Regulatory T Cells Resist Virus Infection-Induced Apoptosis

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ABSTRACT

Regulatory T (Treg) cells are important in the maintenance of self-tolerance, and the depletion of Treg cells correlates with auto-immune development. It has been shown that type I interferon (IFN) responses induced early in the infection of mice can drive memory (CD44hi) CD8 and CD4 T cells into apoptosis, and we questioned here whether the apoptosis of CD44-expressing Treg cells might be involved in the infection-associated autoimmune development. Instead, we found that Treg cells were much more resistant to apoptosis than CD44hi CD8 and CD4 T cells at days 2 to 3 after lymphocytic choriomeningitis virus infection, when type I IFN levels are high. The infection caused a downregulation of the interleukin-7 (IL-7) receptor, needed for survival of conventional T cells, while increasing on Treg cells the expression of the high-affinity IL-2 receptor, needed for STAT5-dependent survival of Treg cells. The stably maintained Treg cells early during infection may explain the relatively low incidence of autoimmune manifestations among infected patients.

IMPORTANCE

Autoimmune diseases are controlled in part by regulatory T cells (Treg) and are thought to sometimes be initiated by viral infections. We tested the hypothesis that Treg may die off at early stages of infection, when virus-induced factors kill other lymphocyte types. Instead, we found that Treg resisted this cell death, perhaps reducing the tendency of viral infections to cause immune dysfunction and induce autoimmunity.

Regulatory T (Treg) cells play an important role in maintaining self-tolerance and limiting an overactive immune response. Immunosuppressive "natural" Treg cells develop in the thymus and are marked by the expression of CD4 and a Forkhead/winged-helix family member, forkhead box P3 (Foxp3; Scurfin), which is an activator and a repressor of transcriptional control (1). Scurfy mice, which have a 2-bp insertion in their exon 8 of the X-linked Foxp3 gene (2), develop a lymphoproliferative disorder and perivascular infiltration of hematopoietic cells in multiple peripheral organs (3). Depletion of the Foxp3-expressing population in mice can lead to autoimmune disorders (4–6). Under the transcriptional control of Foxp3, Treg cells express a high level of interleukin-2 (IL-2) receptor α (CD25) and a low level of IL-7 receptor α (CD127) on the cell surface (7). Treg cells do not produce IL-2 or tumor necrosis factor (TNF) but instead depend on common γ chain cytokines from other cells for survival (7, 8).

CD8 and CD4 T cells, particularly those of the memory phenotype, undergo apoptosis and decline in number early (days 2 to 4) during viral (9–11) and some bacterial (12) infections. This attrition is mediated in part by type I interferon (IFN) and occurs after infection with type I IFN-inducing pathogens or after treatment of mice with the type I IFN-inducer poly(I·C). This attrition and apoptosis can be blocked by antibody to type I IFN (13) and is dramatically reduced in type I IFN receptor (IFNAR) knockout (KO) mice (9, 14). Memory CD8 and CD4 T cells express high levels of CD44, and these CD44hi cells show the highest levels of apoptotic loss at the early stage of infection, perhaps making room for a new immune response to rapidly develop (14).

Infection has been implicated as a potential trigger in autoimmune diseases (15–18), but underlying mechanisms have yet to be clarified. One of the theories is that infection may disrupt the balance of immune regulation, perhaps through Treg cells, in the susceptible organs (18, 19). Foxp3+ CD4+ Treg cells exhibit a partial memory phenotype with an intermediate to high expression of the activation and memory marker CD44 (20). Therefore, we questioned whether Treg cells may behave like the CD44hi CD4 and CD44hi CD8 T cells and get driven into apoptosis and decline in number during early stages of infection. If so, their demise might contribute to the phenomenon of virus-induced autoimmunity.

MATERIALS AND METHODS

Mice and virus stocks. Foxp3-GFP knock-in mice, a kind gift from Vijay K. Kuchroo (21), were bred and maintained in a specific-pathogen-free facility at the University of Massachusetts Medical School (UMMS) (Worcester, MA). C57BL/6 mice between 4 to 6 weeks of age were purchased from the Jackson Laboratory. Experiments were done when mice reached at least 6 to 7 weeks of age.

Lymphocytic choriomeningitis virus (LCMV) strain Armstrong was propagated in baby hamster kidney BHK21 cells (22, 23). Mice were inoculated intraperitoneally (i.p.) with 5×10^4 PFU of LCMV stock. Injection with supernatant from uninfected BHK21 cell cultures (BHK) was used as a sham control in some experiments. Experiments were done in compliance with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical

research, according to protocols approved by the Institutional Animal Care and Use Committee of UMMS.

Surface staining. Splenocytes in suspension were washed in staining buffer (1% fetal calf serum [FCS] in phosphate-buffered saline [PBS]), blocked with anti-CD16/32 monoclonal antibody (MAb; clone 2.4G2; Fc block), stained with anti-CD4 (clone RM4-5), anti-CD8α (clone 53-6.7), anti-CD8β (clone YTS156.7.7), anti-CD44 (clone IM7), anti-CD25 (clone PC61.5), anti-CD127 (clone A7R34), anti-CD122 (clone TM-β1), anti-CD132 (clone TUGm2), and anti-Thy1.2 (clone 30-H12) antibodies, and Live/Dead Fixable Aqua (Invitrogen) for 20 min at 4°C, and fixed with CytoFix (BD Biosciences) for 5 min at 4°C. Samples were resuspended in staining buffer until reading by an LSRII flow cytometer (BD Biosciences). Collected data were analyzed by FlowJo software (Tree Star).

TUNEL assay. To evaluate DNA fragmentation, cells were rested in medium (10% FCS in RPMI 1640 with 2 mM penicillin-streptomycin and 2 mM L-glutamine; Gibco) supplemented with 10 mM HEPES in a 48-well plate for 5 h at 37°C and 5% CO_2, Fc blocked, surface stained, and subjected to CytoFix for 15 min at 4°C. After permeabilization with 70% (vol/vol) ethanol for 3.5 days at −20°C, terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling (TUNEL) was performed using the Apo-BrdU-Red *In Situ* DNA Fragmentation Assay kit (BioVision) according to the manufacturer's instructions. Samples were analyzed within 3 h by LSRII.

Annexin V staining. Annexin V staining was done directly *ex vivo*. After the Fc block, surface staining, and one wash with annexin V binding buffer (eBioscience), cells were stained with annexin V (eBioscience) at room temperature for 15 min. Samples were analyzed immediately by LSRII.

PhosFlow staining. Splenocytes in suspension were stimulated with IL-2 (5 ng/ml; BD Biosciences) for 15 min at 37°C, 5% CO_2. After a quick wash in staining buffer, cells were immediately fixed with CytoFix for 15 min at 4°C, Fc blocked, and surface stained as described above. Cells were then permeabilized with PhosFlow Perm buffer III (BD Biosciences) for 30 min on ice and stained with anti-STAT5 (clone 3H7) or anti-pSTAT5 (clone pY694) for 60 min at room temperature in the dark. Samples were resuspended in staining buffer until analysis by LSRII.

Statistical analysis. Student's *t* test was calculated using Excel or Prism software. Data are presented as means and standard errors of the means (SEM).

RESULTS

Insignificant reduction of Foxp3⁺ CD4⁺ Treg cells after LCMV infection. As an initial screen to determine if there was a loss in Treg cells during viral infection, C57BL/6 mice were infected intraperitoneally with strain Armstrong of LCMV and examined for CD8, CD4, and Treg cell phenotype and number. In a time course study, mice were examined every 4 days for 2 weeks and then every 7 days for 2 more weeks to determine the stability of the Treg cell population. Figure 1 depicts the frequency (Fig. 1a) and total number (Fig. 1b) of Foxp3⁺ CD4⁺ Treg cells as well as CD8 and non-Treg CD4 T cells in the spleen at these different time points. It shows the well-established dramatic rise of the CD8 T cells versus the more stable CD4 T cell number, reflecting the reversal of the CD4 to CD8 T cell ratio during an acute viral infection. The Treg population, however, remained relatively stable during this 4-week time period. This stability of the Treg population was also seen when Treg numbers were separately examined at 0, 2, and 3 days postinfection in many additional experiments as described below.

Normally, at early stages (days 2 to 3) of viral infection, there is a substantial type 1 IFN-induced apoptosis-driven loss in CD8 and CD4 T cells expressing the CD44hi memory phenotype. To examine whether Treg cells, which express intermediate to high
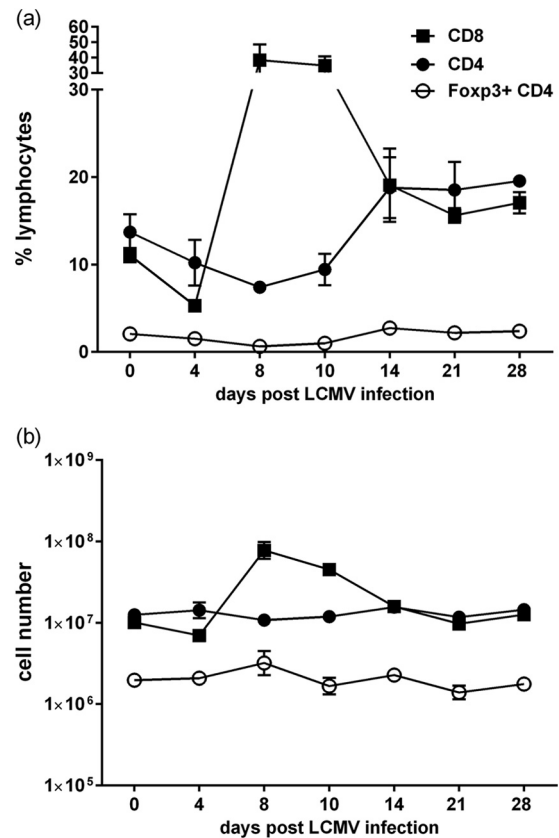

FIG 1 Relatively stable Foxp3⁺ CD4⁺ Treg cell numbers during LCMV infection. C57BL/6 mice were infected i.p. with 5 × 10⁴ PFU of LCMV strain Armstrong, and the spleen was examined every 4 days over 4 weeks for the frequency (a) and total number (b) of CD8 T, Foxp3⁺ CD4⁺ Treg, and Foxp3-negative CD4 T cells. Each time point represents 3 to 14 mice.

levels of CD44, are resistant to apoptosis at these very early time points, we examined their apoptosis and attrition in more detail. LCMV induces type I IFN, which peaks at day 2 postinfection (24). Splenocytes from infected Foxp3-GFP knock-in mice and age-matched naive controls were harvested at this type I IFN peak 2 days postinfection and analyzed by flow cytometry (Fig. 2). While the frequency of Foxp3-negative CD44hi CD4 and CD44hi CD8 T cells among lymphocytes decreased considerably after LCMV infection, the frequency of Foxp3⁺ CD4 T cells was only slightly reduced (Fig. 2b), and the frequency of Foxp3⁺ CD4 T cells among CD4 T cells before and after LCMV infection was not significantly different (11.6% ± 0.75% versus 10.7% ± 2.1%, P = 0.24). The actual number of T cells in the spleen was then calculated for each individual mouse using their total splenocyte counts separately. As expected, CD44hi CD4 and CD44hi CD8 T cells declined significantly in number (Fig. 2c). However, the total number of Foxp3⁺ CD4 T cells did not significantly change after infection (Fig. 2c). At day 3 following LCMV infection, a small but consistent decline in the frequency of Foxp3⁺ CD4 T cells was observed in the spleen (Fig. 2d), but the total number of Foxp3⁺ CD4 T cells in the spleen of infected animals was not significantly different from that of naive animals (Fig. 2e). Therefore, the presence of Foxp3⁺ CD4 T cells in the spleen is relatively stable early during LCMV infection.

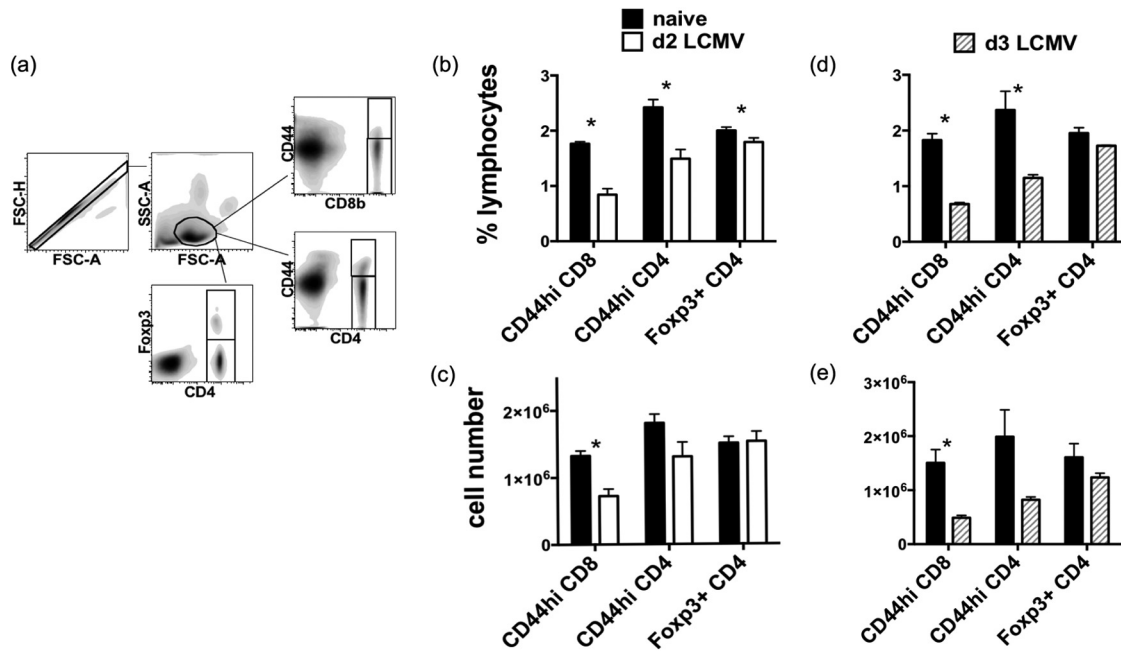Infection can cause Foxp3⁺ CD4 T cells to traffic between the

FIG 2 Foxp3$^+$ CD4$^+$ Treg cells resist loss after LCMV infection in comparison to CD44hi CD8 and CD44hi Foxp3-negative CD4 T cells. Foxp3-GFP mice were infected i.p. with 5 × 10^4 PFU LCMV. Two (b and c) or 3 (d and e) days later, surface-stained splenocytes from infected and uninfected (naive) mice were analyzed by flow cytometry, with gating as shown in panel a. (b and d) The frequencies of CD8 T cells and Foxp3$^+$ CD4$^+$ Treg cells were obtained by direct gating, while the frequency of Foxp3-negative CD4 T cells was calculated by Boolean gating in FlowJo. (c and e) The absolute numbers of the different populations were then calculated from the total live splenocyte counts. (b and c) Combined data from 3 separate experiments ($n = 9$). (d and e) Representative data from seven separate experiments ($n = 3$). *, statistical significance ($P < 0.05$) between naive and LCMV-infected groups.

secondary lymphoid organs. Therefore, the Treg cells from inguinal, axillary/brachial, and mesenteric lymph nodes were examined after LCMV infection, and we found no significant changes in the number and frequency of Treg cells at days 2 and 3 postinfection (data not shown).

Foxp3$^+$ CD4 T cells are resistant to apoptosis during LCMV infection. Cell death by apoptosis is a coordinated process that ultimately leads to DNA fragmentation. The 3′-OH end of the fragmented or nicked DNA can be visualized by TUNEL assay, which covalently attaches a dUTP to the 3′-OH end. Subsequent labeling with a fluorescence-conjugated anti-dUTP antibody allows quantitative assessment of DNA fragmentation by flow cytometry. A previous study showed that both CD44hi CD8 and CD44hi CD4 T cells in the spleen were more TUNEL positive after poly(I·C) treatment (11). Infection with LCMV also increases the frequency of TUNEL-positive CD44hi CD8 T cells in the spleen early after infection (11), and tissue sections have shown two peaks of TUNEL-positive splenocytes after LCMV infection: one at day 3, closely following the peak of the type I IFN response, which we examined here, and the other at day 11, as the response enters the contraction phase (25).

To test if Treg cells were sensitive to virus infection-induced apoptosis, splenocytes were harvested from Foxp3-GFP mice at day 2 after LCMV infection, rested in medium at 37°C for 5 h, and stained with the TUNEL assay (Fig. 3). Without infection, CD44hi Foxp3-negative CD4 T cells and Foxp3$^+$ CD4$^+$ Treg cells appeared to be more TUNEL positive than CD8 T cells (Fig. 3a). Infection with LCMV increased the percentage of TUNEL-positive CD4 and CD8 T cells but had no effect on Foxp3$^+$ CD4 T cells. As expected, the increase in apoptotic events among CD44hi T

cells was more dramatic than that among CD44lo T cells after LCMV infection (Fig. 3b). Although Foxp3$^+$ CD4$^+$ Treg cells from naive mice had a measurable TUNEL background, their levels of apoptosis were similar to those of the relatively resistant CD44lo CD4 and CD44lo CD8 T cells at day 2 after LCMV infection, and they were much less apoptotic than CD44hi CD4 and CD44hi CD8 T cells.

Activated caspase disturbs the asymmetry of the plasma membrane, causing exposure of phosphatidylserine on the cell surface (26). Therefore, apoptosis of splenocytes from LCMV-infected Foxp3-GFP mice was also examined *ex vivo* with amine-reactive live/dead stain and annexin V, which binds to exposed phosphatidylserine. Similar to the data from the TUNEL assay, Foxp3-negative CD44hi CD4 T cells were highly annexin V positive, even among cells from naive spleens (Fig. 4). Annexin V reactivity among CD4 and CD8 T cells increased after LCMV infection but remained low among Foxp3$^+$ CD4$^+$ Treg cells.

Similar results were observed at day 3 after LCMV infection. Foxp3$^+$ CD4 T cells from naive animals displayed some TUNEL and annexin V background reactivities, but their staining intensity was not increased by LCMV infection, while CD8- and Foxp3-negative CD4 T cells, especially their CD44hi subsets, became much more TUNEL and annexin V positive after infection (Fig. 5). Both the TUNEL and annexin V data therefore indicate that Foxp3$^+$ CD4$^+$ Treg cells are much more resistant to virus infection-induced apoptosis than CD44hi CD4 and CD44hi CD8 T cells in the spleen.

Survival factors regulating T cell apoptosis. Cellular apoptosis is generally regulated by a balance between intracellular pro- and antiapoptotic proteins and the availability of survival cyto-
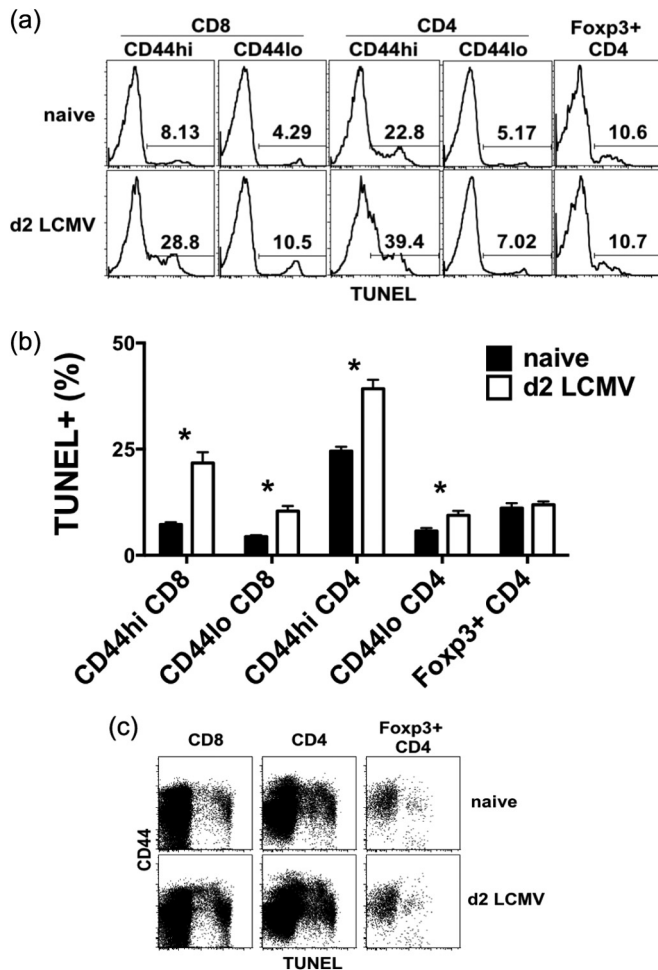
FIG 3 Foxp3$^+$ CD4$^+$ Treg cells are less TUNEL positive than CD44hi Foxp3-negative CD4 and CD44hi CD8 T cells in the spleen at day 2 following LCMV infection. Foxp3-GFP mice were infected i.p. with 5 × 10^4 PFU LCMV. Splenocytes harvested at 2 days postinfection were stained with TUNEL after incubating in medium at 37°C for 5 h. (a) Histograms of TUNEL staining intensity of CD4, CD8, and Treg cells from a naive animal and a day 2 LCMV-infected animal in a representative experiment. (b) Combined data from 3 separate experiments (total $n = 9$). *, statistical significance ($P < 0.05$) between naïve mice and mice at day 2 following LCMV infection. (c) A representative panel of dot plots showing TUNEL reactivity versus CD44 staining among CD4 T cells, CD8 T cells, and Treg cells.

FIG 4 Foxp3$^+$ CD4$^+$ Treg cells are less annexin V positive than CD8 and Foxp3-negative CD4 T cells in the spleen at day 2 following LCMV infection. Foxp3-GFP mice were infected i.p. with 5 × 10^4 PFU LCMV. Splenocytes harvested at 2 days postinfection were stained ex vivo with Live/Dead Fixable stain and annexin V. (a) Histograms of annexin V staining intensity of live CD4, CD8, and Treg cells from a naive animal and a day 2 LCMV-infected animal in a representative experiment. (b) Data are from a representative of three separate experiments ($n = 3$). *, statistical significance ($P < 0.05$) between naive cells and cells at day 2 following LCMV infection. (c) Representative panel of dot plots showing annexin V reactivity versus CD44 staining among CD4 and CD8 T cells and Treg cells.

kines and their receptors. The complete mechanism behind the type I IFN-induced apoptosis of memory T cells during viral infection remains unclear, and it is also not certain if the mechanism of apoptosis for the CD44hi CD8 T cells is the same as that for the CD44hi CD4 T cells. However, it has been shown that the apoptosis of these T cells requires the proapoptotic molecule Bim and that it occurs in T cells lacking CD127, the receptor for the T cell survival factor IL-7 (11, 27). Treg cells are reported to express very high levels of CD25 (7), and their survival is correlated with IL-2-mediated signaling of STAT5 (28, 29). We therefore examined each of these factors in uninfected and day 2 LCMV-activated T cell populations and determined how they correlated with apoptosis.

Although our previous study had shown that the proapoptotic Bim molecule was required for apoptosis of memory T cell popu-
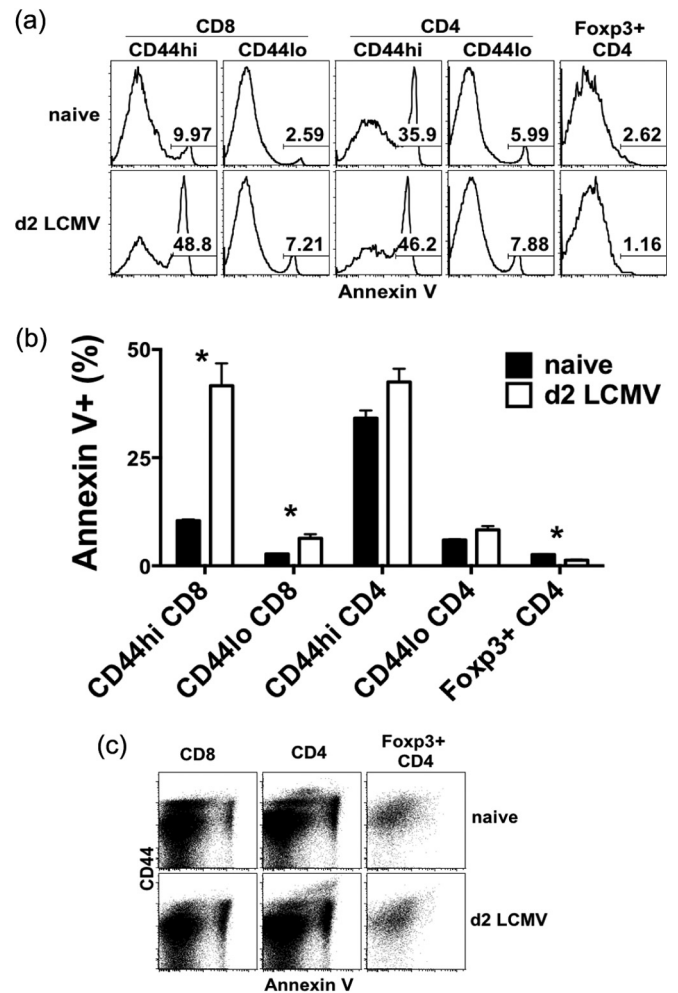
lations (11), examination by flow cytometry found no correlation between proapoptotic Bim or BAK expression and the apoptotic cell populations within the uninfected and LCMV-inoculated naive (CD44lo) and memory (CD44hi) CD4 and CD8 T cell populations and Treg populations. There also was no correlation between expression of the antiapoptotic proteins Bcl-2, Bcl-xL, or Mcl-1 or of the ratio between the pro- and antiapoptotic proteins throughout these five gated cell populations (data not shown).

Failing to find illuminating results by examining pro- and antiapoptotic molecule expression, we focused on the expression of cytokine receptors. It has been shown that IL-7 signaling is critical for the survival of naive (30, 31) and memory (32, 33) T cells in the periphery, whereas IL-2 is important for Treg cell maintenance (34). Therefore, we examined the surface expression of CD127,
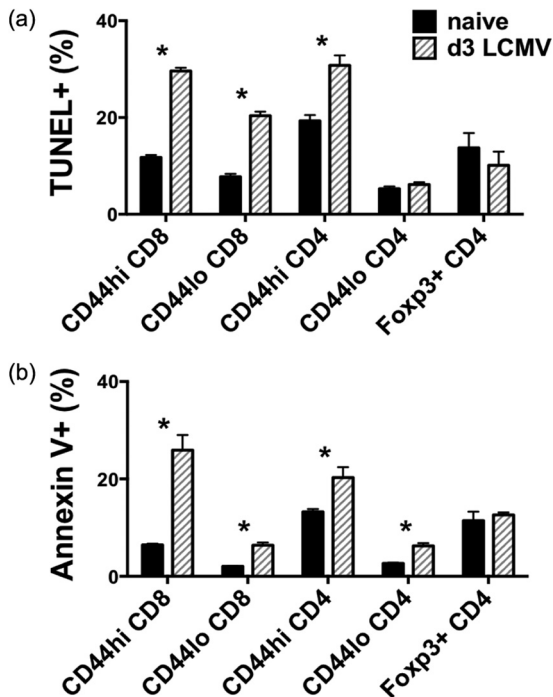
FIG 5 Foxp3$^+$ CD4$^+$ Treg cells are less apoptotic than CD44hi Foxp3-negative CD4 and CD8 T cells in spleen at day 3 following LCMV infection. Foxp3-GFP mice were infected i.p. with 5×10^4 PFU LCMV. Splenocytes harvested at 3 days postinfection were stained with TUNEL after incubating in medium at 37°C for 5 h (a) or *ex vivo* with Live/Dead Fixable stain and annexin V (b). Data are representative of at least three separate experiments ($n = 3$). *, statistical significance ($P < 0.05$) between naive cells and cells at day 3 following LCMV infection.

CD25, IL-2 receptor β (CD122), and the common cytokine receptor γ chain (CD132) on T cells in naive and LCMV-infected Foxp3-GFP mice (where GFP is green fluorescent protein) at day 2 postinfection (Fig. 6). As published previously (35), surface expression of CD127 on Foxp3$^+$ CD4 T cells was much lower than that on Foxp3-negative CD4 T cells in the spleen. CD127 expression on CD44hi Foxp3-negative CD4 T cells was lower than that on CD44hi CD8 T cells (Fig. 6b). After LCMV infection, CD127 was downregulated in all CD8 and CD4 T cells regardless of Foxp3 or CD44 expression status. This downregulation of receptors for this T cell survival cytokine after viral infection may therefore have made conventional CD4 and CD8 T cells more susceptible to apoptosis. Expression of CD25 was high on Foxp3$^+$ CD4$^+$ Treg cells but was negligible on all other CD4 and CD8 T cells (Fig. 6c). LCMV infection, in contrast to its lowering of expression of CD127, upregulated CD25 expression on Treg cells substantially but did not affect the low levels of expression of CD25 on other CD4 or CD8 T cells at day 2 postinfection. As published previously (36), expression of CD122 was the highest on CD44hi CD8 T cells but very low on CD44lo CD4 T cells. We found that Foxp3$^+$ CD4$^+$ Treg cells expressed higher levels of CD122 than Foxp3-negative CD4 T cells and CD44lo CD8 T cells ($P < 0.02$), and CD122 expression was not changed much by LCMV infection (Fig. 6d). All CD4 and CD8 T cells and Treg cells expressed CD132 (Fig. 6e). Although the expression of CD132 was elevated on Foxp3-negative CD4 and CD8 T cells after LCMV infection, it was unchanged on Treg cells. The pattern of receptor expression indicates that

although LCMV infection downregulates the IL-7 receptor on all T cells, the high-affinity IL-2 receptor remains intact or is more highly expressed on Treg cells. This may provide a survival advantage to the Treg cells.

STAT5A and STAT5B mediate IL-2 and IL-7 signaling (28, 37, 38). Phosphorylated STAT5 is involved not only in sustaining Foxp3 expression (39) but also in regulating CD8 T cell homeostasis (40, 41). PhosFlow staining was used to examine the intracellular STAT5 and its phosphorylated form. The percentages of CD4 and CD8 T cells were similar to those obtained by surface staining alone. When the expression levels of STAT5 before and after LCMV infection were compared, we found that the total expression of STAT5 was the highest among Foxp3$^+$ CD4 T cells in naive animals and was marginally increased after LCMV infection (Fig. 7a). In contrast, the total STAT5 expression was slightly reduced among CD8 T cells (both CD44hi and CD44lo) and CD44lo Foxp3-negative CD4 T cells after infection. Consistent with the expression pattern of CD25 (Fig. 6b), only Treg cells were able to respond to IL-2 stimulation *in vitro* and phosphorylate STAT5 (Fig. 7b). A time course experiment also revealed that STAT5 in Foxp3$^+$ CD4 T cells could be phosphorylated in response to IL-2 within seconds of encounter (data not shown). Therefore, the ability of Foxp3$^+$ CD4$^+$ Treg cells to receive survival signals through IL-2 receptor and STAT5 was not diminished early during LCMV infection.

DISCUSSION

Previous work had established that CD44hi CD8 and CD44hi CD4 T cells are driven to apoptosis by virus infection- and poly(I·C)-induced type I IFN (9, 11, 13, 14). The current study extends our knowledge to the Foxp3$^+$ CD4$^+$ natural Treg cells. Treg cells are major regulators of self-reactive T cells and are strong suppressors of autoimmunity. There are several experimental models of virus-induced autoimmunity in animals, and there are epidemiological correlates linking viral infections to the induction or exacerbations of a variety of autoimmune diseases, including diabetes, multiple sclerosis, rheumatoid arthritis, myocarditis, etc. (15, 42). A possible mechanism for virus-induced autoimmunity could be a consequence of the infection-induced apoptosis of Treg cells, because a transient loss of Treg cells is sufficient to initiate an autoimmune disorder (34). However, most of the time viruses do not induce autoimmunity. It is not uncommon to see some self-reactive antibodies generated during infection (43–45), but most of the time the host survives a viral infection without serious autoimmune manifestations. It was, therefore, of interest to determine whether Foxp3$^+$ CD4$^+$ natural Treg cells, present at the beginning of viral infections, were resistant or sensitive to the T cell apoptosis that occurs early during infection. Human Treg cells are more resistant to apoptosis induced by irradiation or a topoisomerase inhibitor than non-Treg CD4 T cells (46), and both murine and human Treg cells are resistant to activation-induced cell death (47, 48). Here we show that Treg cells were substantially more resistant to viral infection-induced apoptosis than were the CD44hi CD4 and CD8 memory T cells with which they coexist. This resistance of Treg cells to apoptosis could be part of the explanation why autoimmunity, which can occur after a virus infection, is relatively rare.

Treg cells might be more resistant to virus infection-induced apoptosis because of their ability to receive signals from IL-2. Both IL-7 and IL-2 have been shown to affect Treg population size in
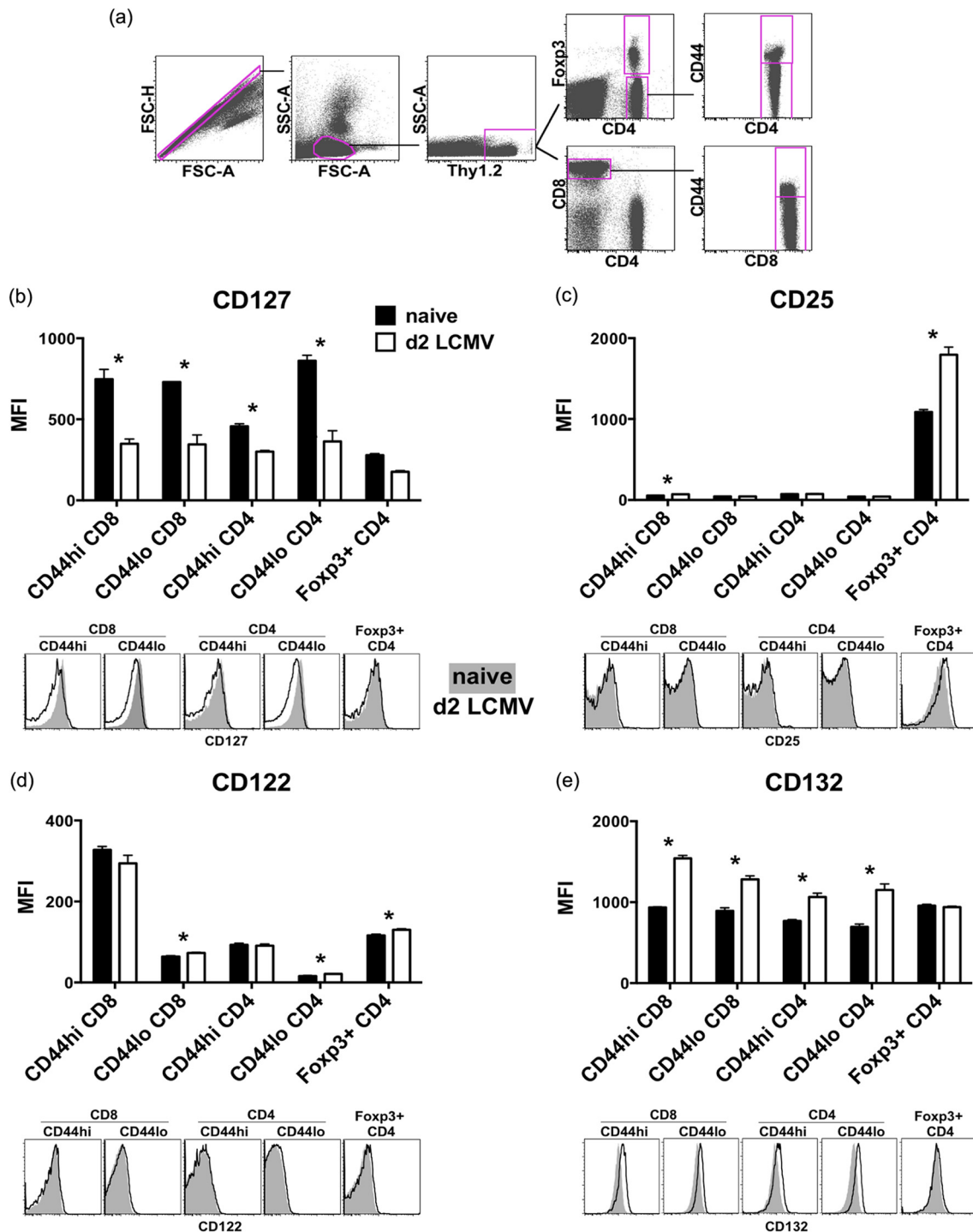
FIG 6 The IL-7 receptor is downregulated on T cells, while the high-affinity IL-2 receptor remains on Foxp3$^+$ CD4$^+$ Treg cells after LCMV infection. Foxp3-GFP mice were infected i.p. with 5×10^4 PFU LCMV. Splenocytes harvested at 2 days postinfection were stained *ex vivo* for CD127 (b), CD25 (c), CD122 (d), and CD132 (e), and the T cell populations were analyzed by flow cytometry (a). Data are representative of at least three separate experiments showing geometric mean fluorescence intensity between naive mice and mice at day 2 following LCMV infection ($n = 3$). *, statistical significance ($P < 0.05$) between naive mice and mice at day 2 following LCMV infection ($n = 3$). Histograms of a representative animal from each group were overlaid for visual comparison.

the periphery through regulating Foxp3 expression (49, 50). Foxp3 as a transcription factor can downregulate the expression of CD127 but upregulate CD25 expression (7), resulting in mature Treg cells being CD127lo and CD25hi. Recent work has also iden-

tified a CD127hi Foxp3$^+$ population in bone marrow and in skin (35), but these were not examined in this study. Upon LCMV infection, CD127 expression was further reduced on Foxp3$^+$ CD4$^+$ Treg cells, but the expression of CD25, CD122, and CD132
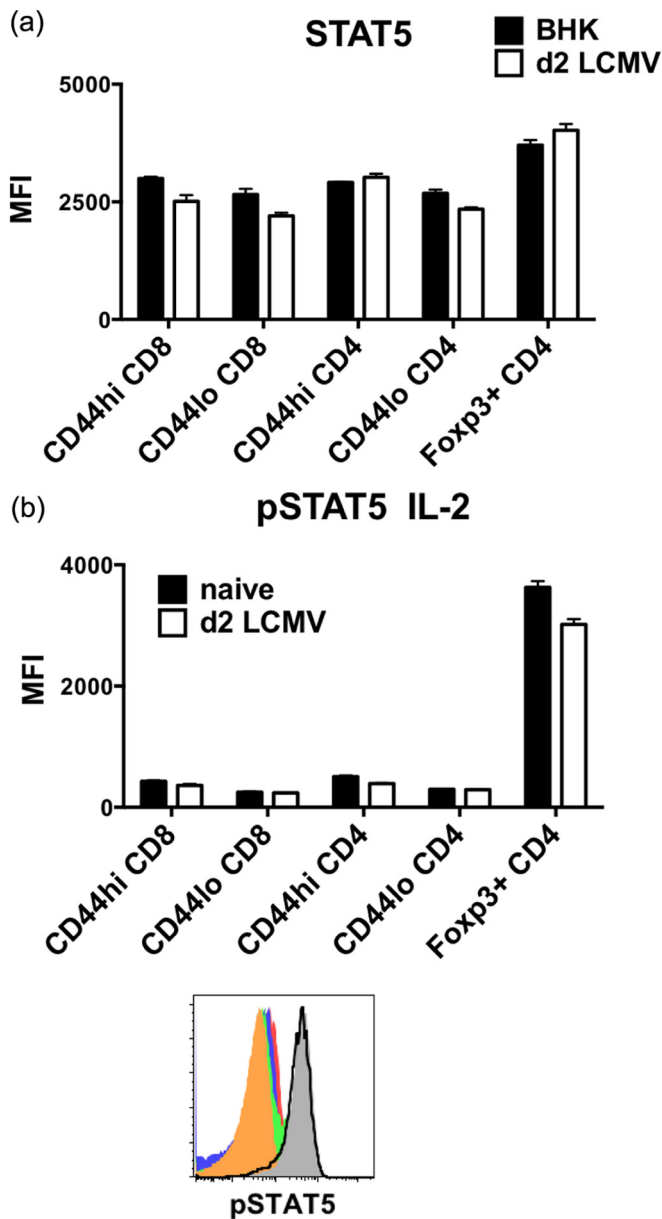
(a)

(b)

FIG 7 Foxp3$^+$ CD4$^+$ Treg cells display the highest level of STAT5 expression and respond well to IL-2 stimulation *in vitro* even after LCMV infection. Foxp3-GFP mice were infected i.p. with 5×10^4 PFU LCMV. (a) Splenocytes harvested at 2 days postinfection were stained *ex vivo* for STAT5. (b) Phosphorylation of STAT5 (pSTAT5) was assessed among CD44hi CD8 (solid red), CD44lo CD8 (solid blue), CD44hi Foxp3-negative CD4 (solid green), CD44lo Foxp3-negative CD4 (solid orange), and Foxp3$^+$ CD4 (solid gray) T cells from a representative naive animal and Foxp3$^+$ CD4 T cells (black line) from a day 2 LCMV-infected animal after stimulating splenocytes with 5 ng/ml IL-2 for 15 min at 37°C. Data are representative of two separate experiments showing geometric mean fluorescence intensity between BHK sham control or naive versus day 2 LCMV-infected groups of 2 or 3 mice.

was either increased or unchanged. The ability to continue to receive survival signals through the IL-2/STAT5 pathway may allow Treg cells to survive and maintain functionality during infection. A high dose (1 mg/mouse) of IL-2-neutralizing antibody has been shown to deplete Foxp3$^+$ CD25$^+$ CD4$^+$ Treg cells over 3 days *in vivo* (34), and the presence of IL-2 (0.1 to 10 ng/ml) has been

shown to protect CD25$^+$ CD4$^+$ Treg cells but not CD25$^-$ conventional CD4 T cells from spontaneous and dexamethasone-induced cell death in a 12-h *in vitro* assay (51). Our preliminary attempt to induce Treg cell death by adding a depleting anti-IL-2 antibody similarly supported the role of IL-2 in Treg cell survival, though more tests are needed to confirm the observation.

The downregulation of CD127 might have made T cells more sensitive to apoptosis. CD127 is thought to be downregulated in response to T cell activation, but CD127 expression on wild-type (WT) donor CD8 T cells is much higher in IFNAR KO hosts than in WT hosts after acute LCMV infection (52). It is noteworthy that the early apoptosis of memory T cells does not occur in LCMV-infected IFNAR KO mice. Thus, type I IFN probably indirectly downregulates CD127 on T cells as it induces their apoptosis. While the expression of CD127 is important for naive and memory CD8 T cell survival, signaling through the IL-7 receptor is not required during the initial proliferative response to an infection or immunization (32). Decreasing the sensitivity of T cells to IL-7 through downregulation of CD127 may be a way to allow the selection and programming of responding T cells by other cytokines to generate specific responses to newly encountered antigens while simultaneously eliminating some preexisting memory T cells.

Two recent reports have examined the effects of type I IFN on Treg cells under inflammatory conditions. One concludes that type I IFN may actually act to maintain Foxp3 expression and Treg activity, a result wholly consistent with our observations of Treg cell resistance to IFN-induced apoptosis (53). A second paper using the LCMV system notes that type I IFN serves to inhibit the proliferation of Treg cells at later stages of infection (54). Clearly, IFN can have many effects on Treg and other types of immune cells, but the early apoptosis of Treg cells does not seem to be one of them.

Previous attempts to decipher the mechanism of memory CD44hi CD8 T cell apoptosis and early attrition induced by type I IFN had not established any connection to NK cells, gamma interferon receptor (IFN-γR), perforin, FasL, granzyme B, TNF-related apoptosis-inducing ligand (TRAIL), TNF receptor 1 (TNFR1), or TNFR2 (9) (K. Bahl and R. M. Welsh, unpublished data). However, Bim is instrumental to the process. In Bim KO mice, the frequency of CD44hi CD8 T cells is relatively stable after LCMV infection, and the CD44hi CD8 T cells are substantially less TUNEL positive at day 3 postinfection with LCMV (11). Bim can physically interact with Bcl-2, Bcl-xL, and Mcl-1 to initiate apoptosis (55, 56). Since Treg cells are more resistant to virus infection-induced early apoptosis, it was expected that the relative abundance of antiapoptotic and proapoptotic molecules might be different between Treg cells and the CD44hi CD8 and CD4 T cells. However, the mean fluorescence intensity (MFI) ratios of Bim to antiapoptotic molecule did not increase with the increased occurrences of apoptosis observed in the CD44hi CD8 and CD4 T cells after LCMV infection (data not shown).

The functions of Treg cells may make them useful to be preserved during infections and yet inevitably problematic in some circumstances. Treg cells play a critical role of regulating effector T cells, NK cells, neutrophils, monocytes, and macrophages early during viral infections, mitigating the pathology incurred by the immune response and prolonging survival of the infected mice (57–60). The presence of virus-specific memory Treg cells can preserve lung function by limiting CD8 T cell response and reduc-

ing pathology during homologous influenza rechallenge (61). However, during heterologous infections, the nature of their persistence may also pose a problem, as demonstrated in the influenza A virus-immune mice during intranasal LCMV challenge, in which the higher number of Treg cells contribute to more-severe immunopathology in the immune mice by preventing exhaustion of responding CD8 T cells and supporting migration of CD8 T cells into the lungs (62).

The association between infection, IFN therapy, and autoimmunity (63–65) had us questioning whether virus-induced type I IFN might drive Treg cells into apoptosis and reduction in number, thereby allowing activation of autoreactive cells. Our study, however, indicates that the Foxp3$^+$ CD4$^+$ natural Treg population is relatively stable early during LCMV infection. Similarly, our preliminary studies with mice treated with type I IFN inducer poly(I·C) for 1 day also showed more stable Foxp3$^+$ Treg cells by TUNEL assay (data not shown), suggesting that type I IFN may not compromise the survival of natural Treg cells. This may explain why the autoimmunity that develops in IFN-treated or infected patients is at a relatively low frequency. Many other factors are involved in the development of autoimmunity, and further investigation will be needed to dissect the specific process for each autoimmune disease.

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