Regulatory T-cell expansion during chronic viral infection is dependent on endogenous retroviral superantigens

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Regulatory T cells (Treg) play critical roles in the modulation of immune responses to infectious agents. Further understanding of the factors that control Treg activation and expansion in response to pathogens is needed to manipulate Treg function in acute and chronic infections. Here we show that chronic, but not acute, infection of mice with lymphocytic choriomeningitis virus results in a marked expansion of Foxp3⁺ Treg that is dependent on retroviral superantigen (*sag*) genes encoded in the mouse genome. Sag-dependent Treg expansion was MHC class II dependent, CD4 independent, and required dendritic cells. Thus, one unique mechanism by which certain infectious agents evade host immune responses may be mediated by endogenous Sag-dependent activation and expansion of Treg.

A hallmark of the mammalian immune system is the ability to distinguish between self and nonself antigens. The mechanisms controlling this outcome begin during T-cell development in the thymus. T cells expressing a fully rearranged T-cell receptor (TCR) with relatively high affinity to antigens presented in the thymus are deleted or rendered anergic during the process of negative selection. As a result, mature T cells in the periphery are largely specific for nonself antigens. Because this process is not completely efficient, additional mechanisms are required in the periphery to maintain homeostasis. Regulatory T cells (Treg) are a subset of CD4⁺ T cells that express the lineage commitment transcription factor Foxp3 and are critical in controlling self-tolerance in the periphery (1, 2). Treg use a number of mechanisms to suppress the activation and effector function of conventional Foxp3[−] T cells (Tconv) (3), and their importance is evident in that mice and humans lacking functional Foxp3 develop multiorgan autoimmunity early in life (4–6).

Treg originate from at least two sources. First, T cells expressing TCR with intermediate affinity to thymic antigens escape negative selection and develop into Foxp3-expressing Treg (natural Treg). It has been shown that natural Treg share a non-overlapping TCR repertoire with Tconv, suggesting that the natural Treg population may be biased toward self-antigens (7–9). Additionally, Treg can differentiate from Foxp3[−] precursors in the periphery in response to TCR stimulation and TGF- β (induced Treg) (10). Although the antigen specificity of induced Treg cells is less clear, these cells may recognize a combination of self and nonself antigens. Nevertheless, the relationship between the antigen specificity of Treg and their effector function is not clear.

After an infection, the balance between Tconv and Treg is critical. Pathogen-specific CD4⁺ and CD8⁺ T cells rapidly expand after infection and, in most cases, clear the infection. Treg may play a role during acute infection, but their importance is still unclear (11). However, during chronic infection, Treg can play critical roles by limiting excessive immune activation and tissue damage, while at the same time facilitating pathogen persistence and maintenance of immunity (12). Further understanding of the role of Treg during chronic infection has been limited by our lack of knowledge regarding the stimuli that drive Treg activation and expansion in these situations. Not only are

pathogen-specific antigens continuously presented during chronic infection, but tissue damage may also result in the presentation of self-antigens. Some studies suggest that Treg that expand after chronic infection can be pathogen specific (13–15), but these findings are not universal (16). Thus, additional information regarding the mechanism of Treg activation and expansion during chronic infection is important.

To examine the role of Treg after infection, we have used the mouse model of infection with lymphocytic choriomeningitis virus (LCMV). Using different strains of the virus, this model allows differentiation between the effects of acute and chronic viral infection. The Armstrong strain of LCMV causes an acute infection that is cleared within 1 wk, whereas the variant clone 13 establishes a chronic infection whereby the mice remain infected virtually for life (17). Treg activation and expansion after clone 13 infection in C57BL/6 mice was most prominent among a subpopulation of cells expressing the TCR V β 5 segment. These cells expanded from a preexisting population of Treg and displayed characteristics of a superantigen (Sag)-mediated response. In addition to the V β specificity, the Treg response was MHC class II dependent, CD4 independent, and entirely dependent on retrovirus-encoded Sags in the mouse genome. Our results describe a unique mechanism by which “self-reactive” Treg expand after chronic infection.

Results

V β 5⁺ Treg Expand During Chronic Viral Infection. We compared the frequency and phenotype of Treg in C57BL/6 mice during the course of Armstrong or clone 13 LCMV infections. Treg dramatically increased in frequency among splenic CD4⁺CD8[−] T cells during clone 13, but not Armstrong, infection. This increased frequency of Foxp3⁺ Treg was transient and peaked at $\approx$ 17 dpi (days post infection) (Fig. 1A). The increase in frequency also translated to an approximately threefold increase in absolute numbers of splenic Treg at the peak. Furthermore, Treg from clone 13-infected mice displayed a more activated phenotype than those from Armstrong-infected animals (Fig. 1B). Specifically, Treg from clone 13-infected mice down-regulated the lymphoid homing marker CD62L and up-regulated the activation marker CD69. Two cell-surface markers that have been described as *in vivo* activation markers of Treg, CD101 (18) and CD103 (19), were also expressed on a higher percentage of Treg from the clone 13-infected group. In addition, Treg from the clone 13-infected mice had higher levels of the costimulatory/inhibitory markers Inducible T cell Costimulator

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Fig. 1. $V\beta 5^{+}Foxp3^{+}$ Treg expand specifically during chronic LCMV infection. (A) Kinetics of Foxp3 expression on C57BL/6 splenic $CD4^{+}CD8^{-}$ T cells after LCMV Armstrong or clone 13 infection. Error bars represent the SD of the mean of at least four animals per time point. (B) Surface expression of activation or costimulatory/inhibitory markers on $CD4^{+}Foxp3^{+}$ cells from uninfected mice (gray) and mice infected with LCMV Armstrong (blue) or clone 13 (red) (22 dpi). (C) Expression of TCR $V\beta$ segments on $CD4^{+}Foxp3^{+}$ or $CD4^{+}Foxp3^{-}$ T cells after infection with LCMV Armstrong and clone 13 (25 dpi). (D) Absolute number of $CD4^{+}Foxp3^{+}$ T cells in the spleen of uninfected and clone 13-infected mice (17 dpi) showing the number of $V\beta 5^{+}$ and $V\beta 5^{-}$ cells. (E) Surface expression of activation or costimulatory/inhibitory markers on $CD4^{+}Foxp3^{+}V\beta 5^{+}$ (green) or $V\beta 5^{-}$ (purple) T cells after clone 13 infection (22 dpi).

(ICOS), Programmed Death 1 (PD-1), OX-40 (CD134), and 4-1BB (CD137).

We next asked whether the increased frequency and number of Treg observed during clone 13 infection was the result of an expansion of a subpopulation of Treg that could be detected by a skewing of the TCR $V\beta$ repertoire. The percentage of Treg expressing different mouse TCR $V\beta$ segments was determined by flow cytometry, and a marked increase in the frequency of Treg expressing TCR $V\beta 5$ was observed specifically during clone 13 infection (Fig. 1C). The percentage of $CD4^{+}Foxp3^{+}V\beta 5^{+}$ cells increased in frequency from 7% in uninfected and Armstrong-infected mice to $\approx 25\%$ in clone 13-infected mice. No change in the percentage of Treg expressing any other $V\beta$ subset was observed, and the frequency of $CD4^{+}V\beta 5^{+}Foxp3^{-}$ T cells was unaltered during either Armstrong or clone 13 infections. At 17 dpi with clone 13, the number of $V\beta 5^{+}$ Treg approximated the total number of splenic Foxp3⁺ Treg in an uninfected mouse (Fig. 1D). A higher percentage of the $V\beta 5^{+}$ Treg were also positive for the Treg activation markers CD101 and CD103 compared with $V\beta 5^{-}$ Treg, and ICOS and PD-1 were up-regulated on both $V\beta 5^{+}$ and $V\beta 5^{-}$ Treg (Fig. 1E). In a standard *in vitro* suppression assay, the expanded $V\beta 5^{+}$ Treg maintained their anergic status and were as suppressive as the $V\beta 5^{-}$ Treg (Fig. S1), indicating that the expanded $V\beta 5^{+}$ Treg were functional.

The failure to observe Treg expansion during Armstrong infection correlated with the absence of viral chronicity and not with the minor sequence differences between the two strains. When perforin^{-/-} mice that lacked functional cytotoxic T cells were infected with Armstrong to establish a persistent infection (20), the magnitude of $V\beta 5^{+}$ Treg expansion was similar to that of wild-type mice infected with clone 13 (Fig. S2A). Conversely, when IL-10^{-/-} mice were infected with clone 13 to allow rapid viral clearance (21), no expansion of $V\beta 5^{+}$ Treg was observed (Fig. S2B), suggesting that chronic infection was both necessary and sufficient to drive $V\beta$ -specific Treg expansion.

$V\beta 5^{+}$ Treg Expand from a Preexisting Pool of Treg. The observation that the $V\beta 5^{+}$ T-cell expansion was Treg specific raised the question of the origin of these cells *in vivo*. To determine whether the expanded Treg were induced in the periphery from Foxp3⁻ precursors, we transferred Foxp3⁻ cells [FACS sorted from Foxp3/GFP reporter mice (22)] into naive congenic C57BL/6 mice 1 d before clone 13 infection (Fig. 2A). After infection, endogenous and transferred $CD4^{+}$ T cells were analyzed to de-

termine the percentage of cells expressing Foxp3 (Fig. 2B). Among the endogenous $CD4^{+}$ T cells, the percentage of cells expressing Foxp3 increased to levels observed previously. However, no induction of Foxp3 was detected in the transferred $CD4^{+}$ T cells, indicating that Treg are not generated *de novo* under these conditions. Because LCMV infects the thymus, it remained possible that the increase in number of $V\beta 5^{+}$ Treg was due to an increase in thymic generation. However, $V\beta 5^{+}$ Treg expansion was completely normal when adult thymectomized mice were infected with clone 13 (Fig. S3).

To test whether the expanded $V\beta 5^{+}$ Treg were derived from the endogenous Treg pool, we transferred GFP/Foxp3⁺ T cells into congenic mice 1 d before clone 13 infection. At 15 dpi, endogenous and transferred Treg were analyzed to determine the percentage of cells expressing $V\beta 5$ (Fig. 2C). The increase in frequency of $V\beta 5^{+}$ Treg was the same among endogenous and transferred Foxp3⁺ cells (2.8- and 2.5-fold, respectively), consistent with the preferential expansion of a preexisting population of Treg. To further test this hypothesis, we examined cell division of the different $CD4^{+}$ T-cell populations early in infection. At 7 dpi, the $CD4^{+}Foxp3^{+}V\beta 5^{+}$ subset had the highest percentage cells incorporating BrdU and expressing the cell cycle marker Ki-67, compared with all other $CD4^{+}$ T cell populations (Fig. 2D).

$V\beta 5^{+}$ Treg Expand in Response to *Mtv* Sag. Studies in other infectious disease models suggest that Treg in infected animals can be specific for the infectious organism. We obtained no evidence for LCMV-specific reactivity of Treg from clone 13-infected mice, including the failure to bind a peptide (GP66-77)-MHC class II tetramer specific for an immunodominant LCMV epitope (Fig. S4).

Because the major fraction of Treg that expanded after clone 13 infection was restricted to a specific $V\beta$ subset, we hypothesized that a Sag was responsible for proliferation. We initially analyzed whether the $V\beta 5^{+}$ Treg expansion was dependent on MHC class II and CD4 molecules. Both C57BL/6 $A^b\beta^{-/-}$ and C57BL/6 $CD4^{-/-}$ mice have a population of TCR⁺Foxp3⁺ cells that have characteristics of Treg (23). We infected both of these strains with clone 13 and analyzed the percentage of $CD3^{+}Foxp3^{+}$ cells that express $V\beta 5^{+}$. For the $A^b\beta^{-/-}$ mice, no change in the percentage of $CD4^{+}$ single-positive (or $CD8^{+}$ single-positive) Foxp3⁺ $V\beta 5^{+}$ cells was observed (Fig. 3A). In the $CD4^{-/-}$ mice, the expansion of $V\beta 5^{+}$ cells among the $CD4^{-}CD8^{-}Foxp3^{+}$ population was completely unaffected (Fig. 3B), together suggesting that the expansion of the

Fig. 2. Vβ5⁺Foxp3⁺ Treg preferentially expand from a preexisting population of Treg after LCMV infection. (A) CD4⁺GFP/Foxp3⁺ or GFP/Foxp3⁻ T cells were FACS sorted from naive CD45.2 reporter mice and transferred into naive CD45.1 recipients 1 d before clone 13 infection. Control uninfected mice were transferred with identical numbers of cells. (B) GFP and Foxp3 expression on splenic CD4⁺ T cells from mice that received CD45.2⁺CD4⁺GFP/Foxp3⁻ T cells (15 dpi). Endogenous (Left) or transferred (Right) cells are shown. Numbers represent the percentage of cells in each quadrant. (C) GFP and Vβ5 expression on splenic CD4⁺Foxp3⁺ T cells from mice that received CD45.2⁺CD4⁺GFP/Foxp3⁺ T cells (15 dpi). Endogenous (Left) or transferred (Right) are shown, and numbers represent the percentage of GFP⁻ or GFP⁺ cells, respectively. (D) Adult C57BL/6 mice were injected with 1 mg BrdU 7 dpi with clone 13, and splenic CD4⁺ T cells were analyzed 24 h later. Numbers represent percentage of cells in each quadrant.

Vβ5⁺ subset was MHC class II dependent and CD4 independent. This pattern is consistent with Sag-mediated activation of CD4⁺ T cells (24, 25). Significantly, in the CD4^{-/-} mice, no expansion of the Vβ5⁺ cells was detected in the CD8⁺ Foxp3⁺ population (Fig. 3B), indicating that CD8 cannot substitute for CD4 as a coreceptor in this mechanism of Treg expansion.

Because LCMV has not been previously shown to express a Sag, a likely explanation for our results was that the expansion of the Vβ5⁺ Treg was secondary to stimulation by an endogenous Sag, such as mouse mammary tumor virus (Mtv)-encoded Sag. Most common strains of laboratory mice carry endogenous Mtv proviruses containing Sag genes in their germline (26). Endogenous Mtv Sag expression in mice expressing MHC Class II I-E leads to intrathymic deletion of specific Vβ subsets in a strain-dependent manner (27). We infected several strains of mice expressing I-E with clone 13 and analyzed the effects of infection on the Vβ repertoire. In every strain, infection with clone 13 resulted in selective expansion of a Vβ-expressing CD4⁺Foxp3⁺ population that was normally deleted by Mtv-encoded Sag in the thymus (Table S1). Specifically in the case of BALB/c (H-2^d, I-E⁺) mice, we observed a significant increase in the percentage of CD4⁺Foxp3⁺Vβ5⁺ and a dramatic increase in Foxp3⁺Vβ12⁺ Treg, but no change in the percentage of CD4⁺Foxp3⁻Vβ5⁺ or Foxp3⁻Vβ12⁺ T cells, after clone 13 infection (Fig. 4A). No changes in the percentages of the other Vβ populations were detected. These Vβ5⁺ and Vβ12⁺ Treg displayed an activated phenotype similar to that seen in the expanded Vβ5⁺ Treg in the C57BL/6 mice (Fig. 4B).

To rule out the possibility that the differences observed between C57BL/6 and BALB/c mice were due to the different genetic backgrounds, we also infected B10.D2 (H-2^d, I-E⁺) and BALB/b (H-2^b, I-E⁻) mice and performed the same Vβ repertoire

Fig. 3. Expansion of Vβ5⁺Foxp3⁺ Treg is MHC class II dependent but CD4 independent. Adult C57BL/6 Aβ^{-/-} or CD4^{-/-} mice were infected with LCMV clone 13, and spleens were harvested 18 dpi. (A) Top: Percentage of CD3⁺Foxp3⁺ T cells in Aβ^{-/-} mice. Middle: Gated on the CD3⁺Foxp3⁺ cells from Top. Bottom: Vβ5 expression on CD4⁺CD8⁻ (Left) and CD4⁺CD8⁺ (Right) cells from Middle. (B) Analysis of cells from CD4^{-/-} mice. Top and Middle: Gated as in A. Bottom: Vβ5 expression on CD4⁺CD8⁻ (Left) and CD4⁺CD8⁺ (Right) cells from Middle. Histograms show cells from uninfected (gray) or clone 13-infected (red) mice. Numbers represent percentage of cells in each region or quadrant.

analysis. The pattern of Treg Vβ expansion in infected BALB/b mice was the same as in C57BL/6 mice, and B10.D2 mice behaved identically to BALB/c mice (Fig. S5). These results are consistent with an Mtv Sag-mediated increase in Treg cells after LCMV infection.

Treg Do Not Expand After Infection of BALB/c Mtv-Null Mice. To directly prove that expansion of the Vβ5 and Vβ12 Foxp3⁺ subsets in BALB/c mice was secondary to activation by endogenous Mtv Sag, we infected congenic BALB/c mice lacking all three of the endogenous Mtv proviruses (Mtv-null) (28) and analyzed their Vβ repertoire. Consistent with a lack of Mtv Sag, these mice had a substantial population of T cells that expressed either Vβ5 or Vβ12 in Foxp3⁺ and Foxp3⁻ subsets. However, we observed no change in the frequencies of either Vβ5⁺ or Vβ12⁺ Treg in these mice after clone 13 infection (Fig. 5A), suggesting that the observed Vβ-specific Treg expansion was due to stimulation by an endogenous retroviral Sag. We also infected F1 crosses between the Mtv-null mice and either wild-type C57BL/6 or BALB/c mice. Vβ5 and Vβ12 T-cell deletion occurred as expected in the F1 hybrids, and Vβ5⁺ and Vβ12⁺ Treg expanded to the same extent as seen in the wild-type BALB/c mice (Fig. S6), thus demonstrating that the presence of the Mtv Sag is dominant.

To determine the relative contribution of each of the Mtv Sag to Treg expansion, we infected congenic BALB/c strains carrying single Mtv proviruses (called BALB/Mtv6, BALB/Mtv8, and BALB/Mtv9). The BALB/Mtv6 animals failed to delete both Vβ5 and Vβ12 T cells, and no expansion of Treg in either population was observed after infection (Fig. 5B). Uninfected BALB/Mtv8 animals had normal numbers of Vβ5⁺ and ~50% of Vβ12⁺ T cells (compared with Mtv-null and BALB/Mtv6 mice), and only the Vβ12⁺ Treg expanded after clone 13 infection (Fig. 5C). However, the BALB/Mtv9 animals were similar to wild-type BALB/c (both in terms of T-cell deletion and Treg expansion after infection) (Fig. 5D), suggesting that Mtv9 Sag is the major contributor to the expansion of Treg observed in wild-type mice.

Fig. 4. Chronic infection of BALB/c mice results in the expansion of Foxp3⁺ Treg populations that are thymically deleted in response to *Mtv*/I-E recognition. (A) TCR Vβ analysis of splenic CD4⁺ T cells from adult BALB/c mice infected with LCMV clone 13. (B) Surface expression of activation or costimulatory/inhibitory markers on CD4⁺Foxp3⁺Vβ12⁺ (green) or Vβ12⁻ (purple) T cells after clone 13 infection (17 dpi).

***Mtv* Sag-Dependent Treg Expansion Requires Dendritic Cells.** To address the mechanism by which chronic infection results in *Mtv* Sag-dependent Treg expansion, we first asked whether LCMV infection resulted in increased expression of the endogenous *sag* genes. Because *Mtv8* and *Mtv9* Sag seemed to be responsible for the Vβ-specific Treg expansion, we determined the relative expression of these genes in splenic MHC class II (I-A/I-E)-enriched cells from BALB/c mice by quantitative real-time PCR using primers specific for the intragenic *env* promoter and 3' LTR sequence (*Mtv6* does not contain an *env* gene and therefore could not be detected using these primers). At 8 dpi, *Mtv8* and/or *Mtv9* *sag* expression was up-regulated ~2- and 25-fold in Armstrong- and clone 13-infected mice, respectively, compared with uninfected controls (Fig. 6A). The inability to detect a product in cells isolated from the *Mtv*-null mice demonstrated that the primers were specific.

Because the I-A/I-E enriched cells were a heterogeneous pool, we sought to further define the type of antigen-presenting cells responsible. Previously, both B cells and dendritic cells (DC) have been shown to be the primary antigen-presenting cells that express several endogenous *Mtvs* in the periphery (29). We examined the role of each of the cell types initially using genetically deficient mice. Infection of C57BL/6 B cell-deficient mice (μMT) with clone 13 resulted in Vβ5⁺ Treg expansion similar to that in wild-type mice. However, Flt3L^{-/-} mice (which are defective in several hematopoietic cell lineages, but primarily DC) infected with clone 13 displayed only a modest (approximately twofold) increase in frequency of Vβ5⁺ Treg (Fig. 6B), suggesting that Sag presentation by B cells was not necessary and that DC were primarily responsible for the Treg expansion.

To further define the role of DC in the expansion of Treg, we used the CD11c-diphtheria toxin receptor (DTR) mice (30). Lethally irradiated C57BL/6 mice reconstituted with CD11c-DTR bone marrow were infected with clone 13 and injected with DT or saline as a control every other day throughout the course of infection. At 17 dpi, DT-treated mice did not contain CD11c/GFP⁺ cells in their spleens (Fig. 6C), and viral titers in the DT-treated mice were similar to those in controls (Fig. 6D). These results indicated that the DC depletion was efficient and that DT treatment did not alter the course of infection. However, mice treated with DT had significantly fewer Vβ5⁺ Treg than controls

Fig. 5. Expansion of thymically deleted Foxp3⁺ Treg in BALB/c mice is *Mtv* Sag dependent. (A) TCR Vβ analysis of splenic CD4⁺ T cells from adult BALB/c *Mtv*-null mice infected with LCMV clone 13. (B) TCR Vβ analysis of splenic CD4⁺ T cells from adult BALB/*Mtv6* mice infected with LCMV clone 13. (C) TCR Vβ analysis of splenic CD4⁺ T cells from adult BALB/*Mtv8* mice infected with LCMV clone 13. (D) TCR Vβ analysis of splenic CD4⁺ T cells from adult BALB/*Mtv9* mice infected with LCMV clone 13. All mice were analyzed at 17 dpi. Numbers represent percentages of Foxp3⁺ or Foxp3⁻ cells in each plot.

(25.5% reduction; Fig. 6E). Finally, *Mtv8* and/or *Mtv9* *sag* expression was assessed in purified splenic DC (defined as TCRβ⁻CD49b⁻CD11c⁺ cells). At 8 dpi, Sag expression levels were highest in DC from clone 13-infected animals (Fig. 6F), suggesting that modulation of Sag gene expression in DC may be important for Vβ-specific Treg expansion.

Discussion

These studies define a unique mechanism of Treg activation and expansion after chronic viral infection. Infection of C57BL/6 mice with LCMV clone 13 resulted in the selective expansion of a preexisting population of Treg expressing TCR Vβ5, whereas infection of mice expressing MHC class II I-E resulted in the selective expansion of Treg expressing particular Vβ subsets that were deleted in the thymus. Taken together, we believe that the Vβ-specific Treg expansion observed after chronic LCMV infection is mediated by *Mtv*-encoded Sag proteins because (i) the Treg response is Vβ specific and corresponds with specific Sag-expressing *Mtvs* found in the germline of multiple mouse strains, (ii) the Treg response is MHC class II dependent and CD4 independent, (iii) the use of a congenic BALB/c mouse strain lacking all endogenous *Mtvs* abolished Vβ-specific Treg expansion, and (iv) F1 hybrids between *Mtv*-null and BALB/c mice revealed that Treg expansion is dominant, typical of Sag-mediated effects. Although C57BL/6 mice do not delete Vβ subsets in the thymus because of the lack of I-E expression, *Mtv*-dependent abortive activation and deletion of Vβ5⁺ T cells in the

Fig. 6. *Mtv* Sag-dependent Treg expansion requires DC. (A) Relative gene expression of *Mtv sag* genes in splenic MHC class II-enriched cells from uninfected BALB/c mice, BALB/c mice 8 dpi with either LCMV Armstrong or clone 13, and uninfected BALB/c *Mtv*-null mice. N.D., not detected. Error bars represent the SD of the mean of at least four animals per group. (B) Analysis of Vβ5-expressing Treg from B cell- (μMT) and Flt3L-deficient mice. Histograms are gated on CD4⁺Foxp3⁺ T cells from uninfected mice (gray) and mice infected with LCMV clone 13 (red; 17–20 dpi). (C) Depletion of DC in CD11c-DTR mice after DT treatment. Flow plots are gated on TCRβ⁺CD49b⁺ splenocytes. (D) Serum viral titers in clone 13-infected CD11c-DTR mice (17 dpi). Each circle represents a single mouse and the horizontal bar the mean. (E) Vβ5 analysis of splenic CD4⁺Foxp3⁺ cells from CD11c-DTR mice. Each circle represents a single mouse and the horizontal bar the mean. **P* < 0.05. (F) Relative gene expression of *Mtv sag* genes in purified DC from uninfected BALB/c mice and BALB/c mice 8 dpi with either LCMV Armstrong or clone 13.

periphery of older mice is observed (31). Therefore, we suggest that a similar *Mtv* Sag-dependent mechanism occurs during chronic viral infection in C57BL/6 mice that leads to the expansion of Treg.

The T-cell repertoire of almost all inbred mouse strains is influenced by the expression of various Sags encoded by *Mtv* proviruses (26, 27). In the present study, we define a major role for *Mtv9* in causing expansion of Treg after chronic LCMV infection. However, it is interesting to note that not all Foxp3⁺ Vβ subpopulations recognized by an *Mtv*-encoded Sag expanded after infection. Although the reason for this observation is not clear, it may be related to several factors. The levels of *sag* mRNA produced by different *Mtv* proviruses are known to differ and may be dependent on the mouse strain. Further, the cell types for *Mtv* Sag expression vary according to the provirus, likely owing to variations in their transcription control regions (32, 33). The levels of MHC class II necessary for Sag expression on DC also may vary after LCMV infection. MHC haplotype and the presence or absence of I-E play a role, because Vβ12 expansion occurs in BALB/c and B10.D2 strains that express H2^d, I-E⁺, but not in congenic strains that express H2^b, I-E⁻, even though the same *Mtv* proviruses are present.

The mechanism by which chronic LCMV infection results in *Mtv* Sag-dependent Treg activation and expansion seems to, at least partially, require DC presentation of the endogenous Sag. Although B cells and DC have been shown to express endogenous *Mtv* genes in the periphery (29), mice genetically lacking DC showed only a modest level of Vβ-specific Treg expansion after infection. The observation that Vβ-specific Treg expansion was limited in mice depleted of DC by DT treatment provides further evidence that DC are important. However, the fact that Vβ5⁺ Treg were 75% of the levels of controls in the latter experiment suggests that other cell types may contribute. Although the presence of B cells was not necessary to drive Vβ-specific Treg expansion, B cells may contribute under normal circumstances.

Indeed, *sag* gene expression levels were highly up-regulated in total MHC class II-enriched cells (a population that largely consists of B cells). The failure to observe Treg expansion after acute LCMV infection is likely due to much lower expression of *Mtv8* and/or *Mtv9 sag* mRNA and, presumably, Sag presentation on DCs compared with that observed during chronic infection by clone 13.

It is unclear from these studies why *Mtv* Sag-specific T cell expansion was confined to the Foxp3⁺ subset, because Sags have not previously been shown to differentiate between different populations of Vβ-specific cells. One explanation may be that some expansion of Foxp3⁺Vβ5⁺ T cells may have occurred during the course of the chronic infection but was balanced by deletion similar to that seen by exogenous mouse mammary tumor virus (MMTV) Sag after infection (34). Previous studies have also shown that Treg are less susceptible to Sag-dependent deletion (35). Alternatively, activated Foxp3⁺ T cells could have functioned to suppress any activation of Foxp3⁺Vβ5⁺ T cells. Further, Sag activation, although occurring in a TCR Vβ-specific fashion, may be enhanced by the presence of costimulatory/adhesion molecules that are not involved in the activation of T cells by conventional antigens (36). Foxp3⁺ Treg may preferentially use such unique costimulatory molecules.

Various chronic infections, including some retroviruses, have been correlated with modest increases in Treg numbers and, in some cases, Treg have been implicated in determining the outcome of infection (37). We have as yet been unable to directly determine whether *Mtv* Sag-mediated Treg expansion is involved in the establishment of chronic infection in this model. Viral titers in wild-type and *Mtv*-null BALB/c mice were similar throughout the course of infection with clone 13. However, the expansion of Treg in BALB/c mice was modest, and many other factors may play roles in the control of viral chronicity. Nevertheless, the absence of *Mtv* proviruses has been linked to altered outcomes to disparate infectious agents. For example, infection

of *Mtv*-null mice with MMTV or a thymotropic variant prevents breast cancer or leukemia (28, 38). It is possible that these results are related to *Mtv* Sag effects on Treg.

Infection with EBV results in the expression of an endogenous retroviral Sag that is encoded in the envelope gene of an endogenous human retrovirus, HERV-18, localized to a region of DNA in the vicinity of an EBV-inducible enhancer (39). Although V β -specific T-cell activation was observed in these experiments, it did not seem to be Treg specific. Additionally, because HERV-18 is normally transcriptionally silent and only expressed after infection (in contrast to *Mtv* Sags that are constitutively expressed), it is unlikely that these phenomena are related mechanistically. However, $\approx 8\%$ of the human genome corresponds to endogenous retroviral or retrovirally related sequences that can encode Sags (40), many with unknown functions. Therefore, the possibility that other HERV-encoded Sags may exert similar effects on Treg activation and expansion in man exists.

Methods

Mice and Infection. Commercially available mice were obtained from Jackson Laboratories or Taconic. *Mtv*-null mice and *Mtv* single-positive mice were generated as previously described (28). Infection of adult mice (6–8 wk old) with LCMV Armstrong or clone 13 was performed as previously described (17).

Adoptive Transfer and Flow Cytometry. Cells for adoptive transfer experiments were FACS sorted using a FACSria cell sorter (BD Bioscience) and transferred i.v. into congenic recipients 1 d before infection. Spleen samples were harvested at the indicated dpi and stained with appropriate fluorescently labeled antibodies. Flow cytometry data were collected using a FACSCalibur or LSR II cytometer (BD Bioscience), and data were analyzed using Flowjo (Treestar).

RT-PCR. Spleens from indicated animals were digested with liberase (Roche) at 37 °C. Splenic MHC class II⁺ cells were enriched by magnetic-activated cell sorting using a PE-labeled anti-I-A/I-E antibody followed by antiphycoerythrin magnetic beads (Miltenyi), and purified DC were FACS sorted. Total RNA was purified from each population of cells, and cDNA was synthesized using RT (Invitrogen). RT-PCR for *Mtv* Sags was performed using SYBR-green (Applied Biosystems) and the following primers: 5' intragenic *env* promoter, ATCGC-CTTTAAGAAGGACGCTTCT; and 3' LTR sequence, GCAAAGCAGAGCTAT-GCC. A dissociation curve was run at the conclusion of the reaction to verify a single product. Control RT-PCR was performed using the 18S rRNA reaction kit (Applied Biosystems).

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- Fontenot JD, Rudensky AY (2005) A well adapted regulatory contrivance: Regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6:331–337.
- Sakaguchi S (2004) Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531–562.
- Shevach EM (2009) Mechanisms of foxp3⁺ T regulatory cell-mediated suppression. *Immunity* 30:636–645.
- Bennett CL, et al. (2001) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20–21.
- Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* 4:330–336.
- Wildin RS, et al. (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27:18–20.
- Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY (2006) An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* 7:401–410.
- Pacholczyk R, et al. (2007) Nonself-antigens are the cognate specificities of Foxp3⁺ regulatory T cells. *Immunity* 27:493–504.
- Wong J, et al. (2007) Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4⁺ T cells. *J Immunol* 178:7032–7041.
- Curotto de Lafaille MA, Lafaille JJ (2009) Natural and adaptive foxp3⁺ regulatory T cells: More of the same or a division of labor? *Immunity* 30:626–635.
- Rouse BT, Suvas S (2007) Regulatory T cells and immunity to pathogens. *Expert Opin Biol Ther* 7:1301–1309.
- Belkaid Y, Tarbell K (2009) Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol* 27:551–589.
- Cabrera G, et al. (2008) Early increases in superantigen-specific Foxp3⁺ regulatory T cells during mouse mammary tumor virus infection. *J Virol* 82:7422–7431.
- McKee AS, Pearce EJ (2004) CD25⁺CD4⁺ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J Immunol* 173:1224–1231.
- Suffia IJ, Reckling SK, Piccirillo CA, Goldszmid RS, Belkaid Y (2006) Infected site-restricted Foxp3⁺ natural regulatory T cells are specific for microbial antigens. *J Exp Med* 203:777–788.
- Antunes I, et al. (2008) Retrovirus-specificity of regulatory T cells is neither present nor required in preventing retrovirus-induced bone marrow immune pathology. *Immunity* 29:782–794.
- Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911–4927.
- Fernandez I, et al. (2007) CD101 surface expression discriminates potency among murine FoxP3⁺ regulatory T cells. *J Immunol* 179:2808–2814.
- Röttschke O, Borsellino G, Battistini L, Falk K, Kleinewietfeld M (2009) In vivo-activated CD103⁺ Foxp3⁺ Tregs: Of men and mice. *Blood* 113:2119–2120, author reply 2120.
- Walsh CM, et al. (1994) Immune function in mice lacking the perforin gene. *Proc Natl Acad Sci USA* 91:10854–10858.
- Brooks DG, Lee AM, Elsaesser H, McGavern DB, Oldstone MB (2008) IL-10 blockade facilitates DNA vaccine-induced T cell responses and enhances clearance of persistent virus infection. *J Exp Med* 205:533–541.
- Korn T, et al. (2007) Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med* 13:423–431.
- Stephens GL, Andersson J, Shevach EM (2007) Distinct subsets of FoxP3⁺ regulatory T cells participate in the control of immune responses. *J Immunol* 178:6901–6911.
- Borrero H, Collazo L, Macphail S (1993) The role of class II molecules in Mls 1a recognition by CD4⁺ T cells is independent of the CD4 molecule. *Cell Immunol* 152:594–604.
- Penninger JM, Schilham MW, Timms E, Wallace VA, Mak TW (1995) T cell repertoire and clonal deletion of Mtv superantigen-reactive T cells in mice lacking CD4 and CD8 molecules. *Eur J Immunol* 25:2115–2118.
- Cohen JC, Varmus HE (1979) Endogenous mammary tumour virus DNA varies among wild mice and segregates during inbreeding. *Nature* 278:418–423.
- Scherer MT, Ignatowicz L, Winslow GM, Kappler JW, Marrack P (1993) Superantigens: Bacterial and viral proteins that manipulate the immune system. *Annu Rev Cell Biol* 9:101–128.
- Bhadra S, Lozano MM, Payne SM, Dudley JP (2006) Endogenous MMTV proviruses induce susceptibility to both viral and bacterial pathogens. *PLoS Pathog* 2:e128.
- Ardavin C, et al. (1996) Expression and presentation of endogenous mouse mammary tumor virus superantigens by thymic and splenic dendritic cells and B cells. *J Immunol* 157:2789–2794.
- Sapozhnikov A, Jung S (2008) Probing in vivo dendritic cell functions by conditional cell ablation. *Immunity* 28:409–415.
- Blish CA, et al. (1999) Chronic modulation of the TCR repertoire in the lymphoid periphery. *J Immunol* 162:3131–3140.
- Barnett A, Mustafa F, Wrona TJ, Lozano M, Dudley JP (1999) Expression of mouse mammary tumor virus superantigen mRNA in the thymus correlates with kinetics of self-reactive T-cell loss. *J Virol* 73:6634–6645.
- Xu L, Wrona TJ, Dudley JP (1997) Strain-specific expression of spliced MMTV RNAs containing the superantigen gene. *Virology* 236:54–65.
- Held W, et al. (1993) Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell* 74:529–540.
- Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Pénit C (1998) Regulatory CD4 T cells: Expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. *Int Immunol* 10:371–378.
- Bueno C, et al. (2006) Bacterial superantigens bypass Lck-dependent T cell receptor signaling by activating a G α 11-dependent, PLC-beta-mediated pathway. *Immunity* 25:67–78.
- Lund JM, Hsing L, Pham TT, Rudensky AY (2008) Coordination of early protective immunity to viral infection by regulatory T cells. *Science* 320:1220–1224.
- Bhadra S, Lozano MM, Dudley JP (2009) BALB/Mtv-null mice responding to strong mouse mammary tumor virus superantigens restrict mammary tumorigenesis. *J Virol* 83:484–488.
- Sutkowski N, Conrad B, Thorley-Lawson DA, Huber BT (2001) Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen. *Immunity* 15:579–589.
- Larsson E, Andersson G (1998) Beneficial role of human endogenous retroviruses: facts and hypotheses. *Scand J Immunol* 48:329–338.