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Early Increases in Superantigen-Specific Foxp3$^+$ Regulatory T Cells during Mouse Mammary Tumor Virus Infection$^{\nabla}$†

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Mouse mammary tumor virus (MMTV) is a milk-borne betaretrovirus that has developed strategies to exploit and subvert the host immune system. Here, we show in a natural model of MMTV infection that the virus causes early and progressive increases in superantigen (SAg)-specific Foxp3$^+$ regulatory T cells (T_{reg}) in Peyer's patches (PP). These increases were shown to be dependent on the presence of dendritic cells. CD4$^+$ CD25$^+$ T cells from the PP of infected mice preferentially suppress the proliferative response of T cells to SAg-expressing antigen-presenting cells ex vivo. We investigated the influence of the depletion of CD25$^+$ cells at different stages of the infection. When CD25$^+$ cells were depleted before MMTV infection, an increase in the number of PP SAg-cognate Foxp3$^-$ T cells was found at day 6 of infection. Since the SAg response is associated with viral amplification, the possibility exists that T_{reg} cells attenuate the increase in viral load at the beginning of the infection. In contrast, depletion of CD25$^+$ cells once the initial SAg response has developed caused a lower viral load, suggesting that at later stages T_{reg} cells may favor viral persistence. Thus, our results indicated that T_{reg} cells play an important and complex role during MMTV infection.

Mouse mammary tumor virus (MMTV) is a betaretrovirus transmitted through milk that causes mammary tumors in mice (7, 32). The MMTV infection model has provided a valuable tool to study how a pathogen can take advantage of the host immune system, and several strategies of virus-host exploitation have been described for this virus (reviewed in references 1 and 13). In neonatal Peyer's patches (PP), MMTV is thought to infect antigen presenting cells (APCs), which then present a virus-encoded superantigen (SAg) to T cells expressing SAg-specific T-cell receptor Vβ chains (9). The resulting interaction is critical since it leads to the amplification of infection in lymphoid cells and induces the proliferation of infected B cells (21). Infected lymphocytes then carry MMTV to the mammary gland, allowing the virus to be passed to the next generation (1, 13). In addition, it has been demonstrated that MMTV interacts with Toll-like receptor 4 (38), and we have shown that this interaction induces recruitment of dendritic cells (DCs) to the PP and increases the expression of the MMTV cell entry receptor on DCs in vivo (8). We have also reported that DCs are capable of producing infectious virus that can be transmitted to other cell types (11). It has also been reported that MMTV can subvert the host immune system by inducing Toll-like receptor 4-dependent secretion of interleukin-10 by splenic B cells (24). However, the involvement of Foxp3$^+$ regulatory T (T_{reg}) cells during MMTV infection has not been studied.

Since the CD4$^+$ CD25$^+$ T-cell population with regulatory capacity was characterized in 1995 (42), numerous reports have postulated the existence of different subsets of regulatory cells (43). The transcription factor Foxp3 is currently considered the most selective marker for CD4$^+$ CD25$^+$ T_{reg} cells (27, 53). CD4$^+$ CD25$^+$ Foxp3$^+$ cells develop either in the thymus or in the periphery and are called "natural" or "adaptive" T_{reg} cells, respectively. Natural T_{reg} cells migrate from the thymus and constitute 5 to 10% of peripheral CD4$^+$ T cells in mice, while adaptive T_{reg} cells are converted from CD4$^+$ CD25$^-$ Foxp3$^-$ T cells under appropriate conditions (43). To date, there is a lack of specific markers to distinguish between these two populations. The T-cell receptor specificity of T_{reg} cells has been controversial since their initial characterization. At first it was assumed that these cells recognized self-antigens, but accumulating evidence suggests that T_{reg} cells are involved in responses to foreign antigens and may function to keep immune responses in check after initial recognition of such antigens (2, 6, 28, 45). In support of this, it has been recently reported that non-self antigens are the cognate specificities of Foxp3$^+$ T_{reg} cells (36). The specificity of T_{reg} cells with regard to their suppressor-effector function is another debated issue. While initial reports postulated that T_{reg} cells activated through their T-cell receptor suppressed immune responses in a nonspecific manner, recent studies suggest that these cells may suppress with specificity both in vivo and in vitro (23, 34, 35, 49).

Recent reports have suggested that T_{reg}-cell induction may be a strategy used by some pathogens to establish and maintain infection (6, 14, 30, 31, 40, 46). As MMTV is known to exploit the immune system, the aim of this study was to investigate whether MMTV causes early alterations in PP CD4$^+$ Foxp3$^+$

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T_{reg} populations and to determine the role of these cells during milk-borne MMTV infection.

MATERIALS AND METHODS

Mice. The following strains of mice were used: BALB/cJ (*H-2^d Mtv7$^-$*) mice; MMTV(LA)-infected BALB/cJ mice (20) carrying three different MMTV exogenous viruses, BALB6, BALB2, and BALB14, encoding SAgs with major specificities for Vβ6, Vβ2, and Vβ14 T cells, respectively; MMTV(BALB6)-infected BALB/cJ mice; C3H/HeN (*H-2^k Mtv-7$^-$*) mice; and AKR/J (*H-2^k Mtv-7$^+$*) mice. Mice were bred in the animal facility of the Instituto de Leucemia Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina. CD11c-DTR mice [C.FVB-Tg (Itgax-DTR/EGFP) 57Lan/J] (25), originally obtained from Dan Littman, were maintained by crossing with BALB/cJ mice at the University of Pennsylvania, Philadelphia, PA. The mice were housed according to the policies of the University of Pennsylvania and Academia Nacional de Medicina (NIH Guide for the Care and Use of Laboratory Animals) (33). *Mtv-7$^+$* and *Mtv-7$^-$* mice were generated by crossing (BALB/cJ × AKR/J)F_1 × BALB/cJ mice, and their phenotype was determined by fluorescence-activated cell sorting (FACS) analyzing the presence or absence of Vβ6$^+$ T cells. These mice were nursed on MMTV(BALB6)-infected females.

Experimental model. Eight-day-old mice were foster nursed on MMTV-infected mothers for the days indicated in the text or in the figures. The mice were sacrificed, their PP were dissected, and single-cell suspensions were prepared by homogenization through a stainless steel mesh. The cells were washed once with RPMI medium–10% fetal bovine serum. FACS analysis was performed as described below.

DC depletion and infection of CD11c-DTR mice. For systemic DC depletion, CD11c-DTR transgenic mice were injected intraperitoneally with 4 ng/g of body weight diphtheria toxin (DT) (Sigma Chemical Co., St. Louis, MO) 1 day prior to injection with virus.

Virus preparation. MMTV(LA) (20) was isolated from tumor tissue, as previously described (19). Dilutions of purified virus were tested for B-cell and SAg-mediated T-cell activation in vivo, as previously described (20). The maximum dilution of virus giving significant activation in the draining versus nondraining lymph node was used in all the experiments. Fifty microliters of diluted MMTV(LA) virus stock was injected into the right-hind footpad of mice; the left hind footpad served as the uninfected control.

Flow cytometry. Cells were incubated with Fc-γ block antibody (anti-mouse CD16/32; Pharmingen, San Diego, CA) to prevent nonspecific binding of antibodies to Fc-γ receptors. Cells (1×10^6) were stained with the following monoclonal antibodies (Pharmingen) and subjected to FACS analysis: fluorescein isothiocyanate (FITC) or Cy-chrome 5-conjugated anti-CD4 (clone H129.19), allophycocyanin-conjugated anti-CD4 (clone RM4-5), FITC or peridinin chlorophyll protein-conjugated anti-CD25 (clones 7D4 and PC61, respectively), FITC-conjugated anti-Vβ6 (clone RR4-7), and FITC-conjugated anti-Vβ10 (clone B21.5). Intracellular staining of Foxp3 was performed using phycoerythrin-conjugated anti-Foxp3 and the Foxp3 staining buffer set (e-Bioscience) according to the manufacturer's protocol. Cells were acquired on a FACScan or FACSaria cytometer (Becton Dickinson, Mountain View, CA). Data were analyzed by using CELLQUEST software (Becton Dickinson Immunocytometry Systems).

MLR. Mixed lymphocyte reactions (MLR) were performed in round-bottom 96-well culture plates (Corning Costar, Cambridge, MA) by coculturing the following cells purified by magnetic cell sorting beads (Miltenyi Biotec, Germany) according to the manufacturer's protocol for 4 days: 2×10^5 BALB/cJ CD4$^+$ CD25$^-$ cells (responders) labeled with 5,6-carboxifluorescein diacetate succinimidyl ester (CFSE) (Molecular probes, Eugene, OR) and 2×10^5 mitomycin C-treated mononuclear spleen cells from AKR/J or C3H/HeN mice (stimulators). CD4$^+$ CD25$^+$ or CD4$^+$ CD25$^-$ cells obtained from PP of infected or uninfected pups were treated with mitomycin C (16), and different numbers of cells (0.5×10^5, 1.0×10^5, or 1.5×10^5) were added to the MLR mixtures from the beginning. Proliferation of CD4$^+$ responder cells was analyzed by CFSE dilution.

Depletion of CD25$^+$ cells. It has been previously shown that depletion of CD25 cells by anti-CD25 antibody (clone PC61) was short term and reversible (31). For depletion before MMTV infection, 2-day-old BALB/cJ mice were inoculated intraperitoneally (i.p.) for three successive days with 0.1 mg of anti-CD25 antibody (clone PC61); i.p. inoculation with isotype-matched rat monoclonal antibody was used as a control. At day 8 after birth, these mice were foster nursed for 15 days on MMTV(LA)-infected mothers.

For depletion during MMTV infection, 8-day old mice were foster nursed on MMTV(LA)-infected mothers for 6 days, and then half of the littermates were injected i.p. daily with 0.1 mg of anti-CD25 antibody from day 6 to day 8 of infection. These mice were used to compare MMTV infection levels between depleted and nondepleted 31-day-old mice and to cross with CD25-depleted littermates to obtain a second generation of mice. Half of the second generation of mice was depleted of CD25$^+$ T cells from day 6 to day 8 of infection, and MMTV infection levels were compared between depleted and nondepleted 31-day-old mice. The efficiency of depletion was assessed by FACS using anti-CD25 antibody (clone 7D4). A 90% reduction in CD4$^+$ CD25$^+$ cells was achieved with these protocols.

Radioactive PCR. DNA was isolated from spleens and mesenteric lymph nodes of 23- to 31-day-old mice. Semiquantitative radioactive PCR analysis was carried out as follows. (i) For MMTV(LA) amplification, the PCR program consisted of 1 min at 94°C, 40 s at 60°C, and 1 min at 72°C for 38 cycles in PCR buffer containing 250 to 500 ng of DNA, 0.5 U *Taq* polymerase, 0.5 μCi of [α-^{32}P]dATP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, a 0.1 μM concentration of each primer, and a 0.2 mM concentration of each deoxynucleotide in a final volume of 50 μl. (ii) For β-actin amplification, the PCR program consisted of 1 min at 94°C, 60 s at 56°C, and 1 min at 72°C for 33 cycles in the same PCR buffer used for MMTV(LA) amplification. These conditions gave linear DNA amplification. The reactions were exponential up to 35 cycles for β-actin and 40 cycles for the virus before the beginning of the plateau (see supplemental material). The following primers were used: to detect virus, 5′-AATTCGGAGAA CTCGACCTTCC-3′ and 5′-CCCCCATGAGTATATTTGA-3′; to detect the mouse β-actin, 5′-TCATGAAGTGTGACGTTGACATC-3′ and 5′-CCTAGAA GCATTTGCGGTGCAACGATG-3′. The PCR products were analyzed by gel electrophoresis in 30% acrylamide gels. The bands corresponding to virus and β-actin amplification were quantified using Scion Image software.

Statistical analysis. Levels of significance were determined using a two-tailed Student's *t* test, and a confidence level of greater than 95% ($P < 0.05$) was used to establish significance.

RESULTS

MMTV infection induces early increases in PP Foxp3$^+$ T_{reg} cells. In order to analyze alterations in the population of T_{reg} cells in neonatal PP at early times after milk-borne MMTV infection, the expression of CD25 and Foxp3 in CD4$^+$ T cells was studied. FACS analysis indicated that the percentage (data not shown) and absolute number of PP CD4$^+$ CD25$^+$ Foxp3$^+$ T_{reg} cells increased progressively in MMTV(LA)-infected mice during the first 2 weeks of infection (Fig. 1A and B). In addition, FACS analysis showed that CD4$^+$ CD25$^-$ Foxp3$^+$ T cells, which have been described either as proliferating T_{reg} cells (18) or as a T_{reg} cell reservoir (52), increased with trends similar to CD4$^+$ CD25$^+$ Foxp3$^+$ T_{reg} cells (Fig. 1C). Of interest, CD4$^+$ CD25$^+$ Foxp3$^-$ activated/effector T cells showed a significant increase in the percentage (data not shown) and absolute number at day 2 of infection and then diminished markedly from day 3 onwards (Fig. 1D). Finally, the CD4$^+$ CD25$^-$ Foxp3$^-$ T-cell population also increased in percentage (data not shown) and absolute number during the first 2 weeks of infection (Fig. 1E), likely due to the recruitment of naive CD4$^+$ T cells to PP that takes place during MMTV infection, as we have previously reported (12).

These results indicate that MMTV infection induced progressive increases in both the CD4$^+$ CD25$^+$ Foxp3$^+$ and CD4$^+$ CD25$^-$ Foxp3$^+$ populations of T_{reg} cells at least during the first 2 weeks of infection. In contrast, CD4$^+$ CD25$^+$ Foxp3$^-$ activated/effector T cells significantly increased at day 2 of infection but then decreased and were maintained at a lower level from day 3 onward.

MMTV infection induces increases in SAg-specific T_{reg} cells. The early response to the MMTV SAg results in an increase in the percentage of SAg-reactive Vβ-specific T-cell

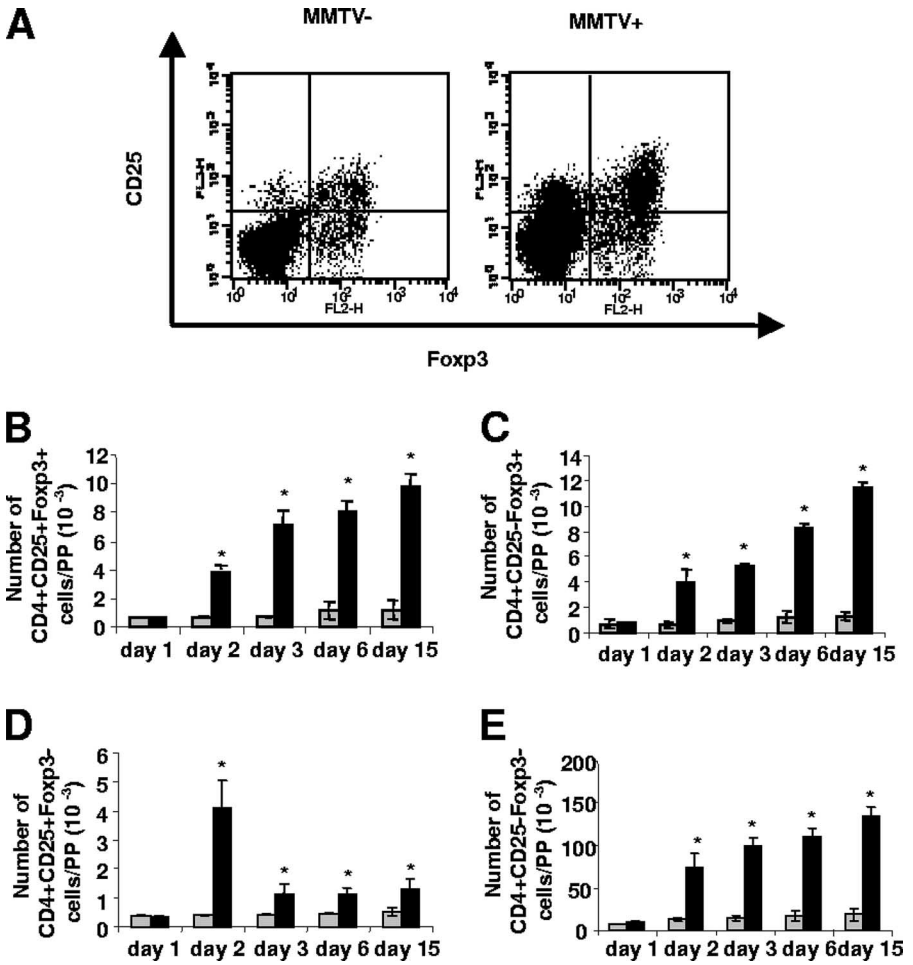

FIG. 1. Changes in PP $CD4^+$ cell populations during the first 2 weeks of MMTV infection. BALB/cJ pups were foster nursed on MMTV(LA)-infected (black bars) or uninfected (gray bars) mothers. Expression of CD4, CD25, and intracellular Foxp3 in PP cells was analyzed by FACS at different days of infection. PP cells were gated for live cells in the forward-sideward scatter (B to E) and for $CD4^+$ cells (A). Cell types are indicated on the *y* axes (B to E). Panel A shows representative dot plots of CD25 and Foxp3 expression in PP $CD4^+$ cells at day 2 of infection. Data in panels B to E are expressed as means ± standard deviations ($n = 4$). *, $P < 0.05$. The experiment was performed three times with similar results.

clones (26). We next addressed whether the $Foxp3^+$ T_{reg} cells that increased in the PP of MMTV-infected neonates were SAg specific. We used BALB/cJ pups infected with MMTV(BALB6), an exogenous virus whose SAg hypervariable region is identical to that of the *Mtv-7* provirus and has primary specificity for Vβ6 T cells (20). We determined that $V\beta6^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ SAg-specific T_{reg} cells increased both in percentage and absolute number from day 2 to day 15 of infection. Figure 2A shows representative dot plots at day 15 of infection; Fig. 2B and C depict the percentages and the absolute numbers. In addition, $V\beta6^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ T_{reg} cells showed increases with similar trends (Fig. 2D and E). Importantly, no significant changes were observed in $V\beta10^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ (Fig. 2F and G) and $V\beta10^+$ $CD4^+$ $CD25^-$ $Foxp3^+$ T cells (Fig. 2A) that were not stimulated by the MMTV(BALB6) SAg. On the other hand, SAg-specific $CD4^+$ $CD25^+$ $Foxp3^-$ activated/effector T cells increased in percentage and absolute number at day two of MMTV(BALB6) infection (Fig. 2I and J) and then decreased with kinetics similar to the total population of $CD4^+$ $CD25^+$ $Foxp3^-$ T cells (Fig. 1D).

Similar results were obtained using the MMTV(LA) virus which carries three different exogenous viruses: BALB6, BALB2, and BALB14 with primary specificities for $V\beta6^+$, $V\beta2^+$, and $V\beta14^+$ T cells, respectively (20). Figure 2H shows representative results indicating that T_{reg} cells were significantly overrepresented within the SAg-cognate but not in SAg-nonreactive T-cell populations during infection.

Taken together, these results showed that MMTV infection induced early and progressive increases in SAg-cognate T_{reg} cells. In addition, since only SAg-reactive T_{reg} cells showed significant increases, these data suggest that the increases in T_{reg} cells were mainly due to increases in SAg-reactive T cells. To further confirm this, we employed neonatal mice in which MMTV(BALB6) SAg-reactive T cells were deleted by the presence of the endogenous *Mtv-7* provirus. *Mtv-7*$^-$ and *Mtv-7*$^+$ mice were generated as described in Materials and Methods and then foster nursed on MMTV(BALB6)-infected

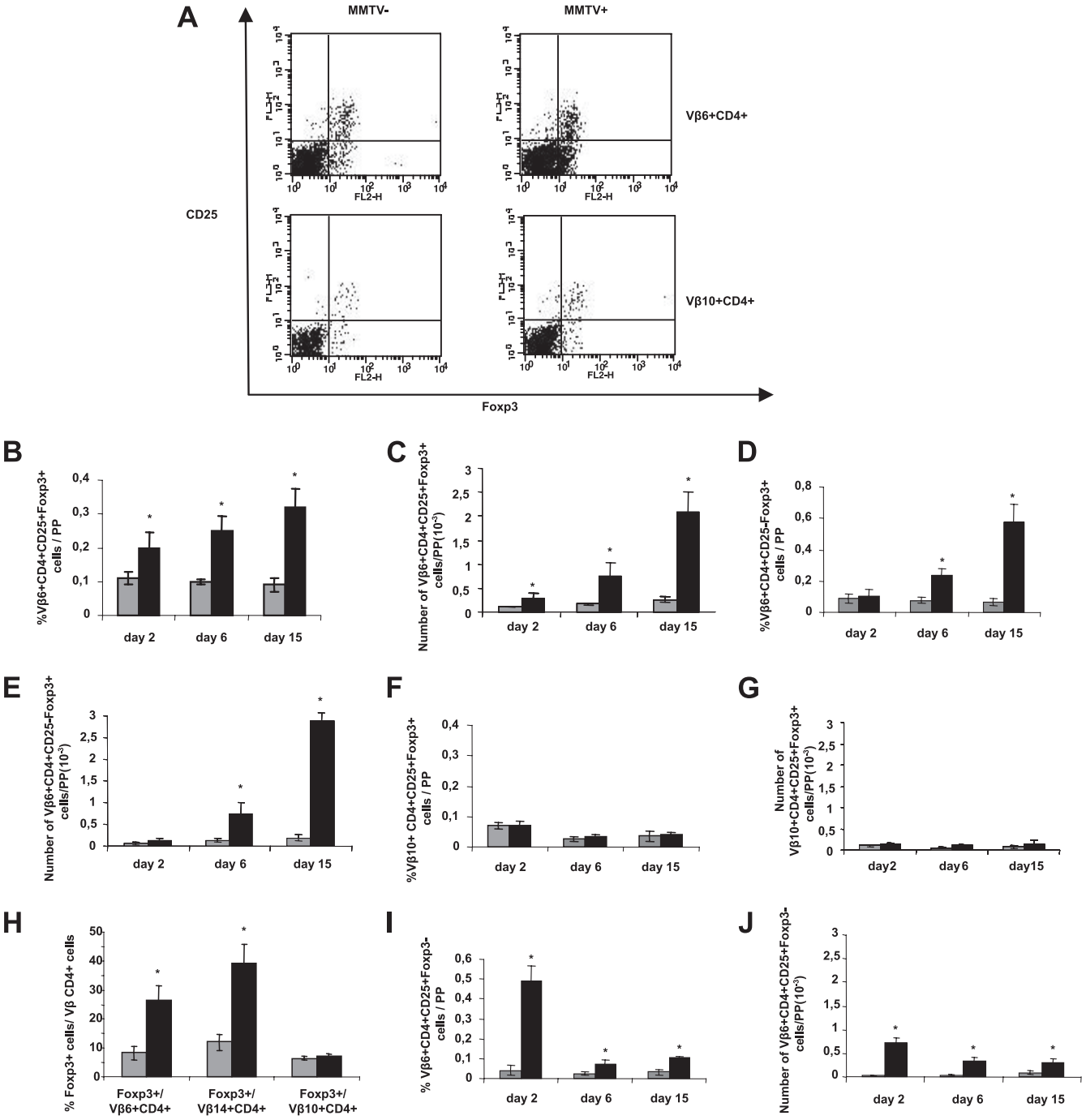

FIG. 2. SAg specificity of PP Foxp3$^+$ T_{reg} cells during the first 2 weeks after MMTV infection. BALB/cJ pups were foster nursed on MMTV(BALB6)-infected (A to G and I and J) or MMTV(LA)-infected (H) (black bars) or uninfected (gray bars) mothers. Expression of Vβ6, CD4, CD25, and intracellular Foxp3 in PP cells was analyzed by FACS at different days after infection. PP cells were gated for live cells in the forward-sideward scatter (B to G and I and J) and for Vβ$^+$ CD4$^+$ cells (A and H). Cell types are indicated along the *y* axes (B to G and I and J). (A) Representative dot plots of CD25 and Foxp3 in PP Vβ6$^+$ CD4$^+$ and Vβ10$^+$ CD4$^+$ cells at day 15 of infection. (H) Percentage of Foxp3$^+$ cells within Vβ6$^+$ CD4$^+$, Vβ14$^+$ CD4$^+$, and Vβ10$^+$ CD4$^+$ cells at day 15 of infection. Data are expressed as the means ± standard deviations ($n = 4$). *, $P < 0.05$. The experiments were performed three times with similar results.

mothers for 6 days. *Mtv-7*$^-$ mice showed increases in the percentage and the absolute number of CD4$^+$ CD25$^+$ Foxp3$^+$ and CD4$^+$ CD25$^-$ Foxp3$^+$ T_{reg} cells, whereas *Mtv-7*$^+$ mice showed no significant increases in these cell populations (Fig. 3). These results confirm that the increases in T_{reg} cells that occurred during MMTV infection were mainly due to increases in SAg-reactive T cells.

T_{reg}-cell increases are dependent on the presence of DCs. DCs play an important role in MMTV infection (8, 11). We recently showed that the SAg response is abrogated in the

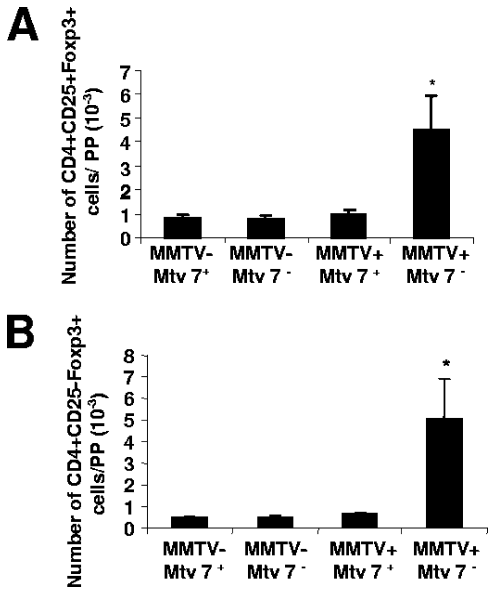

FIG. 3. Increases in PP T_{reg} cells are dependent on the presence of SAg-reactive T cells. *Mtv-7*$^+$ *and Mtv-7*$^-$ mice generated by crossing (BALB/cJ × AKR/J)F_1 × BALB/cJ mice were nursed on MMTV (BALB6)-infected or uninfected mothers for 6 days. The expression of CD4, CD25, and intracellular Foxp3 in PP cells was analyzed by FACS. PP cells were gated for live cells in the forward-sideward scatter. Cell types are indicated along the y axes. Data are presented as the means ± standard deviations ($n = 5$). *, $P < 0.05$. The experiment was performed four times with similar results.

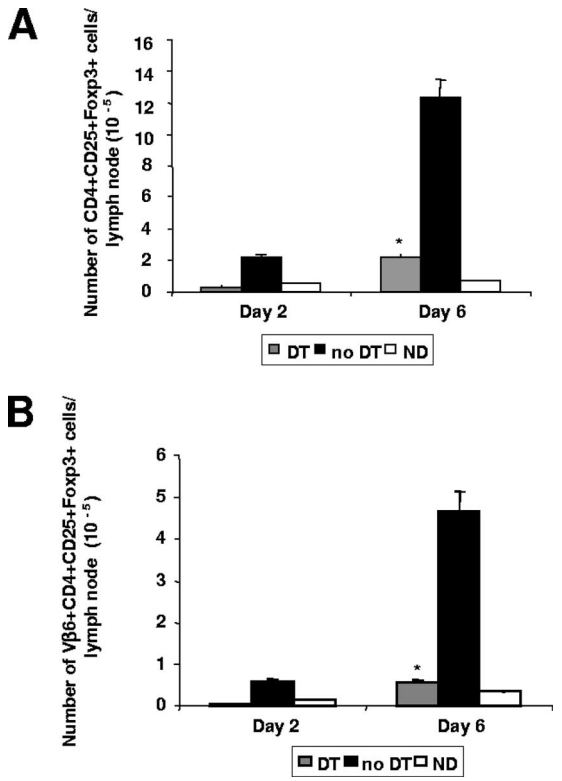

FIG. 4. Increases in T_{reg} cells are dependent on the presence of DCs. CD11c-DTR adult mice were injected i.p. with DT (day −1) and with MMTV(LA) in the footpad (day 0). After 2 or 6 days the expression levels of Vβ6, CD4, CD25, and Foxp3 were analyzed by FACS in the draining and nondraining (ND) popliteal lymph nodes. Lymph node cells were gated for mononuclear cells in the forward-sideward scatter. (A) Number of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in draining popliteal lymph nodes of mice treated with DT or not treated with DT and in nondraining popliteal lymph nodes (ND). (B) Number of $V\beta6^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ cells in untreated (No DT) DT-treated lymph nodes and in nondraining (ND) lymph nodes. Data are presented as the means ± standard deviations ($n = 3$). *, $P < 0{,}05$. One experiment out of two is shown.

absence of these cells (11). To investigate whether DCs were involved in the MMTV-induced increase in $Foxp3^+$ T_{reg} cells, we used an in vivo model of inducible DC ablation, the CD11c-DTR mice (25). Administration of DT to CD11c-DTR mice produces a selective and temporal depletion of $CD11c^+$ cells, generating conditional DC knockouts (25). Although ablation was successfully accomplished in adult mice, our attempts to adapt the model to neonatal mice failed because the DT was lethal for the transgenic pups, even in low doses. Therefore, we carried out these experiments in adult mice. We first tested whether MMTV also affected the $CD4^+$ $CD25^+$ $Foxp3^+$ cell subset in an experimental model of footpad infection. When MMTV(LA) was injected subcutaneously into wild-type adult animals, the draining popliteal lymph node showed an increase in the percentages and absolute numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ and $V\beta6^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} cells, resembling the one observed in the PP (not shown). CD11c-DTR mice were injected i.p. with DT (day −1) and then with MMTV in the footpad (day 0), and the numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ and $V\beta6^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ cells were analyzed in the draining and nondraining popliteal lymph nodes at 2 and 6 days after infection. DC depletion abolished increases in both the $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} cells and the SAg-specific T_{reg} cells (Fig. 4), thus demonstrating that the MMTV-induced increase in T_{reg} cells was DC dependent.

$CD4^+$ $CD25^+$ PP cells from infected mice preferentially suppress the proliferative response of T cells to SAg-expressing APCs. MLR assays are commonly used to test the suppressive activity of T_{reg} cells. For the MLR assays, we purified $CD4^+$ $CD25^+$ cells from the PP of BALB/cJ mice foster nursed for 6 days on MMTV(LA)-infected mothers in order to evaluate their ability to suppress responses ex vivo. We performed the MLR assays by coculturing CFSE-labeled BALB/cJ $CD4^+$ $CD25^-$ cells (responders) with mitomycin C-treated mononuclear spleen cells from AKR/J mice (stimulators). $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ cells obtained from the PP of infected or uninfected pups were added to the MLR mixture from the beginning of the culture. Proliferation of $CD4^+$ responder cells was analyzed by CFSE dilution. As shown in Fig. 5, PP $CD4^+$ $CD25^+$ cells from infected mice strongly inhibited proliferation in the MLR assay using AKR/J APCs and suppressed the proliferative response more efficiently than $CD4^+CD25^+$ cells from uninfected littermates. In addition, the percentage of inhibition increased in a dose-dependent manner (data not shown). These results indicate that $CD4^+$ $CD25^+$ cells obtained from the PP of infected mice had a strong immunosuppressive activity when AKR/J APCs were used in the MLR assays. In addition, the suppressive capacity of these cells was greater than that of $CD4^+$ $CD25^+$ cells from uninfected mice.

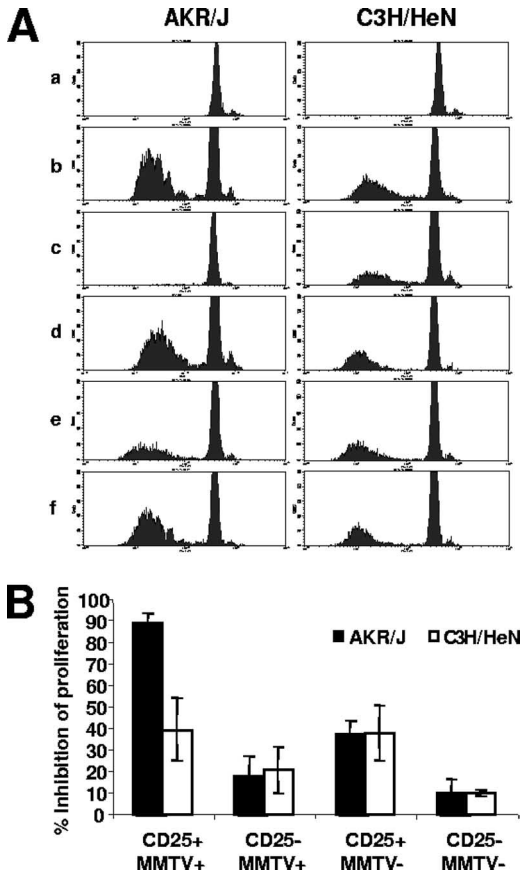

FIG. 5. $CD4^+$ $CD25^+$ cells from PP of MMTV-infected mice are more potent at suppressing the proliferative response of T cells to SAg-expressing APCs. MLR were performed by coculturing CFSE-stained BALB/cJ $CD4^+$ $CD25^-$ responder cells with mitomycin C-treated mononuclear spleen cells from AKR/J (*Mtv-7*$^+$) mice or C3H/HeN (*Mtv-7*$^-$) stimulators. $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ cells (1.5×10^{-5}) obtained from the PP of uninfected pups or those infected for 6 days with MMTV(LA) were added to the MLR mixture from the beginning. Proliferation of $CD4^+$ responder cells was analyzed by CFSE dilution. (A) Representative histogram plots. Except for panel a, where the culture contained only responding cells, PP cells were added to responding and presenting cells as follows: b, none; c, $CD4^+$ $CD25^+$ cells from MMTV-positive mice; d, $CD4^+$ $CD25^-$ cells from MMTV-positive mice; e, $CD4^+$ $CD25^+$ cells from MMTV-negative mice; f, $CD4^+$ $CD25^-$ cells from MMTV-negative mice. (B) Percent inhibition of proliferation observed in MLR; the $CD4^+$ cells added in each case are shown on the *x* axis. Data are presented as the means ± standard deviations ($n = 3$). One experiment out of four is shown.

Recognition of SAgs by T cells is major histocompatibility complex (MHC) dependent but not MHC restricted (44). The magnitude of the proliferative response of the BALB/cJ (*H-2*^{d}) $CD4^+$ T cells to AKR/J (*H-2*^{k} *Mtv-7*$^+$) APCs is mainly due to the SAg encoded by the *Mtv-7* provirus formerly known as Mls-1a (22). MMTV(LA) includes a virus whose SAg hypervariable region is identical to that of the *Mtv-7* provirus. To test whether PP $CD4^+$ $CD25^+$ T cells purified from MMTV(LA)-infected mice preferentially suppressed the proliferative response of T cells to SAg-expressing APCs, we performed MLR assays with stimulator cells from spleens of AKR/J (*H-2*^{k} *Mtv-7*$^+$) or C3H/HeN (*H-2*^{k} *Mtv-7*$^-$) mice. $CD4^+$ $CD25^+$ T cells from uninfected mice inhibited the response to C3H/HeN or AKR/J APCs to the same extent. In addition, $CD4^+$ $CD25^+$ T cells from MMTV(LA)-infected and noninfected mice suppressed the response to C3H/HeN APCs to the same extent. However, the immunosuppressive activity of MMTV(LA)-infected PP $CD4^+$ $CD25^+$ cells was higher upon AKR/J stimulated T-cell proliferation than with C3H/HeN stimulation (Fig. 5). Thus, results reported herein suggest that $CD4^+$ $CD25^+$ T cells from infected mice preferentially suppressed the proliferative response of T cells to SAg-expressing APCs.

Effects of the depletion of $CD25^+$ cells before MMTV infection. The early response to the MMTV SAg has been associated with viral amplification (1, 13). In order to investigate changes in the SAg response in the absence of T_{reg} cells, we depleted mice of $CD25^+$ cells before foster-nursing them for 6 days on MMTV(LA)-infected mothers as described in Materials and Methods. CD25-depleted mice showed a significant increase in the number of responding PP $V\beta6^+$ $CD4^+$ $Foxp3^-$ T cells at day 6 of infection (Fig. 6A).

To determine whether T_{reg} cells affected virus load early during infection, we depleted mice of $CD25^+$ cells before foster-nursing them for 15 days on MMTV(LA)-infected mothers as described in Materials and Methods. The mice were sacrificed at the age of 23 days, DNA was extracted from the mesenteric lymph nodes and spleens, and MMTV integration was studied by radioactive PCR. Figure 6B shows representative results obtained using mesenteric lymph nodes, and Fig. 6C shows the gel quantitation. Mice with $CD25^+$ cell depletion contained at least two times more viral DNA sequences in both organs than nondepleted mice. This difference was small but highly reproducible. Taken together, these results raise the possibility that T_{reg} cells—by attenuating the SAg response—play a role in decreasing viral load at the beginning of MMTV infection.

Depletion of $CD25^+$ cells once the initial SAg response has developed causes lower MMTV viral load. To determine the role of T_{reg} cells once the initial SAg response has occurred, we depleted mice of $CD25^+$ T cells from day 6 to day 8 of infection, i.e., after the $CD4^+$ $CD25^+$ $Foxp3^-$ cells had returned to almost basal levels and the $CD4^+$ $CD25^+$ cells were mostly $Foxp3^+$ T_{reg} cells (Fig. 1). The experiments were performed employing two successive generations of mice. Half of the MMTV(LA)-infected littermates were depleted of $CD25^+$ cells as described in Materials and Methods from day 6 to day 8 of infection, and then these mice were either sacrificed at 31 days to evaluate viral load in the first generation or crossed with BALB/cJ males to obtain a second generation. Half of the second generation was also depleted of $CD25^+$ cells for 3 days (day 6 to day 8 of infection). DNA was extracted from the mesenteric lymph nodes and spleens of 31-day-old mice, and radioactive PCR was performed. A slight decrease in viral load was observed in the first generation of depleted mice (not shown). However, a marked decrease in viral load at the second generation was seen. In this case, the CD25 T-cell-depleted mice contained at least 10-fold less viral DNA in both organs than nondepleted littermates (Fig. 6D and E). These results suggested that after the initial suppression of SAg-mediated T-cell stimulation, T_{reg} cells play a role in increasing viral load.

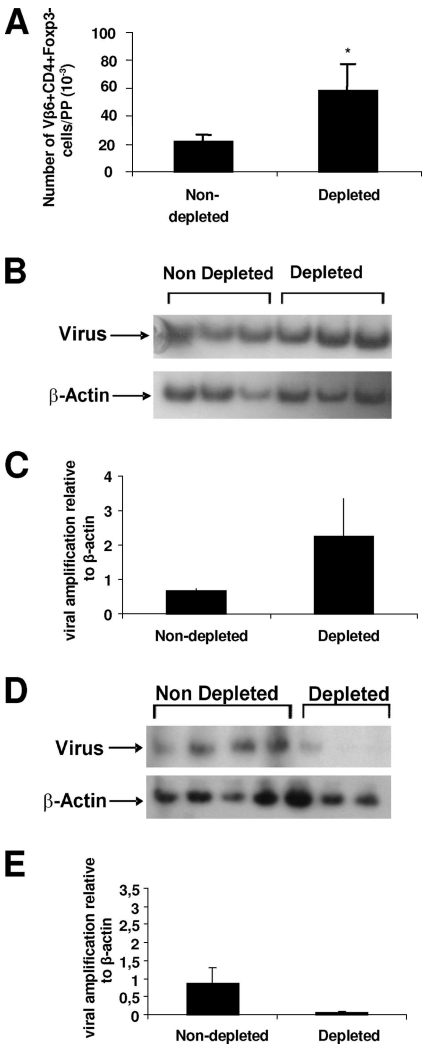

FIG. 6. Effects of the depletion of $CD25^+$ cells during MMTV infection.(A) BALB/cJ mice were depleted of $CD25^+$ cells before infection and foster nursed on MMTV(LA)-infected females from day 8 to day 14, when they were sacrificed. Expression of Vβ6, CD4, and intracellular Foxp3 in PP cells was analyzed by FACS. PP cells were gated for live cells in the forward-sideward scatter. Shown are the number of $V\beta6^+$ $CD4^+$ $Foxp3^-$ cells. Data are presented as the means ± standard deviations ($n = 4$). *, $P < 0.05$. The experiment was performed two times with similar results. (B) BALB/cJ mice were depleted of $CD25^+$ cells before MMTV infection as described in Materials and Methods and foster nursed for 15 days on MMTV(LA)-infected females from day 8 to day 23, when they were sacrificed. DNA from the mesenteric lymph nodes was extracted, and radioactive semiquantitative PCR was performed. The figure shows a representative virus and β-actin amplification in three individual nondepleted and depleted mice each. (C) Quantification of virus amplification relative to β-actin was performed with the software Scion Image. One experiment out of five is shown. (D) Eight-day-old mice were nursed on MMTV(LA)-infected females for 6 days. At this time, mice were depleted of $CD25^+$ cells by three successive i.p. injections of anti-CD25 antibody from day 6 to day 8 of infection. The female mice were bred, and half of their neonatal offspring were depleted of $CD25^+$ cells in a similar manner. Thirty-one–day-old mice were sacrificed, DNA was extracted from spleen, and radioactive semiquantitative PCR was performed. The figure shows a representative virus and β-actin amplification in four nondepleted and three depleted mice. (E) Quantitation of virus amplification relative to β-actin was performed with the software Scion Image. The experiment was performed five times with similar results.

DISCUSSION

The final outcome of an infectious process is influenced by characteristics of both the pathogen and the host immune system. In addition to a well-described number of strategies to evade the immune response, it has been recently reported that some pathogens are able to take advantage of T_{reg} cells to establish infection (14, 30, 31, 40, 46). In particular, the persistence of some retroviral infections, such as feline immunodeficiency virus, human immunodeficiency virus (HIV), and Friend murine leukemia virus infection, has been correlated with increases in T_{reg} cells (4, 5, 46, 49). Here, we investigated whether MMTV infection induces alterations in the population of PP $CD4^+$ $Foxp3^+$ T_{reg} cells during early steps of natural milk-borne infection.

Our results showed that MMTV induced early and progressive increases in the number of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} cells in PP, at least during the first 2 weeks of infection. In addition, we showed that $CD4^+$ $CD25^-$ $Foxp3^+$ cells, which have been described either as proliferating T_{reg} cells (18) or as a T_{reg}-cell reservoir (52), increased with kinetics similar to that of $CD4^+$ $CD25^+$ $Foxp3^+$ cells. The increases in $Foxp3^+$ cells may be due to recruitment of natural $Foxp3^+$ T_{reg} cells (45), conversion of $Foxp3^-$ to $Foxp3^+$ T cells (10, 29), and/or proliferation of natural or converted $Foxp3^+$ T cells (48).

It has been reported that natural T_{reg} cells are significantly exported to the periphery only after day 3 of postnatal life (3) and that 3-day old mice already contain Foxp3-expressing $CD4^+$ $CD25^+$ splenocytes (15). However, there are no data in the literature regarding T_{reg} cells in the PP of neonatal mice. Thus, the possibility exists that natural T_{reg} cells are absent in PP during the first days of life. As in our experimental model we began the foster nursing at day 8 of life, we also foster nursed mice from birth to validate our model. Similar increases in the levels of T_{reg} cells were observed after 15 days of infection (data not shown), thus validating the results of our experimental system.

Accumulating evidence suggests that $CD4^+$ $CD25^+$ T_{reg} cells are able to recognize exogenous antigens derived from microbes, parasites, and allogeneic tissues (2, 6, 31, 45). It is well-known that viral and bacterial SAgs induce increases in CD4 T cells bearing specific receptor Vβ chains (26), but little is known about the involvement of SAg-specific $Foxp3^+$ T_{reg} cells in these increases (17). In an adult model of MMTV(SW) infection, a population of $CD4^+$ $V\beta6^+$ $CD25^+$ cells that remained constant in absolute number throughout the adult life was described. It was suggested that these cells could have a regulatory role during MMTV infection although neither their functionality nor the expression of Foxp3 was examined (37). In addition, it has been reported that when $CD4^+$ $CD25^+$ T_{reg} cells isolated from BALB/c mice were transferred to BALB/c nude mice with DBA/2 (*Mtv-7*$^+$) skin grafts, an increase in the percentage and number of $V\beta6^+$ cells within the transferred $CD4^+$ cell population was observed (35). The experiments performed in our model of natural MMTV infection showed that PP SAg-reactive $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD4^+$ $CD25^-$ $Foxp3^+$ T_{reg} cells increased progressively in percentages and absolute numbers from day 2 onward. No increases were detected in T_{reg} cells that did not respond to the SAgs encoded by the MMTVs used in these experiments. In addi-

tion, SAg-specific activated/effector $CD4^+$ $CD25^+$ $Foxp3^-$ T cells increased at day 2 of infection but then were maintained at lower levels from that day onward. As this decrease correlated kinetically with the increase in SAg-specific T_{reg} cells, we hypothesize that T_{reg} cells were responsible for the decrease in the number of activated/effector T cells.

Importantly, our results indicated that the T_{reg} populations that increased in the PP during MMTV infection not only were able to recognize a foreign antigen, in this case the viral SAg, but also were mainly comprised of SAg-cognate T cells. Further reinforcing this notion, our results showed that infection of neonatal mice in which SAg-reactive T cells were deleted by the presence of an endogenous provirus did not induce alterations in $Foxp3^+$ T_{reg} cells, indicating that the increase in T_{reg} cells that occurred during MMTV infection were due mainly to increases in SAg-cognate T cells.

DCs play a pivotal role in the immune system, and it is becoming evident that these cells are involved in the expansion of T_{reg} cells (28, 51). For example, it has been shown that infected DCs can lead to an expansion of T_{reg} cells during Friend murine leukemia virus infection (4). We recently showed the importance of DCs during MMTV infection, demonstrating that these are the first targets of MMTV infection in vivo and that the SAg-mediated T-cell response is abrogated in absence of this population (11). Supporting the importance of the SAg response, we showed here that the early increases in $Foxp3^+$ T_{reg} cells were also completely abrogated in absence of DCs. Thus, the increase in T_{reg} cells that occurred in response to MMTV infection was likely due to SAg presentation by infected DCs to cognate T cells.

Despite the increasing number of studies concerning T_{reg} cells, the antigen specificity of their suppressor-effector function remains a debated issue. At first it was thought that $Foxp3^+$ T_{reg} cells suppressed without specificity, but there is recent evidence showing that T_{reg} cells induced during certain allogeneic responses are able to suppress in an antigen-specific manner (23, 50). It has also been reported that T_{reg} cells induced during HIV infection are more effective at regulating HIV p24-specific responses than other unrelated immune responses in the same patients (49).

MMTV(LA) includes a virus whose SAg hypervariable region is identical to that of the *Mtv-7* provirus. The use of AKR/J ($H\text{-}2^k$ $Mtv\text{-}7^+$) and C3H/HeN ($H\text{-}2^k$ $Mtv\text{-}7^-$) APCs allowed us to investigate the specificity of the suppressor-effector function of PP T_{reg} cells from MMTV(LA)-infected mice. $CD4^+$ $CD25^+$ T cells from noninfected mice did not distinguish between AKR/J and C3H/HeN APCs. Importantly, PP $CD4^+$ $CD25^+$ cells from infected mice showed greater suppression of the T-cell response to $Mtv\text{-}7^+$ (AKR/J) than to $Mtv\text{-}7^-$ (C3H/HeN) stimulators. T_{reg} cells from infected mice still displayed a certain degree of nonspecificity, as evidenced both by their ability to suppress MLR toward C3H/HeN APCs and by the fact that they were able to almost abrogate both the response to the SAg encoded by the *Mtv-7* provirus and to MHC class II alloantigens expressed by AKR/J cells. Our data suggest that the SAg-cognate T_{reg} cells that increase during infection preferentially suppress the proliferative response of T cells to SAg-expressing APCs.

The mechanisms underlying the ability of T_{reg} cells from infected mice to preferentially suppress the T-cell response to SAg-expressing APCs remain unknown. Our unpublished results indicate that T_{reg} cell suppression in the MLR requires cell contact. It has been postulated that T_{reg}-cell interaction with DCs inhibits efficient antigen presentation to T effector cells (41). It has also been reported that T_{reg} cells are able to down-modulate the expression of costimulatory molecules on DCs, diminishing their capacity to induce proliferation (47). Although other mechanisms cannot be discarded, taking into account that T_{reg} cells which increase during MMTV infection are SAg specific and that AKR/J APCs express the *Mtv-7* SAg on their surface, it is possible that cell contact between T_{reg} cells and SAg-expressing APCs such as DCs inhibits the proliferative response in the MLR. However, it cannot be ruled out that other mechanisms may also be taking place in vivo, such as the production of inhibitory cytokines or the induction of indoleamine 2,3-dioxygenase (41, 47). In support of this, we did detect increased percentages of interleukin-10-producing PP $CD4^+$ $CD25^+$ cells (not shown).

To gain insight into the role of T_{reg} cells during MMTV, infection we performed experiments depleting $CD25^+$ cells. When $CD25^+$ cells were depleted before the beginning of infection, significant increases in the number of PP SAg-cognate $CD4^+$ $Foxp3^-$ T_{reg} cells were measured. In addition, a slight increase in viral load was observed in the mesenteric lymph node and spleen of CD25-depleted mice. Even when the result was highly reproducible, the small difference detected in viral loads does not allow us to formally conclude that T_{reg} cells affect viral load at the beginning of the infection. However, since the SAg response has been associated with viral amplification (1, 13), the possibility exists that T_{reg} cells, by attenuating the SAg response, play a role in decreasing viral load at the beginning of the infection. On the other hand, when $CD25^+$ cells were depleted once the initial SAg response had occurred, virus infection levels decreased at least 10-fold by the second generation. These results suggest that T_{reg} cells favor an increase in virus infection levels at least from day 6 of infection onwards, probably by inhibiting an antiviral response, as has been suggested in other retroviral models (4, 5, 39, 46, 49). Even when the specificity of the T_{reg} cells that favor the increase in viral load at this stage of infection cannot be established from our depletion experiments, it can be hypothesized that the interaction between SAg-specific T_{reg} cells and infected APCs could lead to the generation of a tolerogenic microenvironment that could inhibit not only SAg-stimulated T-cell proliferation but also cytotoxic antiviral responses.

Our results are in agreement with the notion that during infection T_{reg} cells may play a protective role in restraining host responses but may also be detrimental to the host, by dampening the immune responses necessary to avoid pathogen persistence (6). Of interest, it has been proposed that T_{reg} cells may have a dual role in HIV pathogenesis, limiting the onset of immune activation that results in immune exhaustion and immune-mediated tissue damage and also contributing to the onset of immune dysfunction, especially of HIV-specific effector T cells (40).

It is notable that $CD4^+$ $CD25^-$ $Foxp3^+$ cells are not directly affected by administration of anti-CD25 antibody. Thus, the role of this population in MMTV infection remains to be determined.

It has been reported that the generation of T_{reg} cells could

favor the persistence of certain pathogens, but in the great majority of the cases it is unknown whether T_{reg} cells are acting as the cause or the consequence of chronicity (40). Our data showed that MMTV was able to induce early increases in T_{reg} cells, indicating that alterations in these cells were not the consequence of chronicity and strongly supporting the possibility that T_{reg} cells may be playing a role in the cause of chronicity.

In summary, our results indicate that MMTV infection induces early and progressive increases in SAg-cognate $CD4^+$ $Foxp3^+$ T_{reg} cells that are dependent on the presence of DCs. T_{reg} cells would restrict the host responses to the virus at early times, thereby decreasing infection by a virus that requires lymphocyte activation to achieve efficient infection. However, at later stages the virus would be able to take advantage of these cells that are likely dampening a cytotoxic antiviral response. Thus, our results reveal that T_{reg} cells play an important and complex role during MMTV infection.

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