Antigen exposure shapes the ratio between antigen-specific Tregs and conventional T cells in human peripheral blood

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The T-cell receptor (TCR) is required for maturation and function of regulatory T cells (Tregs), but the ligand specificities of Tregs outside the context of transgenic TCRs are largely unknown. Using peptide–MHC tetramers, we isolated rare specific Foxp3$^+$ cells directly ex vivo from adult peripheral blood and defined their frequency and phenotype. We find that a proportion of circulating Tregs recognize foreign antigens and the frequency of these cells are similar to that of self-reactive Tregs in the absence of cognate infection. In contrast, the frequencies of Tregs that recognize some common microbial antigens are significantly reduced in the blood of most adults. Exposure to peripheral antigens likely has a major influence on the balance between Tregs and conventional T-cell subsets because a larger proportion of flu-specific T cells has a regulatory cell phenotype in the cord blood. Consistent with this finding, we show that lymphocytic choriomeningitis virus infection can directly modulate the ratio of virus-specific effectors and Tregs in mice. The resulting change in the balance within an antigen-specific T-cell population further correlates with the magnitude of effector response and the chronicity of infection. Taken together, our data highlight the importance of antigen specificity in the functional dynamics of the T-cell repertoire. Each specific population of CD4$^+$ T cells in human peripheral blood contains a subset of Tregs at birth, but the balance between regulatory and effector subsets changes in response to peripheral antigen exposure and this could impact the robustness of antipathogen immunity.

regulatory T cells | antigen specificity | repertoire | human | influenza

T cells bearing the α/β T-cell receptor are crucial for protection against infectious diseases. To recognize the diverse array of pathogens, T cells express a large repertoire of T-cell receptors (TCRs) (1, 2). However, the need to clear pathogens is balanced with the requirement to remain tolerant to self. It is becoming increasingly appreciated that self-reactive T cells can and do escape clonal deletion (3, 4), and the activity of regulatory T cells (Tregs) is required to maintain immune homeostasis in the presence of autoreactive T cells (5–7). TCR signaling is crucial for Treg development, differentiation, and suppressive function (8). Mutation in several TCR signaling molecules, including Zap70 and LAT, impairs thymic Treg selection and development (9, 10). Mature Tregs deficient in another TCR downstream molecule, SLP-76, were also unable to inhibit proliferation of conventional T cells (11). Furthermore, TCRs on mature Tregs are required for maintaining Treg transcriptional signature (12), and genetic ablation of TCRs abolishes their proliferative capacity and results in loss of suppressive activity (13). These data demonstrate a crucial role for TCR in generating and maintaining functional Tregs. However, despite the importance of this pathway, little is known about the antigens that engage the TCRs to trigger receptor activation. In particular, how the selectivity in Treg suppression is maintained and balanced with the necessity to generate robust antipathogen immunity for immune protection remains poorly understood.

The consensus on the specificity of Tregs is generally that they are more self-reactive. This idea is suggested by Tregs' ability to suppress autoimmunity and supported by multiple lines of evidence that show Tregs compete for stronger self-ligands, and their selection in vivo directly correlates with in vitro TCR affinity for a model self-antigen (14, 15). However, Tregs capable of recognizing microbes have also been identified (16, 17). Microbe-specific Tregs are functionally significant and, under different experimental settings, can either protect host from overly exuberant immunopathology or impede microbial clearance (18). Understanding the prevalence of microbe-specific Tregs and how effective antipathogen immunity can take place in their presence may provide crucial insights into host immunity against infections. To identify microbe-specific Tregs in humans, we used peptide–MHC (pMHC) tetramers to determine T-cell specificity. Moon et al. showed that by combining tetramer staining with the magnetic enrichment method of Wucherpfennig and colleagues (19) one could characterize the preimmune repertoire of murine CD4$^+$ T cells (20). We and others have shown that this approach can identify rare unprimed human T cells as well (3, 21, 22). Using tetramer-based enrichment techniques to directly analyze the preimmune repertoire of CD4$^+$ T cells, we recently found that memory phenotype T cells often make up a large fraction of virus-specific T cells in adults who have never been exposed to these viruses and that at

Significance

Herein we used peptide–MHC tetramer staining and enrichment to identify antigen-specific Tregs directly ex vivo and found that most CD4$^+$ T-cell specificities, self or foreign, contain 5–15% Tregs. A notable exception is that two dominant influenza specificities contain very small proportions of Tregs. We demonstrated using human cord blood cells and murine lymphocytic choriomeningitis virus infection that antigen exposure can alter the balance between Tregs and conventional T cells in an antigen-specific and context-dependent manner. The ratio between specific Tregs and cognate effectors correlates with protective immunity in mice and T-cell dominance hierarchy in humans. Our finding provides insight into Treg specificity and highlights the importance of Treg and effector balance in an antigen-specific context.

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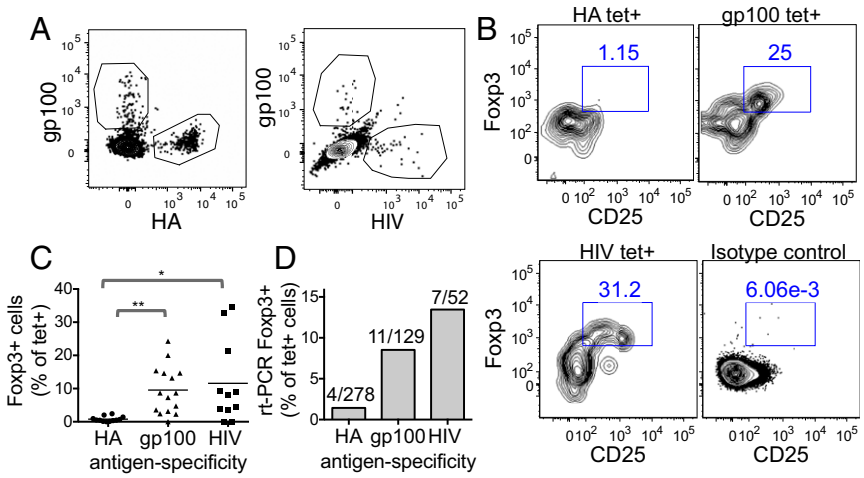

Fig. 1. Identification of antigen-specific Tregs directly ex vivo. Antigen-specific cells were identified by tetramer staining followed by magnetic bead enrichment. All blood samples were obtained from healthy blood donors seronegative for HIV infection. (*A*) Representative tetramer staining for HA, gp100, and HIV-specific T cells. (*B*) Representative Foxp3 and CD25 staining of each Tet$^+$ population. Data are representative of 11–15 individuals, depending on the specificity. (*C*) Frequency of Foxp3$^+$ cells within each tetramer-labeled population. Each symbol represents an antigen-specific population from 1 individual, and the bar indicates the mean. Statistical significance was analyzed using a *t* test. $*P < 0.05$; $**P < 0.005$. (*D*) Single-cell Foxp3 gene expression by RT-PCR. Number above each bar indicates the number of Foxp3$^+$ cells detected over the total number of cells assayed for a given specificity. Data represent cells from 6 individuals.

least some of this phenomenon is likely the result of TCR cross-reactivity (21). Here we used the same approach to identify antigen-specific Tregs and examine the repertoire composition of peripheral Tregs in humans. Tetramer-labeled Tregs were identified by expression of the transcription factor, forkhead box protein P3 (Foxp3), and we combined this assessment with their responses to T-cell stimulation and gene expression analysis by multiplex real-time PCR to firmly establish Treg characteristics.

In total, Tregs for seven microbial peptides and three self-epitopes were examined in the blood of 26 healthy adults. To address the question of whether peripheral Tregs are more self-reactive, we compared the frequency of Foxp3 expression in cells labeled with self- or foreign-peptide MHC tetramers. Interestingly, the two dominant influenza-specific T cells contain very small percentages that are Foxp3$^+$. We showed using cord blood cells that this was not due to a failure to survive positive selection and likely reflects a relative expansion of the cognate Foxp3$^-$ T cells to peripheral antigen exposure. To track the temporal dynamics between Tregs and effector T cells (Teffs) following antigen stimulation, we infected mice with lymphocytic choriomeningitis virus (LCMV) and examined the change in LCMV-specific Tregs and cognate effectors in infected and uninfected mice. We found that the frequency of LCMV-specific Tregs rapidly decreased following both chronic and acute infections, but only a larger and more stable decrease in the relative abundance of LCMV-specific Tregs correlated with viral clearance. Taken together, these data indicate that the balance between Tregs and Teff cells are maintained dynamically in an antigen-specific context and changing this ratio may impact the effectiveness of protective immunity.

Results

Antigen-Specific Tregs in Human Peripheral Blood. We combined pMHC tetramer enrichment with staining for CD25 and Foxp3 to identify Foxp3$^+$ cells that recognize peptides from a melanosomal protein gp100, gag p24 protein of HIV-1, or hemaglutinnin antigen (HA) of influenza virus. These three peptides were selected to provide an initial evaluation for cells that recognize self vs. microbial antigens. Gp100 tetramers identify self-reactive T cells. HA tetramers label memory T cells because nearly all adults have been exposed to influenza antigens by vaccination and/or infection. HIV tetramers identify viral precursor cells because all of the study donors have been tested repeatedly for HIV, as part of routine screening procedure for blood donation, and are HIV negative. Peripheral blood mononuclear cells (PBMCs) from HLA-DRB1*0401$^+$ (for simplicity we will refer to these as DR4$^+$) donors were collected from the Stanford Blood Center. DR4 tetramers were loaded with the particular gp100, HIV, or HA peptides and used to stain CD4$^+$ T cells. Cells that bind were enriched with magnetic beads and stained with Foxp3 and CD25 antibodies using protocols as previously described (20, 21) (Fig. 1 *A* and *B*). Notably, although HA tetramers typically label many more T cells than gp100 or HIV tetramers, Tregs that are HA specific are nearly undetectable in most individuals (Fig. 1*C*). Because the numbers of specific Tregs detected are small, we sought to validate differential Foxp3 expression using a complementary approach. HA, gp100, and HIV tetramer-labeled cells were sorted by single-cell approach and analyzed for Foxp3 gene expression by real-time PCR. Foxp3 gene transcripts were detected in 4/278 HA-specific T cells, 7/52, HIV-specific T cells, and 11/129 gp100-specific T cells (Fig. 1*D*). These data are consistent with flow-based protein-level analysis and demonstrate that peripheral Tregs may be quite rare for certain antigen-specific populations.

To more broadly survey the peripheral Treg repertoire, we examined T cells that recognize a peptide from tetanus toxin or self-peptides from fibrinogen (Fib) or preproinsulin (PPins). Additional precursor specificities to pp65 from cytomegalovirus (CMV) or VP16 protein of herpes simplex virus (HSV) from uninfected individuals were also analyzed. These T-cell epitopes have been previously described and were selected based on T-cell stimulation and evidence that they are the product of natural processing and presentation (21). Tregs generally range from 5% to 10% in bulk CD4$^+$ T cells, but the antigen-specific T cells characterized here have Treg frequencies across a wider range, spanning from 0.8% to 14.4% (Fig. 2*A*). Past studies in mice have demonstrated that higher affinity for self-antigens promotes the selection of a thymocyte into the Treg lineage (14, 15). To determine whether a larger proportion of self-reactive T cells in the periphery are Foxp3$^+$, we separated Tregs into two groups by their ability to recognize a self or nonself peptide and compared their frequencies. We find that, whereas the mean frequencies of self-reactive Tregs is higher than that of T cells specific for foreign antigens, this difference is mostly driven by HA-specific T cells and is diminished after excluding this population (Fig. 2 *B* and *C*). Thus, Tregs are present in all specific populations we analyzed, but there is a wide range of variation. Certain microbe-specific T cells are mostly Foxp3$^-$, whereas a robust Foxp3$^+$ population similar to the frequency in self-reactive T cells can be detected in T cells for other foreign antigens.

Here, we equate Foxp3 expression with Treg lineage in cells that have been isolated directly ex vivo. However, because activated T cells in humans may transiently express Foxp3 (23, 24), we sought to confirm Treg characteristics of tetramer-labeled T cells by response to T-cell stimulation. Antigen-specific T cells were stimulated with phorbol myristate acetate (PMA) and ionomycin to assay for IL-2 and IFN-γ secretion. Analysis of HIV-specific

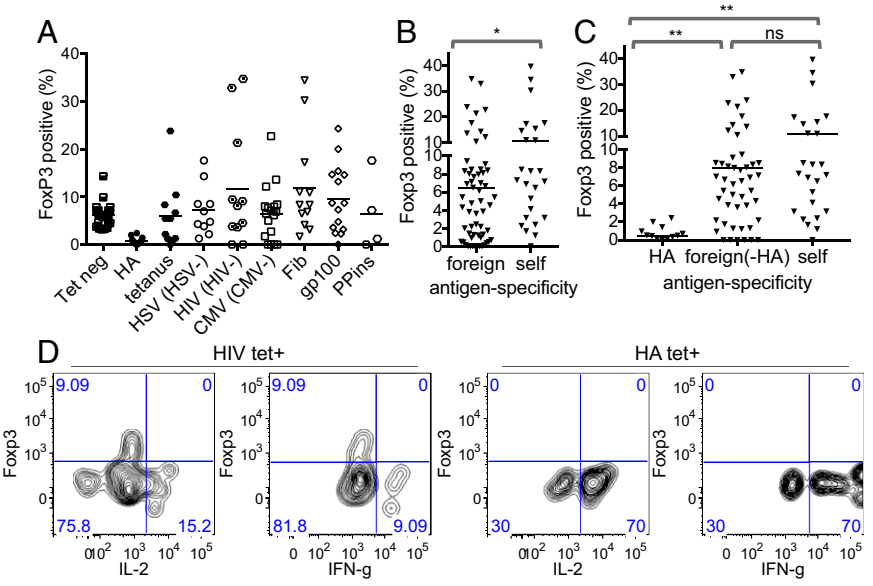

Fig. 2. Tetramer$^+$Foxp3$^+$ cells do not produce IL-2 or IFN-γ in response to T-cell stimulation. (*A*) Percentage of Foxp3$^+$ cells within each tetramer-tagged population. Blood donors are negative for HSV, HIV, and CMV infection by antibody tests. Each symbol represents an antigen-specific population from one individual, and the bar indicates the mean of experiments performed independently with blood obtained at different times. (*B*) The percentage of Foxp3$^+$ cells among T cells recognizing foreign (HA, tetanus, HSV, HIV, CMV) or self-antigens (Fib, gp100, PPins). *$P < 0.05$. (*C*) Foreign antigen-specific T cells are separated into HA alone (HA) or the remaining specificities [foreign(-HA)]. Statistical significance was analyzed using a *t* test. **$P < 0.005$. (*D*) CD4$^+$ T cells were stimulated with PMA and ionomycin for 4 h before tetramer labeling and intracellular staining for cytokine production. Data is representative of experiments from six individuals.

T cells showed that Foxp3$^+$ cells do not produce either cytokine, in contrast to the high levels of IL-2 and IFN-γ production by Foxp3$^-$ cells (Fig. 2*D*). Furthermore, we performed gene expression analysis comparing HA-specific T cells with self-reactive T cells that recognize gp100 and fibrinogen using the Fluidigm Biomark system. In total, we sorted 46 HA-specific T cells, 22 fibrinogen-specific T cells, and 36 gp100-specific T cells from three individuals into 96-well plates and analyzed each cell for 42 cell-lineage and activation-related gene transcripts (25, 26). Differential gene expression was assessed by Wilcoxon rank-sum test and adjusted for multiple comparisons using the Benjamini–Hochberg method. Consistent with low Foxp3 protein expression in HA-specific T cells, none of the 46 HA-specific T cells expressed the Foxp3 transcript. In addition, we also found lower expression of other Treg-associated genes in these cells, including BLIMP1, HELIOS, CD25 (IL2RA), IRF4, PDE3B, and TGFβ1 (Fig. 3*A*). The variation in this dataset was further distilled using principal component analysis (PCA). A PCA plot showed a clear separation of HA-specific T cells from self-reactive lymphocytes in the PCA space, and the greatest variance tracked with several Treg-associated genes (Fig. 3 *B*–*D*). Thus, in addition to the absence of proinflammatory cytokine response to T-cell stimulation, antigen-specific Foxp3$^+$ T cells from healthy human PBMCs also exhibit the typical features of Tregs by the expression of characteristic genes.

HA-Specific Tregs Are Detectable in the Cord Blood. The frequency of HA-specific Tregs is notably low by multiple measurements in adult blood. We hypothesize that this may be the result of coming into contact with HA by flu infection and/or vaccination. To examine the effect from antigen exposure, we also analyzed umbilical cord blood to determine the frequency of influenza-specific Tregs that are present at birth. Five DR4$^+$ umbilical cord blood samples were identified and the characteristics of HA, gp100, and HIV–specific T cells were analyzed and compared with those of adult blood cells. Because the availability of cord blood cells is more limited, Tregs in this set of experiments were identified by high CD25 and low CD127 surface staining (CD25^{hi}CD127^{lo}) to minimize cell loss from fixation and permeabilization procedures necessary for Foxp3 staining (27). We then confirmed that these markers largely detect an equivalent population of T cells as by using Foxp3 (Fig. S1). As expected, the frequency of CD25^{hi}CD127^{lo} HA-specific T cells was low in adult PBMCs (Fig. 4*A*). In contrast, cord blood contains HA-specific Tregs in similar frequency as HIV- or gp100-specific T cells (Fig. 4*B*), suggesting that postnatal exposure to HA likely contributes to their diminished frequency in adult blood. We also examined the number of HA-specific Tregs and found this number to be comparable between the adult PBMCs and cord blood cells (Fig. 4*C*). Thus, HA-specific Tregs are present at birth and their low frequency in adult blood likely reflects a dyssynchronous response from preferential expansion of nonregulatory subsets.

LCMV Infection Reduces gp66-Specific Tregs and Inversely Correlates with Effector T-Cell Response. Our data on influenza-specific T cells suggest that peripheral exposure to antigens may modify the relative ratio of specific Tregs. Despite this example, we find that not all forms of antigen exposure result in reduced Treg frequency in the immune system of adults. We compared HSV-specific T cells between HSV-infected and -uninfected individuals and, whereas there is a trend toward a lower frequency of specific Tregs in HSV$^+$ blood donors, the retention of HSV-specific Tregs in some HSV-infected individuals was also observed (Fig. S2*A*). Influenza infections are acute, whereas HSV causes a chronic life-long infection. We hypothesize that the balance between regulatory and effector subsets for a given specificity could impact the effectiveness of protective immunity. Consistent with this hypothesis, HA-specific T cells are generally more abundant than HSV-specific T cells from the same individual (Fig. S2*B*). To determine how the abundance of specific Tregs relates to the effectiveness of pathogen clearance, we used the mouse model of LCMV to track Treg response following infection (28). This model was selected because acute or chronic infection can be induced by viral strains that differ only by 2 amino acids that do not affect the relevant T-cell epitopes (29). Thus, the same specific T cells can be examined in distinct infectious contexts and at different time points to provide information on Tregs dynamics following acute or chronic viral exposure. We used pMHC tetramers to identify T cells that recognize the major I-A^b-restricted CD4$^+$ T-cell epitope, gp66-77 and combined it with magnetic enrichment to examine rare preimmune precursors and gp66-specific Tregs post-Armstrong (acute) or post-clone 13 (chronic) LCMV infection (Fig. 5*A*). Here we found a rapid and sharp decline in the relative proportion of gp66-specific Tregs by 8 days following infection. The decrease in virus-specific Tregs was greater for self-limited Armstrong infection compared with chronic infection caused by clone 13 and reflects a change in both relative frequency and the numbers of specific Tregs detected (Fig. S3 *A*–*C*). No enrichment for Vβ5 expression was detected among Foxp3$^+$ gp66

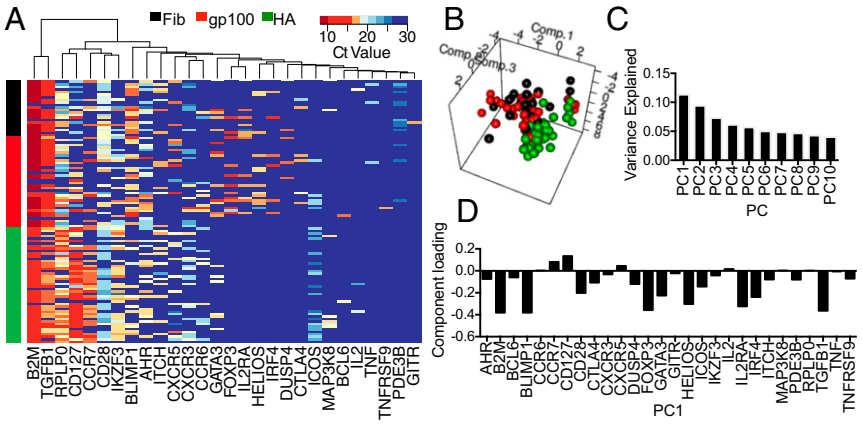

Fig. 3. HA-specific T cells have a distinct transcriptional profile and lack Treg signatures. HA, Fib, or gp100 tetramer-labeled T cells from three healthy subjects were individually sorted and analyzed for the expression of 42 cell lineage and activation-related gene transcripts using the Fluidigm BioMark system. (*A*) Heatmap showing single cell gene expression for Tet$^+$ cells. (*B*) PCA plot showing separate clustering of HA-specific T cells from Fib- and gp100-specific T cells. Each dot represents a single cell in the space of the first three components based on the PCA score. (*C*) The percent variation explained by the first 10 components. (*D*) The PCA parameter loadings (weighting coefficients) for the first component.

tetramer-labeled cells (Fig. S3*D*). We tested for this possibility because endogenous retroviral superantigens (SAGs) had been previously shown to induce expansion of Tregs enriched for Vβ5 usage in mice infected with clone 13 virus (30). The absence of Vβ5 enrichment suggests that Tregs detected in our study are unlikely to be driven by a SAG-mediated process. To further evaluate how antigen-specific Tregs might impact antiviral CD4$^+$ T-cell immunity, we examined the relationship between gp66-specific Tregs and gp66-specific Teff cells. We showed that the response to LCMV Armstrong is typically more robust and generates larger numbers of gp66-specific T cells that contain fewer Tregs, whereas a less robust expansion of gp66-specific population with a higher Foxp3$^+$ proportion is generally observed in clone 13 infected mice (Fig. 5*B*). A nonstatistically significant trend is also present between background level of Tregs (tetramer negative) and gp66-specific effector response, suggesting that by-stander Treg suppression from other LCMV specificities could also influence the antiviral response, but the effect is minor (Fig. S3*E*). Notably, infection by clone 13 results in a stable decline in gp66-specific Tregs that persists months after viral clearance, whereas this decrease in clone-13–infected mice appears to be short lived and rebounded 2–3 months after viral inoculation that coincides with contraction of Teff cells (Fig. 5*C*). These changes in specific Tregs reflect differences by relative proportions and also by the numbers of Foxp3$^+$ gp66-specific T cells (Fig. 5*D*). Together, these data suggest that pathogen exposure alters the balance between peripheral Treg and Teff cells in an antigen-specific context, and in some conditions, the effect may be long lasting and persist after the resolution of initial infection.

Antigen-Specific Treg Frequency Tracks with the Hierarchy of Memory Cells to Influenza Virus in Humans. The inverse correlation between the magnitude of antimicrobial response and Treg frequency remained stable after the effector phase of the response, suggesting that we may be able to examine this relationship in humans using T cells that recognize common microbial antigens to which exposure is nearly certain but the timing of contact is unknown. To test this possibility, T cells that recognize several influenza epitopes, tetanus toxin, and HSV in people seropositive for HSV infection were analyzed with the corresponding antigen-specific Tregs. Here we see that, similar to findings in mice, the population size of tetramer-labeled T cells inversely correlates with Foxp3 expression (Fig. 6*A*). Additionally, the proportions of memory cells within each specific population is inversely associated with Treg frequency, consistent with a role for antigen exposure and ligand recognition in modulating the abundance of microbe-specific Tregs in the periphery (Fig. 6*B* and Fig. S4). Because the magnitude of cellular immunity typically has a distinct pattern of hierarchy that focuses on a limited numbers of antigenic epitopes, we extended the analysis to determine whether Treg abundance predicts the immunodominance of a particular antigen-specific population. We chose to examine T cells that recognize three distinct epitopes from the influenza virus, HA, PB1, and PA. These influenza peptides have been previously characterized and were found to stimulate T-cell proliferation in vitro (31). We found a distinct hierarchy of cellular abundance across different individuals for these flu-specific populations. T cells that recognize HA are the most highly expanded, followed by PB1, then PA (Fig. 6*C*). Significantly, the order of T-cell frequency inversely correlates with Foxp3 expression within each population, which is the greatest for the least expanded PA-specific T cells (16%), lower in the next abundant PB1-specific T cells (1.5%), and lowest in the most highly expanded and antigen-experienced HA-specific T cells (0.8%) (Fig. 6 *C*–*E*). Taken together, these data indicate that the balance between Foxp3$^+$ and Foxp3$^-$ cells for a given

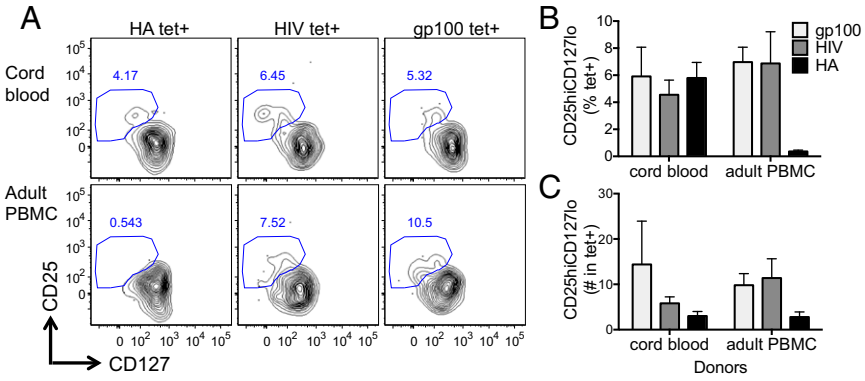

Fig. 4. Antigen-specific Tregs are identified in the cord blood and adult PBMCs. (*A*) Representative contour plots showing HA, HIV, and gp100 tetramer-labeled T cells in adult PBMCs or cord blood. Five adult PBMC and five cord blood samples were assayed in parallel. Tregs are identified by high CD25 and low CD127 surface expression. (*B*) Summary of Treg percentage among T cells that recognize gp100, HIV, or HA in the cord blood and adult PBMCs. (*C*) The numbers of Tregs detected by gp100, HIV, or HA tetramers in the cord blood or adult PBMCs. Error bars represent SEM.

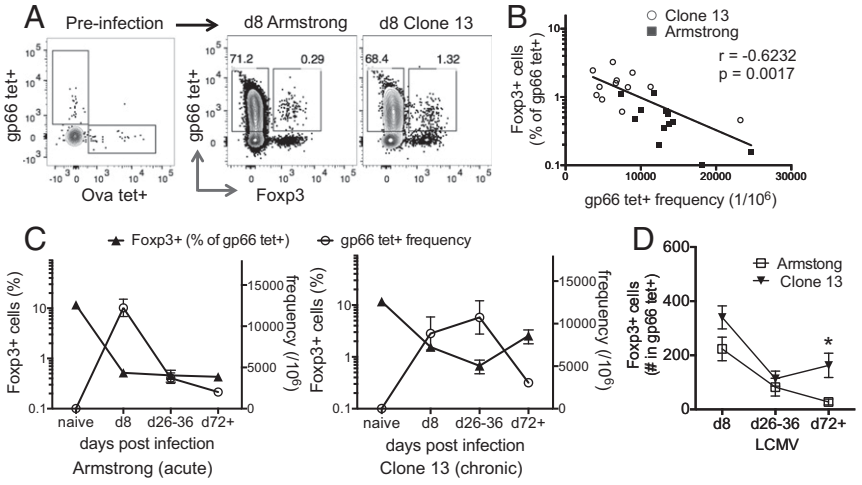

Fig. 5. LCMV infection reduces gp66-specific Tregs and inversely correlates with the effector T-cell response. (*A*) Representative flow plots of I-A^b gp66 tetramer staining preinfection and 8 days post-LCMV Armstrong (acute) or clone 13 (chronic) infection. (*B*) Scatterplot showing the correlation between the frequency and the percentage of Foxp3$^+$ cells within each gp66-specific population 8 days postinfection. (*C*) T-cell frequency and the corresponding Foxp3 expression for gp66-specific T cells at the indicated time points after infection with LCMV Armstrong (*Left*) or clone 13 (*Right*). (*D*) The number of gp66 tetramer-labeled Foxp3$^+$ T cells at each time point following Armstrong or clone 13 infection. Data represent 9–18 mice in each group performed at least three times. *$P < 0.05$; error bars represent SEM.

specificity is shaped by past antigen exposure and tracks with the hierarchy of T-cell dominance.

Discussion

The specificity of Tregs in a polyclonal repertoire remains a major unresolved question. Here, we analyzed Tregs in the blood of healthy adults using class II pMHC tetramers and find results that suggest Treg repertoire is broad and its breadth is dynamically maintained. We find that tetramers stain Foxp3$^+$ CD4$^+$ T cells that are authentic Tregs by analyzing their functional responses to T-cell stimulation and by single cell multiplex real-time PCR, which we performed because rare T-cell populations are difficult to assess using the standard Treg suppression assay or demethylation analysis of the Treg-specific demethylated region of *FOXP3*. The advantage of our approach is that we can directly assess the repertoire and characteristics of the human Tregs to self or microbial antigens. Whereas tetramers only capture the most avid T cells with a given specificity (32) and will miss some cells if Tregs recognize antigens differently from conventional T cells, the results are quite striking. Most specificities we surveyed had a significant fraction of typical Tregs based on the expression of characteristic genes and their response to T-cell stimulation. Notable exceptions are CD4$^+$ T cells that recognize two dominant influenza peptides, HA and PB1, which have very low frequencies of Foxp3$^+$ cells in many individuals. In contrast, for other T cells, such as those that recognize an HIV-1 epitope, there is little difference in the proportions of Foxp3$^+$ cells compared with those that recognize self-peptides. Whereas our result depends on the choice of T-cell specificities examined, it clearly demonstrates that a substantial fraction of most foreign or self-specific CD4$^+$ T cells have a Treg phenotype.

One possible explanation for the very low frequency of HA and PB1-specific Tregs might be that the thymus selects for a distinct Treg repertoire lacking T cells that recognize these specific epitopes. Alternatively, past studies have demonstrated selection of unique colonic Tregs by commensal bacteria (17), and exposure to specific antigens in the periphery may similarly alter Treg frequency in circulation. To evaluate these possibilities in our system, we used cord blood cells to approximate an antigen-naïve state. Our data support a role for peripheral antigen exposure and not deficiencies in the development of HA-specific Tregs. In newborns, Tregs that recognize HA are present in similar frequency as HIV and gp100-specific Tregs. Although adult blood contains fewer HA-specific Tregs by percentage, the actual numbers of specific Tregs are similar between adult PBMC and cord blood cells, suggesting that their low frequency is largely due to a relative expansion of conventional T cells in adult PBMCs. Whereas cord blood cells provide a valuable window into the prior exposure state, these cells are immature and may not accurately represent adult cells (33). Thus, we sought to further define the impact of antigen exposure on Treg and effector T-cell response using LCMV infection in mice. We show that the frequency of gp66-specific Tregs decreased following infection, and the trajectory of this decline differs for acute or chronic infections. For mice infected with a self-resolving viral strain, there is a greater and more stable decrease in gp66-specific Tregs. In contrast, gp66-specific Tregs are maintained at a higher level throughout the course of clone 13 infection and increased further with prolonged viral persistence. Thus, the proportions of Tregs and Teff cells appear to be in a dynamic balance and this ratio correlates with the effectiveness of protective antiviral immunity.

By analyzing specific Tregs, our findings also provide insight into how TCR specificity relates to suppressive function. Activated Tregs can suppress the proliferation and activity of effector T cells that recognize a different specificity (34, 35). What remains less clear is whether the suppression is more effective when Tregs and Teff cells recognize the same antigen. Evidence for the latter includes in vitro studies that showed the potency of suppression is higher when Teffs and Tregs are matched for antigen specificity (36). In vivo, transfer of Tregs specific for an islet autoantigen is more effective at preventing islet inflammation than polyclonal Tregs in a mouse model of type I diabetes (37). Recently, Moon and colleagues described Cre-specific Tregs that increase with respect to effectors of the same specificity when Cre is expressed in the lungs or intestinal tissues of mice (4). Ablating Tregs in these mice generally made the Cre-specific effectors more reactive, suggesting that Tregs can act to control cognate effectors. Consistent with these findings in mice, studies using cells from patients with colorectal cancer demonstrated that the effect of depleting Tregs appears to be antigen dependent and impacts the expansion of some, but not all, antitumor specificities (38). We add to these studies and show an inverse relationship between the abundance of LCMV-specific Tregs and the magnitude of the cognate antiviral effector response. Our work also extends beyond murine models of infection to cells directly analyzed from human PBMCs. Although the number of specific Tregs that can be isolated from blood is insufficient to test antigen specificity of suppressive activity in vitro, ex vivo analysis of antigen-specific T cells shows that the ratio between Foxp3$^+$ and Foxp3$^-$ T cells correlates with the abundance of cells to distinct influenza epitopes, suggesting that the availability of specific Tregs could also impact the hierarchy of T-cell dominance on a more global level.

Changes in the cytokine milieu may explain the decrease in specific Tregs. For example, the type I IFN response induced by LCMV infection has been shown to directly inhibit Treg cell activity, proliferation, and impede viral clearance (39). Tregs specific for gp66 were not particularly expanded in this study, but

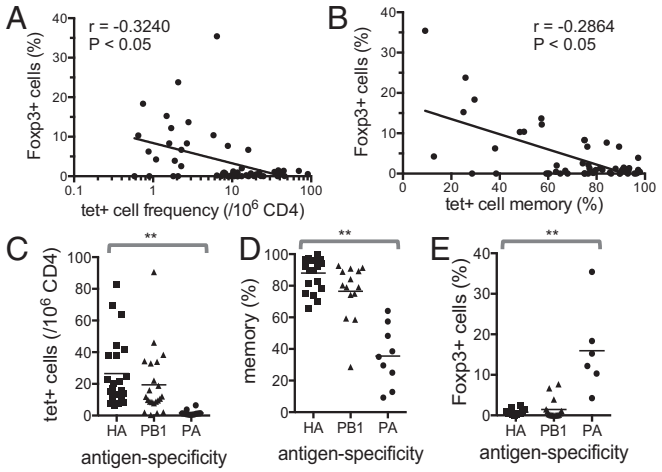

Fig. 6. Treg frequency is inversely associated with immunodominance hierarchy. (*A* and *B*) Scatterplot showing correlation between Foxp3 expression and the frequency or the percentage of memory cells that recognize the same peptide from influenza virus, tetanus, and HSV (in HSV seropositive donors). (*C–E*) HA, PB1, and PA-specific T cells were identified by tetramer enrichment from healthy individuals and stained for CD45RO and Foxp3 expression. Each symbol represents an antigen-specific population from one individual, and the bar indicates the mean. $^{**}P < 0.005$. Statistical significance was analyzed using a *t* test.

the interpretation may be complicated by the paucity of total gp66-specific T cells in the absence of IFN. Selective pruning of Tregs has also been observed in mice infected with *Mycobacterium tuberculosis*, which is driven, in part, by IL-12 induction of T-bet (40). In addition, Belkaid and colleagues have used a lethal *Toxoplasma gondii* infection model to show that Treg cell numbers decrease in the context of a strong inflammatory signal via a shutdown of IL-2 and enhanced IFN-γ signaling (41). In addition to mechanisms that directly inhibit Treg cells, selective tissue sequestration may also contribute to the disappearance of certain specific Tregs in the blood in humans and lymphoid tissues in mice. Tregs could also convert into other cell fates in an inflammatory environment and this may be another mechanism that could lead to a decrease in the frequency and the numbers of gp66-specific Tregs after LCMV infection (42). These mechanisms likely also contribute to Treg homeostasis in humans, and our data further point to the importance of effector response in determining the balance between Tregs and nonTreg subsets. Arguably, the most interesting aspect of our data is the implication that circulatory Treg repertoire is dynamic and the relative abundance of specific Tregs can be changed by antigen exposure during the life of an individual. Additionally, our data showing a stable decrease in gp66-specific Tregs many months after acute LCMV infection suggest that certain transient exposures may leave an imprint on the Treg repertoire that are stable and long lasting.

In summary, our survey of the antigen-specific Treg repertoire shows that it includes both self and foreign specificities in the peripheral blood. We also find a key role for antigen exposure in decreasing antigen-specific Treg frequencies in the midst of a major T-cell response or with chronic exposure. These changes likely shift the balance in favor of effector T cells to generate a more vigorous response to microbial infection and/or vaccination. These data suggest that interventions that decrease specific Treg frequencies versus cognate effectors could potentiate effector T-cell responses in cancer immunotherapy, infectious diseases, or vaccination.

Materials and Methods

PBMCs were from deidentified DR4$^+$ blood donors from the Stanford Blood Center. Informed consents were obtained and study subject recruitment was conducted in accordance with the rules and regulations of the Stanford Institutional Review Board. Human HLA-DR4 umbilical cord blood was purchased from AllCells and from the New York Blood Center. C57BL/6J mice were purchased from The Jackson Laboratory. Mice were infected with either Armstrong (Arm) (2×10^5 pfu) intraperitoneally or clone 13 (2×10^6 pfu) intravenously. All mice were used in accordance with University of Pennsylvania Institutional Animal Care and Use Committee guidelines. Tetramer analyses were performed according to standard protocol as previously described (21). For identification of Tregs, cells were stained with anti-CD25 and anti-CD127 antibodies and/or anti-CD25 and anti-Foxp3 antibodies. Single cell gene expression analyses were performed according to manufacturer instructions (CellDirect, Invitrogen) with TagMan Gene Expression Assay (Applied Biosystems).

Additional information about the experimental methods may be found in *SI Materials and Methods*.

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