–IL-10R (1B1.3a) at the time of infection. Seven days later, (**a**) ovary cells and splenocytes (SPL) were stained. Left panels: Representative dot plots of gated $CD8^+$ cells stained for CD44 and B8R-tetramer. Numbers indicate the percentages of $CD8^+CD44^+$B8R-tetramer-positive cells. Right panels: Total numbers of $CD8^+CD44^+$B8R-tetramer-positive cells per organ. (**b**) Ovary cells were stimulated with B8R peptide and stained for intracellular IFN-γ and TNF. Top panels: Representative plots of IFN-γ (left), TNF (middle), and IFN-γ/TNF (right), gating on $CD8^+CD62L^-$ cells. Percentages for each cytokine are indicated. Bottom panels: Total numbers of $CD8^+$IFN-γ^+ (left), or $CD8^+TNF^+$ (middle), and $CD8^+$IFN-γ^+TNF^+ cells (right). (**c**) Splenocytes were stimulated with B8R and stained for intracellular IFN-γ and TNF. Total numbers of $CD8^+$IFN-γ^+ (left), $CD8^+TNF^+$ (middle), and $CD8^+$IFN-γ^+TNF^+ cells (right). (**d**) VACV titers were determined in ovaries (left), and other indicated organs (right). (**e**–**g**) WT and IL-10 knockout mice were infected IP with VACV-Western Reserve (2×10^5 PFU/mouse). (**e**) Seven days later, total numbers of $CD8^+CD44^+$B8R-tetramer-positive cells (left) and $CD8^+$IFN-γ^+ cells (right) in the ovaries were quantified. (**f**) VACV titers were determined on day 7 (left) and day 15 (right). (**g**) The level of VACV-specific antibodies in serum was quantitated on day 15. Data represent mean ± SEM from 4 to 6 mice. Similar results were reproduced in two separate experiments ($^*p<0.05$ as determined by Student's *t*-test).

induction of mRNA for IFN-γ was also detected (Fig. 3d). As experiments in mice have demonstrated that the forced expression of IFN-γ can strongly enhance VACV clearance and promote immune responses (20–22), this suggested that concomitant induction of IL-10 and/or TGF-β might limit the activity of CD8 T-cell-derived IFN-γ, which would antagonize the clearance of the virus from the ovary.

The anti-VACV CD8 T-cell response and VACV replication in the ovaries is independent of IL-10 and TGF-β

To address IL-10, we used an antagonist IL-10R antibody (anti–IL-10R) that we previously used to block IL-10 signaling after MCMV infection (13). Seven days after infection and

antibody treatment, the number of VACV-specific CD8 T cells in both ovaries and spleen were quantified using a tetramer of B8R, the immunodominant class I epitope of VACV. Anti–IL-10R did not alter the percentage and total number of B8R-reactive CD8 T cells that accumulated in either organ (Fig. 4a). Furthermore, VACV-specific CD8 T cells in the ovaries displayed similar reactivity in producing IFN-γ, TNF, or both cytokines (Fig. 4b). Similar results were found in the spleen (Fig. 4c). More importantly, blocking IL-10R signaling did not abrogate or impair VACV replication in the ovaries or in other visceral organs (Fig. 4d). This antibody, and the other antibodies used below, did penetrate the ovary, as we could detect significant levels of rat IgG in ovarian homogenates (data not shown). A previous study reported that VACV replication in the ovaries, measured at 6 days following infection, was dramatically impaired in IL-$10^{-/-}$ mice (23). To address if the discrepancy with our data was due to the use of a neutralizing antibody, we directly infected IL-$10^{-/-}$ mice with VACV. Although the average number of ovarian VACV-specific CD8 T cells making IFN-γ was slightly higher in IL-$10^{-/-}$ mice, it was not statistically significantly different compared to WT mice (Fig. 4e). We did find that the viral titer was marginally but significantly reduced in the ovaries of IL-$10^{-/-}$ mice at day 7 post-infection, by three to fourfold (Fig. 4f). However, this is in contrast with the 10,000-fold reduction reported in the prior report (23). Virus titers were not significantly different in IL-$10^{-/-}$ mice in the ovaries at day 15 post-infection (Fig. 4f). We also determined VACV-specific neutralizing antibody levels in the serum of infected IL-$10^{-/-}$ mice to identify whether IL-10 plays any modifying role on the antibody response. A comparable serum level of anti-VACV IgG was found in IL-$10^{-/-}$ and WT mice at day 15 (Fig. 4g), and day 20 (data not shown) after infection. Thus our data suggest that IL-10 plays a minor role in regulating VACV replication, and this is largely independent of activity on the CD8 response and neutralizing antibody response.

To determine the role of TGF-β, naive B6 mice were treated with a TGF-β-neutralizing antibody, 1D11, before VACV infection. This antibody has been shown to be effective in blocking TGF-β activity in a number of different experimental settings (24–26). At day 7 post-infection, neutralizing TGF-β did not promote the expansion and accumulation of VACV-specific CD8 T cells. Additionally, anti-VACV activity of these cells as assessed by cytokine production was similar in both ovaries and spleen (Fig. 5a–c). Furthermore, virus titers in the ovaries and other visceral organs were all unaffected by TGF-β neutralization

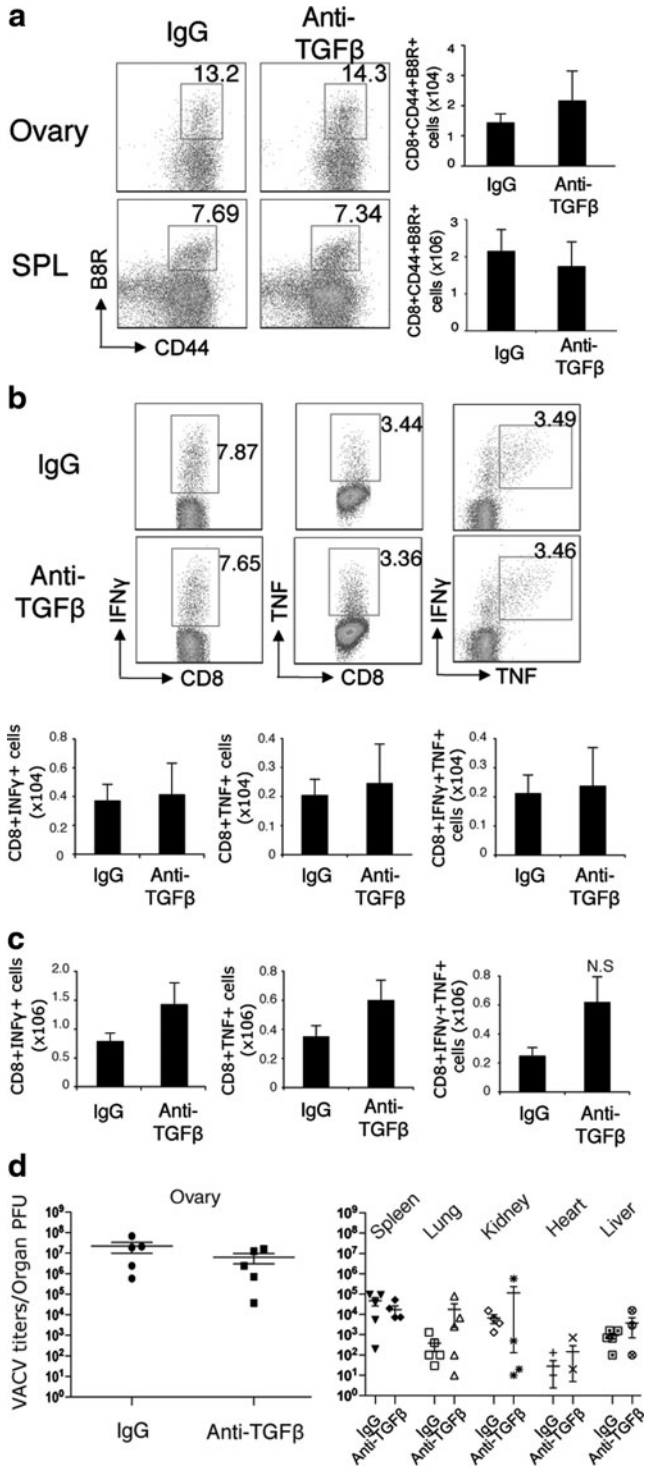
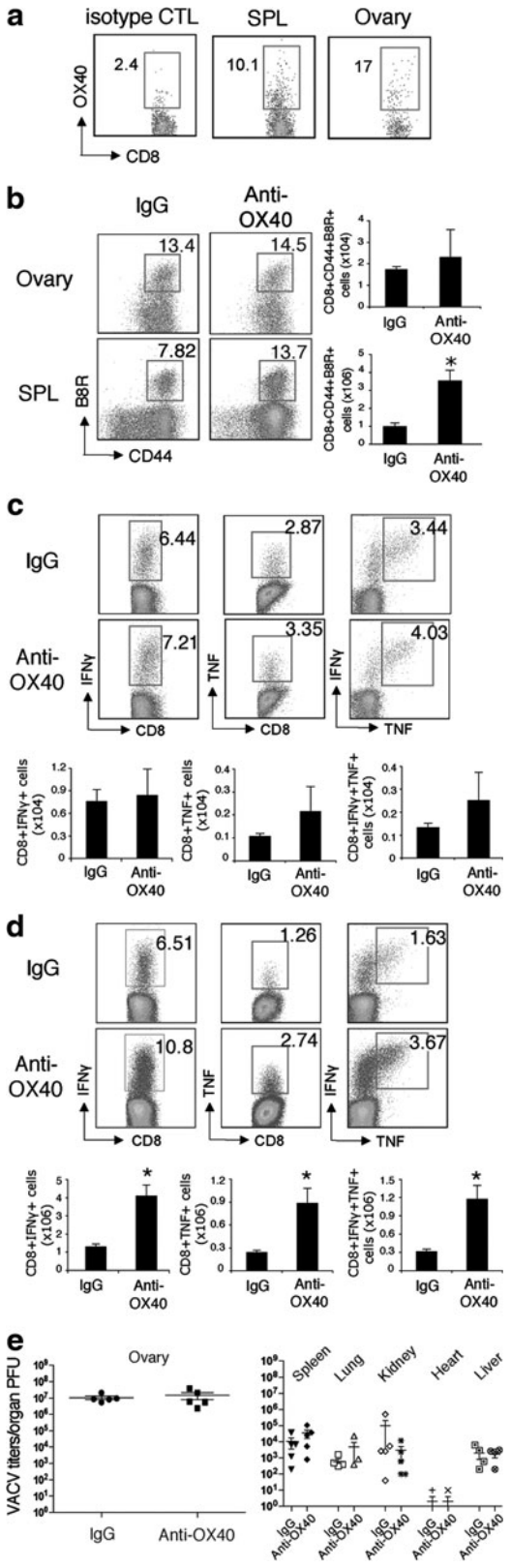


FIG. 5. Blocking TGF-β does not impact VACV replication in the ovaries. WT mice were infected IP with VACV-Western Reserve (2×10^5 PFU/mouse), and treated with 100 μg rat IgG or anti-TGF-β on days -3, -2, -1, and 0 of infection. Seven days after infection, (**a**) ovary cells and splenocytes (SPL) were stained. Left panels: Representative plots of gated $CD8^+$ cells stained for CD44 and B8R-tetramer. Numbers indicate the percentage of $CD8^+CD44^+$B8R-tetramer-positive cells. Right panels: Total number of $CD8^+CD44^+$B8R-tetramer-positive cells per organ. (**b**) Ovary cells were stimulated with B8R peptide. Top panels: Representative plots of IFN-γ (left), TNF (middle), and IFN-γ/TNF (right) staining, gating on $CD8^+CD62L^-$ cells. Positive percentages for each cytokine are indicated. Bottom panels: Total numbers of $CD8^+$IFN-γ^+ (left), $CD8^+TNF^+$ (middle), and $CD8^+$IFN-γ^+TNF^+ cells (right). (**c**) Splenocytes were stimulated with B8R peptide. Total numbers of $CD8^+$IFN-γ^+ (left), $CD8^+TNF^+$ (middle), and $CD8^+$IFN-γ^+TNF^+ cells (right). (**d**) VACV titers were assessed in ovaries (left), and other indicated organs (right). Data represent mean ± SEM from 4 to 6 mice. Similar results were reproduced in two separate experiments.

(Fig. 5d). Similar results were also observed when TGF-β was blocked at the time of infection and at a later time point (data not shown). These data also suggest a limited role for TGF-β in regulating organ-specific anti-VACV immunity.



Augmented IFN-γ expression does not promote VACV clearance

Lastly, to determine whether increasing T-cell activity could block virus replication in the ovaries, we targeted the TNFR family molecule OX40 with an agonist antibody. Our previous studies have shown that this can strongly increase priming of both CD8 and CD4 effector T cells (27,28), and in the context of MCMV infection, anti-OX40 augmented accumulation of CD4 T cells making IFN-γ in the salivary glands, and also led to a substantial reduction in virus titer in this organ (13). OX40 is expressed on a proportion of B8R-tetramer-reactive CD8 T cells in the spleen at days 4–5 post-infection with VACV (29), and a comparable level of OX40 was also detected on ovarian VACV-specific CD8 T cells (Fig. 6a). Although this indicated that ectopic targeting of OX40 on ovary-accumulating CD8 T cells was feasible, the numbers of VACV-specific CD8 T cells producing IFN-γ and TNF in mice treated with anti-OX40 were quite variable, and no significant increase was found over several experiments (Fig. 6b and c). Targeting OX40 did significantly increase the number of IFN-γ- and TNF-producing VACV-specific CD8 T cells in the spleen (Fig. 6b and d); however, no difference in VACV replication in the ovary, spleen, or other organs was found (Fig. 6e).

Discussion

Although previous reports have demonstrated that VACV strongly accumulates and replicates in the ovaries, there has been little research into whether there is an adaptive immune response against VACV in this organ. We show here that there is dramatic infiltration of multiple immune cell populations into the ovaries following VACV infection. However, manipulation of several anti-inflammatory cytokines and targeting T-cell activity had no appreciable effect on

FIG. 6. Increased IFN-γ production by VACV-specific CD8 T cells does not reduce viral load. WT mice were infected IP with VACV-Western Reserve (2×10^5 PFU/mouse), and treated with 100 μg IgG or anti-OX40 (OX86) 1 day later. (**a**) OX40 expression on spleen and ovary $CD8^+CD44^+B8R$-tetramer-positive cells on day 5. (**b**) After 7 d, ovary cells and splenocytes (SPL) were stained. Left panels: Representative dot plots of gated $CD8^+$ cells stained for CD44 and B8R-tetramer. Numbers indicate the percentage of $CD8^+CD44^+$ B8R-tetramer-positive cells. Right panels: Total number of $CD8^+CD44^+B8R$-tetramer-positive cells per organ. (**c**) Ovary cells were stimulated with B8R peptide on day 7. Top panels: Representative plots of IFN-γ (left), TNF (middle), and IFN-γ/TNF (right) staining, gating on $CD8^+CD62L^-$ cells. Positive percentages are indicated. Bottom panels: Total numbers of $CD8^+$IFN-γ^+ (left), $CD8^+TNF^+$ (middle), and $CD8^+$IFN-γ^+ TNF^+ cells (right). (**d**) Splenocytes were stimulated with B8R peptide on day 7. Top panels: Representative plots of IFN-γ (left), TNF (middle), and IFN-γ/TNF (right) staining, gating on $CD8^+CD62L^-$ cells. Positive percentages are indicated. Bottom panels: Total numbers of $CD8^+$IFN-γ^+ (left), $CD8^+$ TNF^+ (middle), and $CD8^+$IFN-γ^+TNF^+ cells (right). (**e**) VACV titers were assessed in ovaries (left) and other indicated organs (right) on day 7. Data represent mean ± SEM from 4 to 6 mice. Similar results were reproduced in two separate experiments (*$p<0.05$ as determined by Student's t-test).

reducing virus accumulation in this organ. Neutralizing antibody is ultimately responsible for clearing VACV from the ovaries (30), but our data question whether immune regulation and cell influx into this organ has any bearing on why the virus preferentially replicates in this site.

Two categories of ovarian leukocytes have been reported: residential and infiltrative. Macrophages, the best characterized residential leukocyte population in the ovaries, distribute in the interstitium and corpus luteum (31). In addition, a small number of lymphocytes, including $CD4^+$ and $CD8^+$ T cells, have been found in naive ovaries (32). Similarly, we detected low percentages of $CD4^+$ and $CD8^+$ T cells and B cells, but few macrophages, in naive ovaries by immunofluorescence staining. It has been reported that macrophages and neutrophils dramatically infiltrate the ovaries during the ovulatory process, likely in response to a luteinizing hormone surge (33), and an equally important influx of monocytes into the corpus luteum occurs at the late post-ovulatory phase (34). In another study, a novel $CD8\alpha\alpha^+$cell population was further observed to influx into ovulating follicles (15). These data suggest that there is a dynamic immune environment within the ovaries that is associated with ovarian function. The potential role of lymphoid cells in regulating infection in the ovaries was studied in a poultry model after *Salmonella* infection. Populations of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were increased in the stroma and the theca of follicles, although further mechanistic studies were absent (14). In our experiments, immune cell populations did infiltrate into the ovaries following VACV infection, including CD8 T cells, NK cells, macrophages, granulocytes, and B cells. This was accompanied by enhanced levels of mRNA for IL-10 and TGF-β, which may be produced mainly by infiltrating macrophages. IFN-γ was also dramatically elevated, and we found that infiltrating effector CD8 T cells were the main source, confirming that there is a potential immune response against this virus in the ovaries.

Older literature demonstrated that a recombinant VACV expressing IL-10 resulted in less natural killer cell activity at 3 days post-infection compared to a control virus, and also suppressed VACV-specific cytotoxic T-cell activity measured after 1 wk (35). However, our study revealed that VACV-specific CD8 T-cell activity in the ovaries or spleen of mice injected with neutralizing anti–IL-10R, or in IL-$10^{-/-}$ mice, was normal. More importantly, blocking IL-10 signaling did not abrogate or impair VACV replication in the ovaries and the other visceral organs, and did not positively affect the generation of antibodies against VACV. The reasons for the discrepancy noted between our data and those of another publication (23), that claimed that IL-$10^{-/-}$ mice had reduced replication of VACV in the ovaries, is not clear. The onset and severity of both spontaneous and experimentally-induced inflammation in IL-$10^{-/-}$ mice may be strongly influenced by the conditions in which these mice are kept. Thus it is possible that the gene-deficient mice in the former study were already exhibiting a hyperresponsive immune system before infection, which could have led to reduced viral replication that was not connected with any regulation in the ovaries. In addition to IL-10, TGF-β was also elevated in the ovaries during VACV infection. The effect of viral infection on TGF-β expression has been described for several viruses, including HIV, CMV, RSV, and HSV-1, and earlier studies have suggested that TGF-β might be important in the pathology of various disease processes involved with these viral infections (16–18,36,37). TGF-β is capable of preventing CD8 cytotoxicity by inhibiting perforin expression, and can also inhibit IFN-γ production in T cells (38). However, again we found that blocking TGF-β did not enhance anti-VACV CD8 T-cell responses, or promote virus clearance in the ovaries and the other organs. Although we did not perform co-blocking experiments, given the positive effects of blocking IL-10 or TGF-β individually in other viral responses, we feel it is unlikely that a role for these molecules would be revealed only when both are blocked.

In vivo studies have demonstrated that the role of IFN-γ in antiviral immune responses is more complicated than previously thought. Its functionality actually varies with the type and conditions of infection. Mice with a disrupted gene for IFN-γR1 or IFN-γ, or mice treated with anti-IFN-γ, are more susceptible to ectromelia virus, CMV, and mouse hepatitis virus infection (39–43), due to the loss of direct antiviral activity via IFN-γ. In contrast, IFN-γ plays a redundant role in controlling acute infection with vesicular stomatitis virus, pseudorabies virus, γ-herpesvirus, and influenza virus (39,43,44). We found that targeting the TNFR family molecule OX40 with an agonist antibody significantly enhanced IFN-γ- and TNF-producing VACV-specific CD8 T cells in the spleen, but this was not evident in the ovaries, and targeting OX40 did not impair VACV replication in any organs. Our data are likely in agreement with those of a report showing that the clearance of VACV was not severely affected in IFN-$\gamma^{-/-}$ mice (23), and with studies showing that depletion of CD8 T cells did not impair VACV clearance in the ovaries (30).

One possible caveat of our study is whether the null effect of the antibodies used here is a genuine biological effect or a failure of the antibodies to penetrate the ovaries. First, the antibodies used in this study have shown positive functionality in several systems used by our group, and other groups have used the same clones with the effects mentioned above. Also, the data in IL-$10^{-/-}$ mice essentially confirmed the results seen with anti–IL-10R. Second, by ELISA, the antibodies could be easily detected in the supernatant from homogenized ovaries of recipients of anti–IL-10R, anti-TGF, and anti-OX40. Furthermore, previous studies of whole human ovary tissue homogenates have found evidence of circulating ovarian antibodies, which strongly correlated with ovarian autoimmunity and/or infertility (45,46). Although this does not directly address the functionality of antibodies that enter the ovary, these data do suggest that circulating antibody can penetrate the ovary and target ovarian cells.

Therefore, overall these results suggest that if there is regulation of VACV replication in the ovaries through innate or adaptive cellular immunity, it is via mechanisms other than induction of regulatory cytokines by VACV and the impairment of CTL responses.

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Author Disclosure Statement

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