



# Effects of Friend Virus Infection and Regulatory T Cells on the Antigen Presentation Function of B Cells

Tyler C. Moore,<sup>a\*</sup> Ronald J. Messer,<sup>a</sup> Lorena M. Gonzaga,<sup>a\*</sup> Jennifer M. Mather,<sup>a\*</sup> Aaron B. Carmody,<sup>b</sup>  Wibke Bayer,<sup>c</sup> Elisabeth Littwitz-Salomon,<sup>c</sup> Ulf Dittmer,<sup>c</sup>  Kim J. Hasenkrug<sup>a</sup>

<sup>a</sup>Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

<sup>b</sup>Research Technologies Branch, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

<sup>c</sup>Institute for Virology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

**ABSTRACT** Friend virus (FV) is a naturally occurring mouse retrovirus that infects dividing cells of the hematopoietic lineage, including antigen-presenting cells (APCs). The infection of APCs by viruses often induces their dysfunction, and it has been shown that FV infection reduces the ability of dendritic cells (DCs) to prime critical CD8<sup>+</sup> T cell responses. Nonetheless, mice mount vigorous CD8<sup>+</sup> T cell responses, so we investigated whether B cells might serve as alternative APCs during FV infection. Direct *ex vivo* analysis of B cells from FV-infected mice revealed that infected but not uninfected B cells upregulated expression of the costimulatory molecules CD80, CD86, and CD40, as well as major histocompatibility complex class II (MHC-II) molecules. Furthermore, *in vitro* studies showed that, compared to uninfected B cells from the same mice, the FV-infected B cells had significantly enhanced APC function, as measured by their capacity to prime CD8<sup>+</sup> T cell activation and proliferation. Thus, in contrast to DCs, infection of B cells with FV enhanced their APC capacity and ability to stimulate the CD8<sup>+</sup> T cell responses essential for virus control. FV infections also induce the activation and expansion of regulatory T cells (Tregs), so it was of interest to determine the impact of Tregs on B cell activation. The upregulation of costimulatory molecule expression and APC function of B cells was even more strongly enhanced by *in vivo* depletion of regulatory T cells than infection. Thus, Tregs exert potent homeostatic suppression of B cell activation that is partially overcome by FV infection.

**IMPORTANCE** The primary role of B cells in immunity is considered the production of pathogen-specific antibodies, but another, less-well-studied, function of B cells is to present foreign antigens to T cells to stimulate their activation and proliferation. Dendritic cells (DCs) are considered the most important antigen-presenting cells (APCs) for CD8<sup>+</sup> T cells, but DCs lose APC function when infected with Friend virus (FV), a model retrovirus of mice. Interestingly, B cells were better able to stimulate CD8<sup>+</sup> T cell responses when they were infected with FV. We also found that the activation status of B cells under homeostatic conditions was potently modulated by regulatory T cells. This study illustrates an important link between B cell and T cell responses and illustrates an additional mechanism by which regulatory T cells suppress critical T cell responses during viral infections.

**KEYWORDS** B-cell responses, CD8<sup>+</sup> T cells, antigen presentation, regulatory T cells, retroviruses

As originally shown for lymphocytic choriomeningitis virus (LCMV) (1–4), viral infection of antigen-presenting cells (APCs) can disrupt their function and subsequent CD8<sup>+</sup> T cell responses (5–8). APCs process viral proteins into small peptide fragments

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Address correspondence to Kim J. Hasenkrug, [khasenkrug@nih.gov](mailto:khasenkrug@nih.gov).

\* Present address: Tyler C. Moore, Bellevue University, Bellevue, Nebraska, USA; Lorena M. Gonzaga, University of Nebraska, Lincoln, Nebraska, USA; Jennifer M. Mather, Bellevue University, Bellevue, Nebraska, USA.

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that are presented to cognate T cells in the context of major histocompatibility complex (MHC) molecules to provide signal 1 for T cell priming. Completion of T cell priming requires a second signal such as the ligation of T cell CD28 by the costimulatory molecule CD86 and/or CD80 on the APC (9–11). Cytokine signaling by interleukin-12 (IL-12) and type 1 interferons (IFNs) provides additional stimuli to further the differentiation and development of functional CD8<sup>+</sup> T cells (12). Many viruses have evolved mechanisms to disrupt these activation pathways in order to establish persistent infections. For example, murine gammaherpesvirus-68 (MHV-68) infection of dendritic cells (DCs) downregulates CD86 cells so that they only weakly stimulate CD8<sup>+</sup> T cells (13). Herpesviruses also downregulate MHC class II (MCH-II)-mediated help for CD8<sup>+</sup> T cell responses (14). AKR/Gross murine leukemia virus infection of APCs inhibits antiviral CD8<sup>+</sup> T cells in a Fas/FasL-dependent mechanism (15), and in the retrovirus Friend virus (FV) model, it has been reported that infection of DCs impairs their maturation and their ability to stimulate T helper type 1 responses and preferentially promotes the induction of regulatory T cells (Tregs) over effector cells (16, 17).

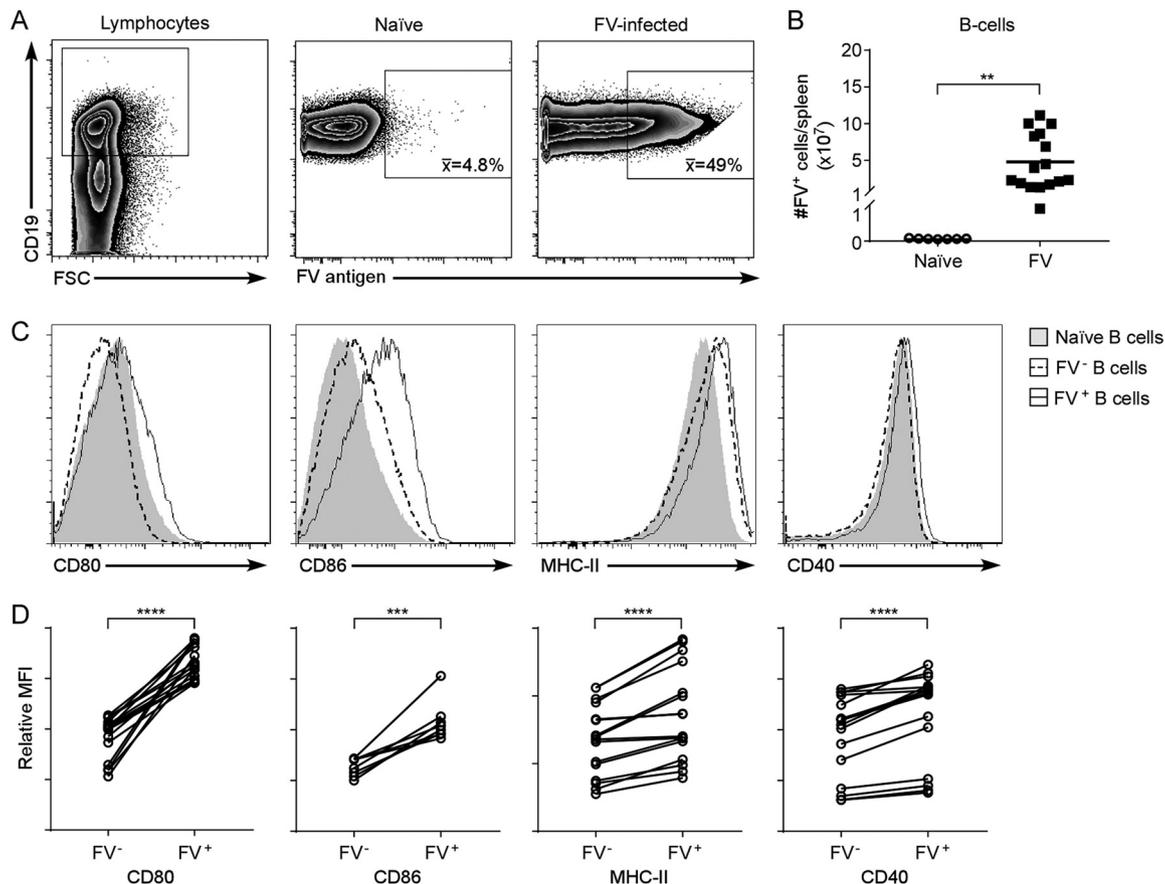
Despite the reported dysfunction of DCs and induction of immunosuppressive Tregs during FV infection (18), mice with a resistant MHC H-2<sup>b/b</sup> haplotype mount vigorous virus-specific CD8<sup>+</sup> T cell responses that are critical for recovery (19–21). Therefore, we investigated whether B cells might be acting as alternative APCs to promote CD8<sup>+</sup> T cell responses. Approximately one-third of splenic lymphocytes in the mouse are B cells, which are infected with FV during the acute phase of infection (22). FV is a gammaretrovirus, which requires active cell division for nuclear integration and productive infection. Thus, the infection of B cells indicates that they are proliferating in response to the FV infection, either through stimulation of their B cell receptors at an antigen-specific level or through nonspecific mechanisms, such as Toll-like receptor (TLR) signaling (23). It has been shown that complement opsonization of FV virions plays a significant role in the uptake of virus by B cells (24), uptake that could trigger TLR signaling. In addition, B cells loaded with complement-opsonized FV virions *in vitro* were able to activate CD8<sup>+</sup> T cells in an antigen-specific manner (24). It was therefore of interest to analyze whether *in vivo* infection of B cells altered their APC function.

Important for APC function is the upregulation of costimulatory molecules, which is influenced by factors including the following: TLR signaling (25); ligation of cytokines such as type I interferons (26), IL-4 (27), and IL-13 (27); signaling through the B cell receptor (28); and signaling through CD40 (27). Not all viral infections disrupt APC function, and in some cases, infection can even induce the upregulation of costimulatory molecules by signaling through pattern recognition receptors (29). This suggests the possibility that *in vivo* infection of B cells could upregulate costimulatory molecules, thereby promoting their APC capacity and ability to prime CD8<sup>+</sup> T cells. To investigate that possibility, we examined how infection of B cells with FV *in vivo* affected their costimulatory molecule expression and their APC function, especially with respect to the activation of cytotoxic CD8<sup>+</sup> T cells, which are critical for control of acute FV infection (30, 31).

In addition to effects from FV infection, we also sought to determine whether B cells might be subject to direct or indirect suppression by CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (Tregs), which are known to be induced during FV infections (32, 33). It has been shown that Tregs directly inhibit the function of cytotoxic CD8<sup>+</sup> T cells (34). Tregs also suppress antibody responses against FV (35), but Treg-mediated effects on B cells as APCs have not yet been studied. Thus, in the present studies, we also examined the influence of Tregs on B cell phenotype and capacity to prime antiviral CD8<sup>+</sup> T cells.

## RESULTS

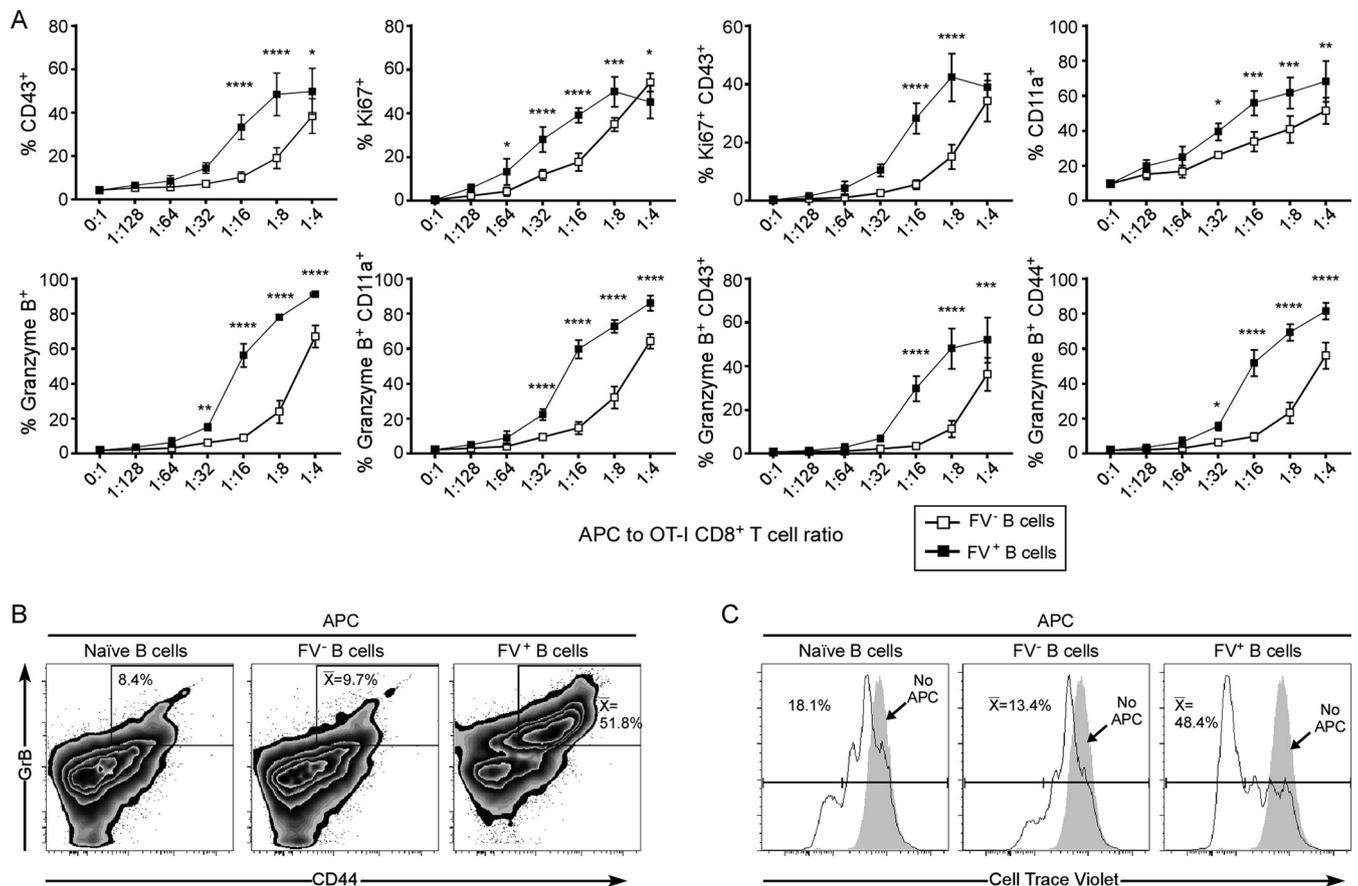
**FV infection of B cells stimulates expression of costimulatory molecules.** The level of FV infection of B cells was examined by flow cytometric detection of surface expression of the viral antigen, glycosylated gag (glycogag), as previously described (22). An example of the gating strategy for B cells and detection of FV glycogag antigen is shown in Fig. 1A. At 5 days postinfection (dpi), an average of 48 million B cells per



**FIG 1** Infected B cells upregulate CD80, CD86, MHC class II, and CD40 during infection with Friend virus. Y10 mice were infected with FV, and at 5 dpi, splenocytes were processed, stained, and analyzed by flow cytometry. (A) Representative FACS plots of FV antigen (FV glycoprotein; MAb 34 staining) on B cells. Live splenic lymphocytes were gated on forward scatter by CD19. The mean percentage of FV<sup>+</sup> B cells among B cells from naive or FV infected mice at 5 dpi is shown. The slight background level of FV<sup>+</sup> B cells in the naive mice is an artifact of the two-step staining procedure requiring an anti-mouse IgG2b secondary stain. (B) Absolute numbers of B cells per spleen that stained positive for surface expression of FV glycoprotein in naive and FV-infected mice at 5 dpi. *n* = 7 and 16, respectively, for two separate experiments. Horizontal lines indicate the mean of each group. \*\*, *P* < 0.01 by unpaired *t* test. (C) Representative histograms of CD80, CD86, MHC class II, and CD40 expression on B cells from naive mice, or FV<sup>-</sup> or FV<sup>+</sup> B cells from FV-infected mice. (D) Comparisons of relative CD80, CD86, MHC class II, and CD40 median fluorescence intensities (MFI) on uninfected (FV<sup>-</sup>) and infected (FV<sup>+</sup>) B cells from FV-infected mice. The cells from individual mice are connected with a line. Each pair of connected dots represents an individual mouse. Data are from two or four pooled experiments. \*\*\*, *P* < 0.001, and \*\*\*\*, *P* < 0.0001, as determined by paired *t* test.

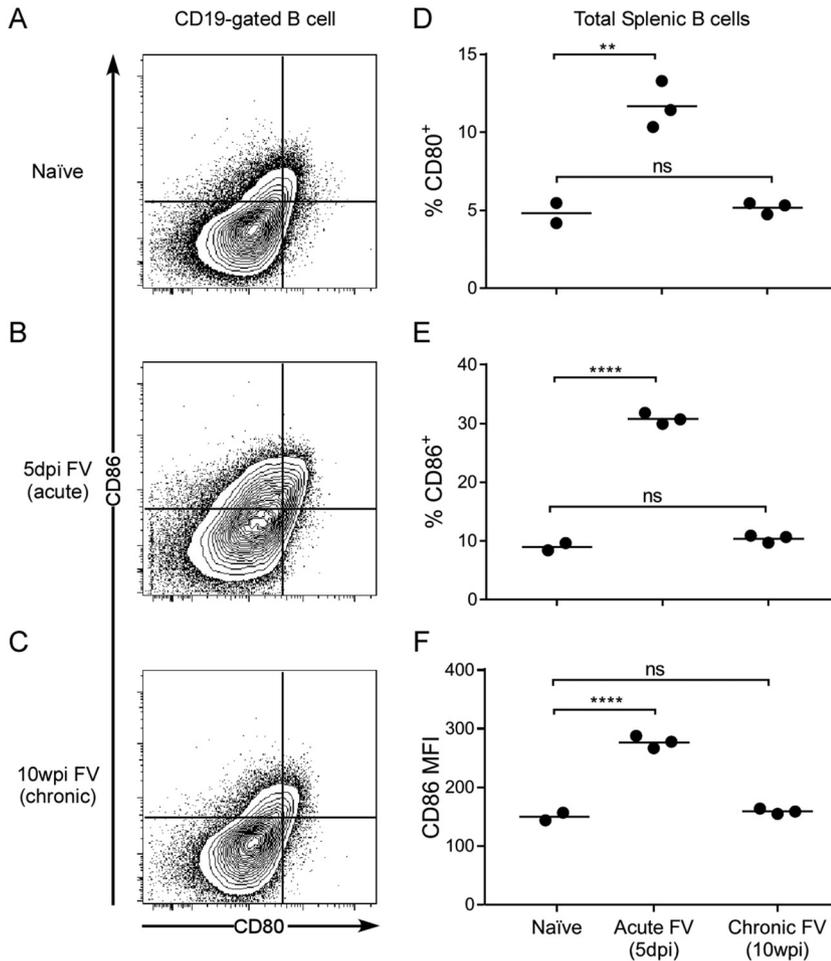
spleen were infected (Fig. 1B). To determine whether FV infection impacted expression of costimulatory molecules, the cell surface expression (median fluorescence intensity [MFI]) of CD80, CD86, MHC class II, and CD40 was examined directly *ex vivo* at 5 dpi. The levels of expression were compared between B cells from naive mice and both the infected and uninfected B cells from FV-infected mice (Fig. 1C). Compared to uninfected B cells, infected B cells most strongly upregulated CD86, but CD80, MHC class II, and CD40 were also slightly but significantly upregulated (Fig. 1D). These results indicated that FV infection of B cells induced increased expression of costimulatory molecules and MHC class II expression, suggesting that FV infection might positively rather than negatively affect APC function.

**FV-infected B cells are better APCs for CD8<sup>+</sup> T cell responses than uninfected B cells.** CD8<sup>+</sup> T cell responses are critical for the resolution of acute FV infection, become primed in a CD4<sup>+</sup> T-cell-independent manner, and require APCs for priming (30, 31). Thus, we next investigated whether infection with FV *in vivo* impacted the ability of B cells to stimulate CD8<sup>+</sup> T cells *in vitro*. At 5 dpi, FV-infected and uninfected B cells were cell sorted based on expression of CD19 for B cells and expression of cell surface FV glycoprotein (monoclonal antibody [MAB] 34) for infection. Because the



**FIG 2** FV-infected B cells are better at priming naive CD8<sup>+</sup> T cells *in vitro*. (A to C) Y10 mice were infected intravenously with 6,000 spleen focus-forming units of FV. At 5 dpi, spleens were harvested and FV antigen (MAb 34)-positive (FV<sup>+</sup>) or antigen-negative (FV<sup>-</sup>) B cells were sorted by FACS to greater than 90% purity, incubated with OVA peptide, and used as APCs for priming of naive OT-I CD8<sup>+</sup> T cells (as described in Materials and Methods). (A) CD43, CD11a, CD44, Ki-67, and GrB expression on OT-I CD8<sup>+</sup> T cells was examined following priming with either no APCs (0:1), uninfected (FV<sup>-</sup>) B cells, or infected (FV<sup>+</sup>) B cells from FV-infected mice at APC/OT-I CD8<sup>+</sup> T cell ratios from 1:128 to 1:4, as indicated. (B) Representative FACS plot of GrB and CD44 expression on OT-I CD8<sup>+</sup> T cells activated by naive B cell APCs, uninfected B cells from FV-infected mice (FV<sup>-</sup> B cells), or infected B cells from FV-infected mice (FV<sup>+</sup> B cells). The numbers in the upper-right corners represent the mean percentages of GrB and CD44 double-positive cells at a 1:16 APC/T cell ratio. (C) Representative FACS histograms showing proliferation of OT-I CD8<sup>+</sup> T cells based on loss of CellTrace Violet stain (as described in Materials and Methods) following activation by naive B cell APCs, uninfected B cells from FV-infected mice (FV<sup>-</sup> B cells), or infected B cells from FV-infected mice (FV<sup>+</sup> B cells). The numbers are mean percentages of cells to the left of the vertical bar in the graph. The shaded histogram represents CellTrace Violet staining of OT-I CD8<sup>+</sup> T cells cultured without any APCs as a no-priming control. The data shown are from one experiment representative of 3 independent experiments with at least 4 mice per group per experiment. In panel A, \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ , and \*\*\*\*,  $P < 0.0001$ , as determined by ordinary one-way analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons. In addition, two-way ANOVA showed statistically significant differences between the curves from infected versus uninfected B cell APCs ( $P < 0.0001$ ).

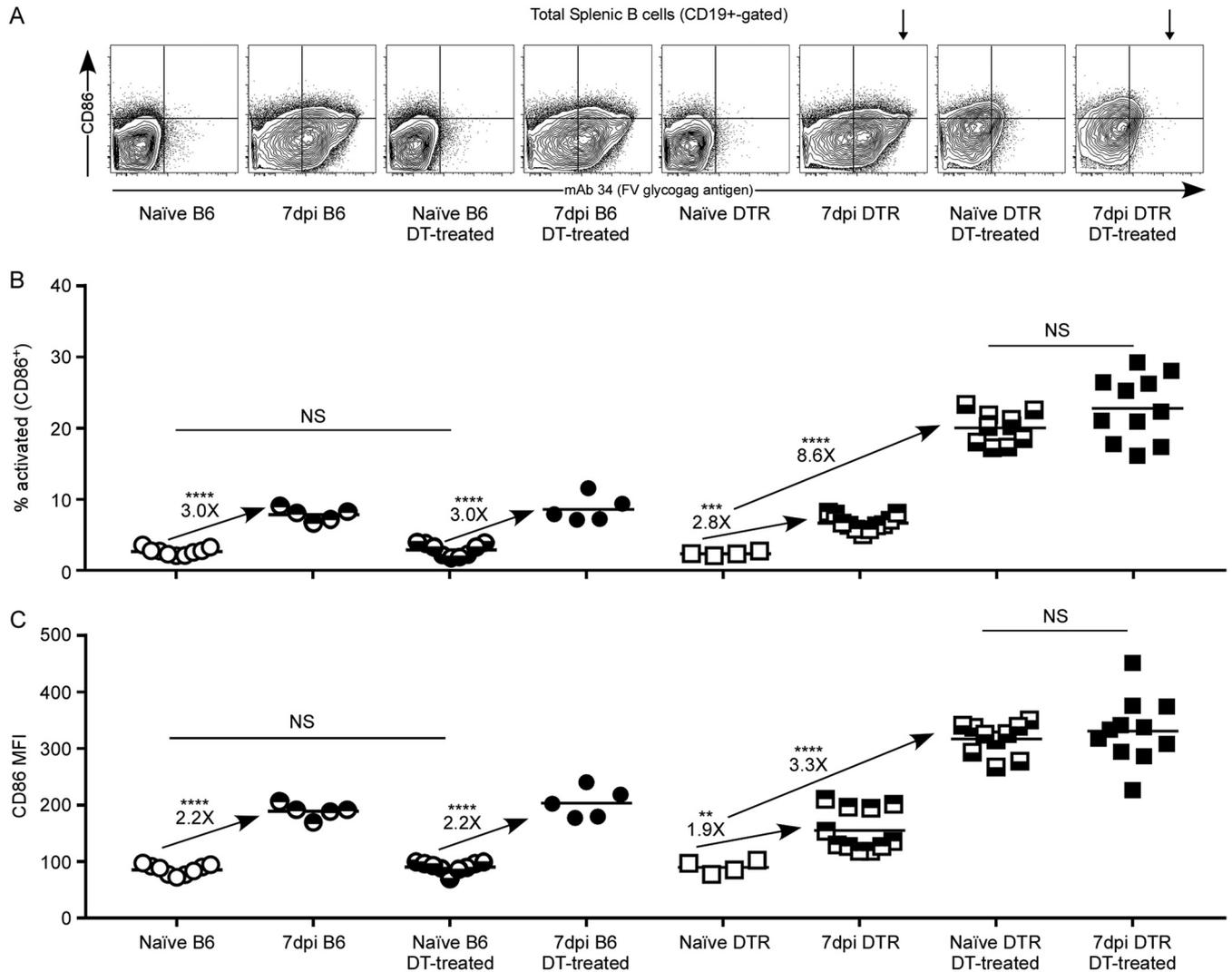
FV<sup>+</sup> B cells would already have FV antigen, whereas FV<sup>-</sup> B cells would not, measurements of FV-specific CD8<sup>+</sup> T cell responses using FV peptide-loaded APCs could be biased. Thus, we switched to the ovalbumin (OVA) SIINFEKL peptide as the APC test antigen and measured the responses of SIINFEKL-specific CD8<sup>+</sup> T cells from OT-I T cell receptor (TCR) transgenic mice using controlled peptide antigen concentrations. Sorted B cells were incubated for 1 h with SIINFEKL peptide and then cocultured with naive OT-I CD8<sup>+</sup> T cells for 3 days at APC/T cell ratios ranging from 1:128 to 1:4 or in the absence of APCs (0:1). The activation of OT-I CD8<sup>+</sup> T cells was determined by flow cytometric measurement of the expression levels of the activation-induced isoform of CD43, CD11a, and CD44, granzyme B (GrB), which is a functional marker for cytotoxicity, and Ki-67, a proliferation marker. For all parameters of CD8<sup>+</sup> T cell activation, as well as coexpression of activation and functional markers, FV-infected B cells were significantly better APCs for OT-I CD8<sup>+</sup> T cells than uninfected B cells from the same mouse (Fig. 2A and B). The uninfected B cells from infected mice performed similarly to B cells from naive mice (Fig. 2B). To confirm the results from the proliferation marker Ki-67, the



**FIG 3** B cell activation during chronic infection. Y10 mice were intravenously infected with ~6,000 SFU of FV, and spleens were harvested for examination of B cell activation by flow cytometric analysis of CD80 and CD86 on total CD19<sup>+</sup> cells at 7 days postinfection and 10 weeks postinfection as indicated. Statistical analysis was done by one-way ANOVA with Tukey's posttest for multiple comparisons. \*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ ; ns, not significant. Panels A to C show representative flow plots for each group, and panels D to F show the cumulative results.

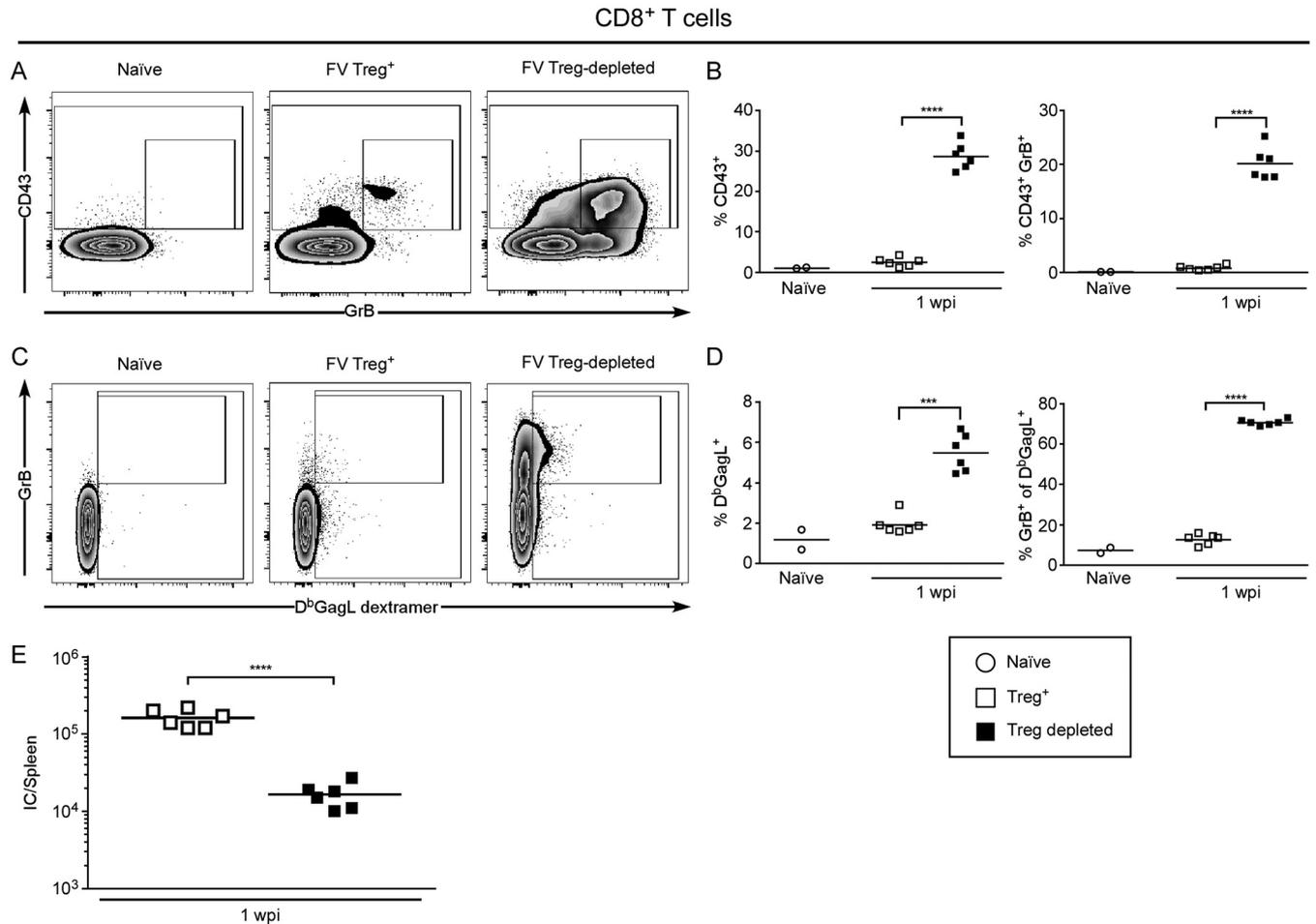
proliferation of the activated OT-I CD8<sup>+</sup> T cells was measured by dilution of CellTrace Violet stain following cell division. In addition to a more activated and functional phenotype, T cells stimulated by FV-infected B cells proliferated significantly more than those stimulated by uninfected B cells (Fig. 2C). Thus, the increased expression of costimulatory molecules on FV-infected B cells (Fig. 1) was associated with significantly better *in vitro* APC activity compared to uninfected B cells (Fig. 2).

**B cell activation during chronic FV infection.** High-recovery mice such as the (B10 × A.BY)F<sub>1</sub> mice used in these experiments resolve acute FV infections but maintain low-level chronic infections for life (36). To determine whether the activation of B cells was sustained through chronic infection, we analyzed CD80 and CD86 activation markers on total splenic B cells. We used total B cells because chronic FV infection levels are too low (as few as 1 in 10<sup>4</sup> to 10<sup>5</sup> splenic B cells) to distinguish infected from uninfected B cells (37, 38). Representative flow cytometry plots are shown in Fig. 3A to C. As expected from previous results, the percentages of activated total B cells in mice acutely infected with FV were significantly higher than in naive mice, both by the proportions of CD80<sup>+</sup> (Fig. 3D) and CD86<sup>+</sup> (Fig. 3E) B cells and by CD86 mean fluorescence intensity (Fig. 3F). The activated phenotype of total B cells was not maintained through chronic infection (Fig. 3D to F), but it remains possible that low numbers of chronically infected B cells remained activated.



**FIG 4** Treg depletion enhances CD86 expression on B cells. (A to C) B6 and B6.FOXP3-DTR-GFP (DTR) mice were left uninfected or were infected with a high dose (~20,000 SFFU) of FV and either treated with DT on days 0, 3, and 6 to deplete Tregs or left untreated. On day 7 postinfection, spleens were harvested and B cells were analyzed by flow cytometry. (A) Representative FACS plots showing CD86 expression versus FV glycogag levels on B cells from uninfected (Naïve) or FV-infected (7dpi) B6 or DTR mice, either untreated or Treg depleted (DT-treated). (B and C) The percentage of (B) CD86<sup>+</sup> and (C) CD86 MFI on B cells from the specified groups of mice. In panels B and C, each symbol represents an individual mouse. Numbers above arrows indicate fold differences between indicated groups. Results are compiled from 3 separate experiments. Lines indicate the mean from each group. \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ , and \*\*\*\*,  $P < 0.0001$ , as determined by one-way ANOVA with Tukey's posttest for multiple comparisons. NS, not significant.

**Treg depletion induces expression of CD86 costimulatory molecules *in vivo*.** It has been shown that FV infections induce Tregs through two distinct mechanisms during early acute infection (18, 39, 40), but no effects on B cell APC function by FV-induced Tregs have yet been reported. To examine this issue, we used B6 mice that expressed the diphtheria toxin receptor (DTR) under the control of the endogenous FOXP3 promoter, such that only Tregs expressed DTR and were subject to depletion by treatment with diphtheria toxin (DT). Of note, these mice do not carry the FV2 susceptibility gene (41), so they do not become as highly infected as the mice used in the previous experiments. B6.DTR and B6 control mice were infected with FV and treated with DT at 0, 3, and 6 dpi to specifically deplete Tregs. Tregs were >90% depleted at 1 week postinfection (wpi) in the DTR mice (data not shown). CD86 was chosen as the marker for APC activation, as previous experiments showed it to be the most sensitive marker and consistent with expression of CD80, MHC-II, and CD40 (Fig. 1C). Total splenic B cells were stained for activation (CD86) and infection (MAb 34), and representative flow cytometric plots from each group are shown (Fig. 4A). As



**FIG 5** Treg depletion during FV infection enhances CD8<sup>+</sup> T cell activation. (A to E) B6.FOXP3-DTR-GFP mice were infected with FV and treated with DT on days 0, 3, and 6 (Treg-depleted), or left untreated (Treg<sup>+</sup>). At 1 week postinfection (wpi), spleens were harvested, CD8<sup>+</sup> T cells were analyzed by flow cytometry, and FV infectious centers (IC) were determined. (A and B) CD8<sup>+</sup> T cell activation as measured by expression of the activation-induced isoform of CD43 and granzyme B (GrB) expression. (C and D) Analysis of CD8<sup>+</sup> T cells specific for FV D<sup>b</sup>-GagL peptides as measured by dextramer binding and activation as measured by GrB expression. The cells in panels B and D, left, were gated on live CD8<sup>+</sup> T cells. The cells in panel D, right, were gated on live dextramer-positive CD8<sup>+</sup> T cells. (A and C) Representative FACS plots. (B and D) Quantification of pooled data. In panels B and D, each dot represents an individual mouse from one of two separate experiments. (E) The numbers of FV infectious centers per spleen were determined at 1 wpi in mice left untreated or depleted of Tregs by treatment with DT on days 0, 3, and 6. Each dot represents an individual mouse. Lines indicate the mean of each group. \*\*\*,  $P < 0.001$ , and \*\*\*\*,  $P < 0.0001$ , as determined by Student's *t* test.

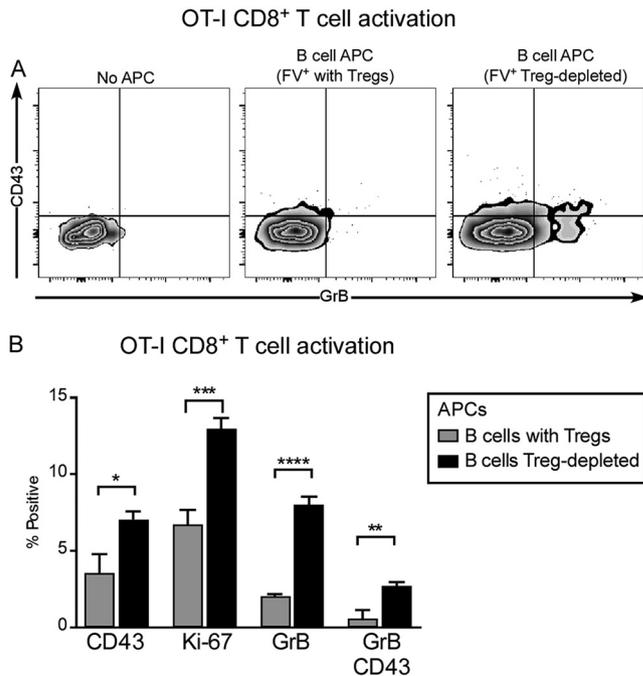
expected in control B6 mice, DT treatments did not deplete Tregs (data not shown) and had no effect on B cell activation (Fig. 4A to C). However, FV infection significantly increased the activation of B cells, as indicated both by increased percentages of CD86<sup>+</sup> B cells (Fig. 4B) and by increased expression levels of CD86 (Fig. 4C). Infection of DTR mice with FV caused an increase in B cell activation—about on par with wild-type B6 mice (Fig. 4B and C). DT treatment (Treg depletion) of DTR mice had a very potent effect on B cell activation, increasing the proportion of activated B cells by 8.6-fold (Fig. 4B) and the CD86 median fluorescence intensity by 3.3-fold (Fig. 4C). FV infection on top of Treg depletion only slightly increased the percentage of activated B cells above Treg depletion alone, and the increase was not statistically significant (Fig. 4B). There was also no significant increase in CD86 MFI when Treg-depleted mice were infected (Fig. 4C). It has previously been shown that Treg depletion strongly enhanced CD8<sup>+</sup> T cell responses and virus clearance (42, 43). As indicated by loss of FV antigen staining with MAb 34, Treg depletion of FV-infected DTR mice strongly reduced FV infection in this experiment also (compare 7-dpi DT-treated and untreated DTR mice, indicated under the arrows in Fig. 4A). There was a corresponding 10-fold decrease in spleen infectious centers as well (Fig. 5E).

**Treg depletion expands the activation and proliferation of CD8<sup>+</sup> T cells in FV-infected mice.** The finding that Treg depletions increased the activation status of B cells made it of interest to determine whether the higher activation of B cells was associated with enhanced CD8<sup>+</sup> T cell activation. As previously demonstrated (43), Treg depletion during acute FV infection of DTR mice strongly enhanced the activation of CD8<sup>+</sup> T cells, as indicated by CD43 expression, GrB expression, or both (Fig. 5A and B). In addition, the proportion of CD8<sup>+</sup> T cells staining with D<sup>b</sup>GagL dextramers (44), which label CD8<sup>+</sup> T cells specific for an immunodominant FV epitope (45), more than doubled in Treg-depleted mice (Fig. 5C and D). Furthermore, the proportion of those FV-specific CD8<sup>+</sup> T cells that expressed granzyme B increased from only about 10% to 70% (Fig. 5D). Thus, Treg depletion not only increased CD8<sup>+</sup> T cell proliferation, but also had a very potent effect on the development of function in the virus-specific CD8<sup>+</sup> T cells. Associated with increased proportions of activated CD8<sup>+</sup> T cells was an approximately 10-fold reduction in mean numbers of infected spleen cells at 7 dpi (Fig. 5E). These values are consistent with previously reported decreases in infection following Treg depletion (42). Studies using combined Treg depletion and CD8<sup>+</sup> T cell depletion have shown that the increased control of FV following Treg depletion was primarily due to increased CD8<sup>+</sup> T cell activity (46). Thus, in addition to direct Treg-mediated suppression of CD8<sup>+</sup> T cells, as shown previously (34), the ability of Tregs to suppress the upregulation of costimulatory CD86 on APCs such as B cells (Fig. 4) could also be an important factor in regulating CD8<sup>+</sup> T cell responses and limiting the rapid control of FV infection. However, the present experiments do not differentiate between direct Treg-mediated effects on CD8<sup>+</sup> T cells and indirect effects mediated through APCs. An obvious experiment would be to test B-cell-deficient or -depleted mice during Treg depletion, but we and others have shown that B cells are required for both homeostatic levels and function of Tregs, as well as Treg responses during FV infection (47–49). Thus, two-way interactions between Tregs and B cells have potent effects on both cell subsets, and testing them independently *in vivo* is not yet feasible.

**B cells from Treg-depleted mice are better APCs for CD8<sup>+</sup> T cells *in vitro*.** To determine whether B cells from Treg-depleted mice were more functional APCs *in vitro*, we performed a priming experiment using OT-I CD8<sup>+</sup> T cells stimulated with total B cells from either FV-infected B6.DTR mice or from FV-infected/Treg-depleted B6.DTR mice. Total B cells from FV-infected mice induced activation of naive CD8<sup>+</sup> T cells (Fig. 6A and B), but B cells from FV-infected/Treg-depleted mice induced significantly more, as measured by expression of CD43, GrB, and the proliferation marker Ki-67 (Fig. 6B). Thus, the dramatic elevation of CD86 expression on B cells from Treg-depleted/FV-infected mice (Fig. 4) was associated with significantly enhanced capacity to prime naive CD8<sup>+</sup> T cells. These results support the idea that in addition to direct effects on CD8<sup>+</sup> T cells, FV-induced Tregs may also indirectly suppress CD8<sup>+</sup> T cell responses by modulating costimulatory molecules on B cells.

## DISCUSSION

These results show that FV infection of B cells induced their activation, based on expression of the costimulatory molecules CD80, CD86, CD40, and MHC-II (Fig. 1). When tested in an *in vitro* priming assay to activate naive CD8<sup>+</sup> T cells, the higher expression of CD80 and CD86 on FV-infected B cells was associated with a greater capacity to prime (Fig. 2). These results are consistent with a study of influenza infection of mice, which showed that virus strains that induced CD86 expression on B cells promoted antiviral CD8<sup>+</sup> T cell responses even in the absence of CD4<sup>+</sup> T cell help (50). In addition, we show that Treg depletion greatly enhanced the expression of CD86 on both infected and uninfected B cells, which was associated with enhanced CD8<sup>+</sup> T cell responses against FV (Fig. 4 and 5). Finally, B cells from Treg-depleted mice more potently primed naive CD8<sup>+</sup> T cells (Fig. 6). Altogether, these results demonstrate that FV infection of B cells enhances their expression of costimulatory molecules and their capacity to prime naive CD8<sup>+</sup> T cells. In contrast, Tregs limited the magnitude of costimulatory molecule expression on B cells and reduced their capacity to prime naive CD8<sup>+</sup> T cells. Although



**FIG 6** B cells from Treg-depleted mice more potently prime naive CD8<sup>+</sup> T cells. (A and B) Naïve or FV-infected B6.FOXP3-DTR-GFP mice were treated with DT (Treg-depleted) or left untreated (Treg<sup>+</sup>). At 7 dpi, spleens were harvested, and CD19<sup>+</sup> B cells were bead purified, incubated with OVA peptide, and used as APCs for naive OT-I CD8<sup>+</sup> T cells as described in Materials and Methods. (A) Representative FACS plots for CD43 and GrB expression by OT-I CD8<sup>+</sup> T cells following priming with B cell APCs from FV-infected, or FV-infected DT-treated mice at a 1:8 APC/T cell ratio. The leftmost panel of OT-I CD8<sup>+</sup> T cells without APCs was used as a no-priming control. (B) Summary of OT-I CD8<sup>+</sup> T cell activation based on percentage of CD43<sup>+</sup>, Ki-67<sup>+</sup>, GrB<sup>+</sup>, or CD43<sup>+</sup> GrB<sup>+</sup>. Bars represent the mean ± standard error of the mean (SEM) from 3 to 8 independent APC/T cell replicates from 5 to 10 pooled mice for APCs or 5 mice for OT-I CD8<sup>+</sup> T cells. In panel B, \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ , and \*\*\*\*,  $P < 0.0001$ , between indicated groups as determined by Student's *t* test.

the role of Tregs in suppressing the APC function of B cells has not been well studied, Treg-mediated suppression of antibody production by B cells has been demonstrated. A subset of Tregs that specifically suppress the follicular T helpers required for T-dependent antibody responses has been described (51–54), and direct suppression of B cell antibody production has also been described both *in vivo* and *in vitro* (55, 56). Furthermore, both mice and humans with genetic Treg deficiencies have highly dysregulated antibody responses (57), including numerous autoantibodies (58).

Our experiments examined the role of B cells as APCs during FV infection, and such a role for B cells in the activation of CD8<sup>+</sup> T cells during FV infections was previously indicated in studies using complement C3-deficient mice (24). In that study, it was demonstrated that FV infection of B cells was significantly enhanced by complement opsonization of the virus. In the absence of complement opsonization, B cell infection was reduced and CD8<sup>+</sup> T cell responses were diminished. A recent study showed that dendritic cells from TLR3 knockout mice failed to upregulate CD86 and had impaired FV-specific antiviral CD8<sup>+</sup> T cell responses (59). Thus, TLR3, a sensor for double-stranded RNA, appears to be important for the activation of FV-infected dendritic cells and their subsequent capacity to activate T cells. Furthermore, FV-infected myeloid cells that were differentiated into DCs (bone marrow-derived dendritic cells [BMDCs]) poorly induced activation of CD4<sup>+</sup> T cells *in vitro* (17). Subsequent studies showed that FV-infected BMDCs (FV-BMDCs) failed to upregulate CD80 or CD86 costimulatory molecules or CD40 or MHC-II. Instead, they preferentially induced Th2 CD4<sup>+</sup> T cells and failed to induce robust CD8<sup>+</sup> T cell activation (16). These results are consistent with other viruses that infect APCs, downregulate expression of costimulatory molecules, and impair their capacity to activate CD8<sup>+</sup> T cells (reviewed in reference 60). In contrast

to the impaired function of FV-BMDCs, our data indicate that FV infection enhances the activation and APC function of B cells. On the other hand, Tregs induced during FV infection appear to limit the activation and APC function of FV-infected B cells (Fig. 3 and 4). In hepatitis C virus (HCV) infections, B cells also become infected, and HCV-infected B cells upregulate CD86 (61). Although B cells from HCV patients appear hyporeactive to mitogenic stimuli, they stimulate *ex vivo* allogeneic T cell responses better than B cells from healthy donors (61). Interestingly, Ayers et al. also showed that B cells from HCV-infected patients enhanced the generation of Tregs *in vitro*. Similarly, the FV-induced activation and expansion of Tregs during *in vivo* infection are dependent on B cells (47).

Impaired CD8<sup>+</sup> T cell responses in the absence of APC costimulation have been shown in numerous models (9–11, 62). These studies support our current findings, which showed that CD86 upregulation following FV infection or Treg depletion was associated with better capacity to prime naive CD8<sup>+</sup> T cells. A recent study found that type I interferon (IFN-I) can partly compensate for lack of costimulatory molecules in priming CD8<sup>+</sup> T cells during viral infection (63). However, FV infection does not induce very strong IFN-I responses (64, 65), so the regulation of costimulatory molecule expression is likely a major factor dictating the magnitude of anti-FV CD8<sup>+</sup> T cell responses. In the presence of Tregs, there was little or no upregulation of CD86 on uninfected B cells from FV-infected mice, indicating that cell extrinsic factors such as cytokines were not involved (Fig. 4B). In contrast, infection of B cells led to significant upregulation of CD86 even in the presence of Tregs. This finding suggests that Treg-mediated suppression of CD86 expression can be partly overcome by positive signaling events that occur in an infected APC. However, Treg depletion significantly enhanced the CD86 expression on both infected and uninfected B cells, indicating that both cellular populations are susceptible to Treg suppression. A recent study found that Tregs can downregulate CD86 expression on B cells, thus, limiting B cell priming of CD4<sup>+</sup> helper T cells and germinal center formation (54). Furthermore, previous results demonstrated that FV-induced Tregs directly or indirectly suppressed antiviral B cell responses (35). Specifically, Treg depletion resulted in elevated CD86 on B cells, increased class switching to IgG, more robust FV-specific antibody titers, and better control of FV viremia. The current results indicate that Treg-mediated suppression of CD86 expression on B cells is also associated with impaired capacity to prime naive CD8<sup>+</sup> T cells. However, FV-infected B cells appear to partly overcome Treg suppression in order to prime antiviral CD8<sup>+</sup> T cell responses essential for recovery (19–21). It is likely that the enhanced APC activity of infected B cells also affects CD4<sup>+</sup> T cell responses as it was previously shown that the clonal diversification of CD4<sup>+</sup> T cells was dependent on B cell activation and APC function (66).

Our results also indicate that CD8<sup>+</sup> T cells during FV infection are susceptible to not only direct suppression by Tregs (34) but also indirect suppression via the effects of Tregs on APCs. Thus, unlike some viruses that establish chronicity by directly disrupting APC function to prevent the CD8<sup>+</sup> T-cell-mediated cytolysis of infected cells, FV has evolved mechanisms to coopt the activity of Tregs, which suppress both APCs and CD8<sup>+</sup> T cells. Altogether, our findings provide new insight into the factors dictating the activation and function of APCs during FV infection.

## MATERIALS AND METHODS

**Mice.** Experiments were conducted using female mice of the (C57BL/10 × A.By)F<sub>1</sub> (abbreviated Y10), C57BL/6-Tg(TcraTcrb)1100Mjb/J (abbreviated OT-I), B6.129(Cg)-*Foxp3tm3(DTR/GFP)Ayr/J* (B6.FOXP3-DTR-GFP [abbreviated FDG]) lines (obtained from Jackson Laboratory and bred at the Rocky Mountain Laboratories, Hamilton, MT) (67). All of the mice were 10 to 20 weeks old at the beginning of the experiments and were treated in accordance with an Institutional Animal Care and Use Committee-approved protocol and the regulations and guidelines of the National Institutes of Health.

**Virus, diphtheria toxin, and injections.** The FV stock used in these experiments was an FV complex containing replication-competent Friend murine leukemia helper retrovirus and replication-defective polycythemia-inducing spleen focus-forming retrovirus (free of lactate dehydrogenase-elevating virus) (22). Mice were infected by intravenous (i.v.) injection of 20,000 spleen focus-forming units (SFFU) (for resistant B6.FOXP3-DTR-GFP mice) or 6,000 SFFU (for Y10) in 0.2 ml via the retroorbital sinus. Tregs were

depleted by injecting mice intraperitoneally (i.p.) with 0.5  $\mu\text{g}$  of DT (Sigma) on 0, 3, and 6 days postinfection. For coculture assays, spleens were harvested at 5 or 7 days postinfection (as indicated in the figures), homogenized, and processed for *in vitro* coculture assays. For analysis of *in vivo* responses, spleens were harvested at 5 or 7 days postinfection, homogenized, processed, and stained for flow cytometric analysis following red blood cell lysis with ammonium chloride potassium (ACK) for 5 min.

**In vitro coculture assays.** B cells from naive mice or FV-infected mice were purified by CD19<sup>+</sup> magnetically activated cell sorting (MACS) columns per the manufacturer's recommendations followed by fluorescence-activated cell sorter (FACS) sorting to purify CD19<sup>+</sup> MAb 34<sup>-</sup> cells or to enrich for CD19<sup>+</sup> MAb 34<sup>+</sup> cells. B cells were cultured for 1 h at 37°C with 1  $\mu\text{g}/\text{ml}$  SIINFEKL OVA peptide. Following peptide loading, B cells were washed to remove excess OVA peptide. T cells were harvested from naive female OT-I mice, purified by CD8<sup>+</sup> MACS column (Miltenyi Biotec), and labeled with CellTrace Violet (Thermo Fisher Scientific) according to the manufacturers' recommendations. Because differences in APC function could be lost if APCs are in excess, B cells and CD8<sup>+</sup> T cells were cocultured at B cell/CD8<sup>+</sup> T cell ratios from 1:128 to 1:4 and cultured for 3 days at 37°C. CD8<sup>+</sup> T cells cultured in the absence of APCs were used as a negative control (0:1 ratio).

**Surface and intracellular antibody staining and flow cytometry.** The following antibodies were used for cell surface staining: FV glycogag was stained with MAb 34 followed by anti-mouse IgG2b conjugated with fluorescein isothiocyanate (FITC) (R12-3; BD Biosciences). All other antibodies were fluorescently labeled: anti-CD8a conjugated with Pacific Blue or Alexa Fluor 700 (53-6.7), anti-CD40 conjugated with BV711 (3/23), and anti-I-A/I-E conjugated with BV786 (MHC class II) (2G9) from BD Biosciences; anti-CD19 conjugated with phycoerythrin (PE) CF594 or Alexa Fluor 700 (1D3), anti-CD80 conjugated with V450 (16-10A1), anti-CD86 conjugated with PE or Alexa Fluor 700 (G11), anti-CD43 conjugated with PE-Cy7 (1B11), anti-CD11a conjugated with FITC (LFA1), and anti-CD44 conjugated with Alexa Fluor 700 (1M7) from Biologend; or D<sup>b</sup>-GagL dextramer conjugated with PE from Immudex. Following surface staining, intracellular staining was performed with the FXP3 permeabilization and fixation kit from eBiosciences according to the manufacturer's recommendations using the following antibodies: anti-Ki-67 conjugated with peridinin chlorophyll protein (PerCP)-Cy5.5 (Sola15; BD Biosciences) and anti-granzyme B conjugated with allophycocyanin (GB11; Invitrogen). Lymphocyte populations were initially gated on the basis of forward scatter versus side scatter. Flow cytometric data were collected with an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star). FACS sorting was performed on a FACSAria (BD Biosciences).

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