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Requirement for Diverse TCR Specificities Determines Regulatory T Cell Activity in a Mouse Model of Autoimmune Arthritis

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CD4^+CD25^+Foxp3^+ regulatory T cells (Tregs) are required to restrain the immune system from mounting an autoaggressive systemic inflammatory response, but why their activity can prevent (or allow) organ-specific autoimmunity remains poorly understood.  We have examined how TCR specificity contributes to Treg activity using a mouse model of spontaneous autoimmune arthritis, in which CD4^+ T cells expressing a clonotypic TCR induce disease by an IL-17–dependent mechanism.  Administration of polyclonal Tregs suppressed Th17 cell formation and prevented arthritis development; notably, Tregs expressing the clonotypic TCR did not.  These clonotypic Tregs exerted Ag-specific suppression of effector CD4^+ T cells using the clonotypic TCR in vivo, but failed to mediate bystander suppression and did not prevent Th17 cells using nonclonotypic TCRs from accumulating in joint-draining lymph nodes of arthritic mice.  These studies indicate that the availability of Tregs with diverse TCR specificities can be crucial to their activity in autoimmune arthritis.  The Journal of Immunology, 2012, 188: 000–000.

Both mice and humans that congenitally lack Foxp3 develop a severe autoimmune disorder characterized by widespread lymphocytic infiltration, and elimination of CD4^+Foxp3^+ T cells from adult mice similarly precipitates a fatal systemic pathology (1–3).  These findings have indicated that CD4^+CD25^+Foxp3^+ regulatory T cells (Tregs) can play a dominant role in regulating autoreactive lymphocytes.  However, many autoimmune diseases develop despite the presence of Tregs, and why disease develops despite their presence is currently unknown (4, 5).  Tregs can be generated intrathymically based on interactions between the thymocyte TCR and self-peptides, and one possibility is that affected individuals produce Treg TCR repertoires lacking specificities that are required for recognition of critical disease-related self-peptides (6).  TCR specificity can play a role in determining Treg function, as targeting Tregs to particular autoantigens could suppress disease in mouse models of diabetes, experimental autoimmune encephalitis, and autoimmune ovarian disease (7–10).  However, there is also evidence that Tregs can exert regulatory activity via bystander mechanisms (11), and the extent to which Treg recognition of disease-related autoantigens is required to prevent autoimmune disease remains poorly understood.  One reason that it has been difficult to assess the role of TCR specificity in directing Treg activity in autoimmune settings is that in many cases the Ags that are recognized are poorly defined.

Studies in rheumatoid arthritis (RA) patients have made the seemingly paradoxical observation that arthritic individuals typically possess elevated frequencies of CD4^+CD25^+ Tregs at an inflammatory site (i.e., in the synovial fluid of arthritic joints) (12–14).  In vitro suppression studies have shown that exposure to TNF-α (which is elevated in RA patients) can impede CD4^+CD25^+ Treg function, and Tregs isolated from RA patients undergoing infliximab (anti–TNF-α) treatment exhibited improved suppressor function (15–17).  These studies have led to the suggestion that Tregs might be incapable of mediating suppression in the inflammatory environment of RA as an explanation for their ineffectiveness in preventing disease despite their high frequency.  Consistent with the findings in RA patients, CD4^+CD25^+ Tregs are present in the synovial fluid, joints, and joint-draining lymph nodes (LNs) in murine models of inflammatory arthritis (18, 19).  Murine models have also been used to show that Treg deficiency can impact arthritis development; thus, K/B × N mice that had been crossed to scurfy mice to eliminate Foxp3-expressing cells developed an accelerated and more severe arthritis than did conventional K/B × N mice (19).  In the collagen-induced arthritis model, Ab depletion of CD4^+CD25^+ Tregs results in increased disease severity, and prophylactic transfer of exogenous CD4^+CD25^+ Tregs could ameliorate arthritis (20, 21).  Although these mouse models have provided evidence that Treg activity can affect arthritis development in some settings, it remains unclear why the Tregs that are present in diseased mice and humans fail to prevent arthritis from developing.  Whether and how the Treg repertoire might be able to be manipulated to prevent arthritis development is similarly not understood.

In this study, we examine how TCR specificity affects the ability of Tregs to suppress autoimmune arthritis in a mouse model in which CD4^+ T cell recognition of a systemically expressed neo–self-peptide drives disease (22).  We show that exogenously administered polyclonal Tregs suppress arthritis, demonstrating that increasing the representation of Tregs in prearthritic mice can prevent disease.  Unexpectedly, however, Tregs that are specific for
the surrogate autoantigen do not prevent arthritis; even though they mediate effective Ag-specific suppression in vivo, they fail to exert bystander suppression and do not prevent the accumulation of effector cells using nonclonotypic TCRs in the draining LNs of arthritic mice. This study provides novel insights into the role that TCR specificity plays in directing Treg activity in vivo, and may shape their use as therapeutic approaches to autoimmune disease treatment.

Materials and Methods

Mouse strains

TS1, HA28, HACII, TS1 × HA28, and TS1 × HACII mice have been previously described (22–27) and backcrossed with BALB/c mice for at least 10 generations. Foxp3GFP mice (28) are on a BALB/c background and were purchased from The Jackson Laboratory. TcrΔΔΔ mice (29) were bred to homozygosity for the H-2d haplotype and then backcrossed at least four generations with BALB/c mice before breeding with HACII transgenic mice. All mice were maintained in specific pathogen-free conditions in the Wistar Institute Animal Facility, and experiments were conducted under protocols approved by the Wistar Institute Institutional Animal Care and Use Committee.

Assessment of arthritis

Mice were assessed weekly for signs of arthritis for a minimum of 9–10 wk. Distal joints were examined for limb swelling (visual assessment and measurement with micrometer caliper) and mice were assigned a score based on one of two indices: A) 0, no arthritic limbs; 1, one arthritic limb; 2, two arthritic limbs; 3, three arthritic limbs; and 4, arthritis in all four limbs; or B) 0, no visible swelling or discoloration; 1, visible swelling but no discoloration; and 2, severe swelling accompanied by skin discoloration. In scoring model B, the minimum score per mouse is 0 (no affected limbs) and the maximum score per mouse is 8 (four limbs with a value of 2).

Flow cytometry

Single-cell suspensions of LNs (either popliteal or pooled axillary, brachial, cervical, and mesenteric LNs) or spleens were stained with Abs at 4°C for 30 min. The following Abs were purchased from eBioscience: anti-CD4 PE-Cy7, allophycocyanin, allophycocyanin-eFluor780 (L3T4); anti-CD25 PE, PerCp-Cy5.5 (PC61); anti-CD44 PE (eBio17B7). Anti-CD69 PE, PE-Cy7, allophycocyanin (XMG1.2); anti–IFN-γ PE, PE-Cy7, allophycocyanin (eBio17B7). Anti-CD69 PE (H1.2F3) was purchased from BD Pharmin- gen. Anti–6.5-biotin (27) was detected using streptavidin-allophycocyanin (eBioscience) or streptavidin-Qdot655 (Invitrogen). Intracellular Foxp3 was detected according to the eBioscience protocols. Samples were col- lected on a FACSCalibur or LSR II flow cytometers (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Cell sorting

Single-cell suspensions from LNs or spleens were stained with Abs at 4°C for 30 min and sorted using a DakoCytomation MoFlo or a BD FACSAria cell sorter (BD Biosciences).

Adaptive transfers

Purified cells (0.5–1 × 10⁶) in sterile PBS were injected into the tail veins of 5- to 6-wk-old recipient mice, or i.p. into 2-d-old neonatal mice.

Intracellular cytokine staining

Cells were incubated in supplemented IMDM with 10% FBS, 50 ng PMA (Sigma-Aldrich), 1 µM ionomycin (Sigma-Aldrich), and a 1:1000 dilution of brefeldin A (eBioscience) for 4 h. Cells were then stained with cell surface Abs, fixed, permeabilized, and stained with anti-cytokine Abs. In cases where intracellular cytokine staining was analyzed in conjunction with surface staining for the 6.5 TCR, the amounts of PMA and ionomycin used were titrated to achieve optimal cytokine staining with minimal TCR downmodulation.

Anti–IL-17 Ab treatment

Mice were treated from 5 to 14 wk age with three i.p. injections per week of 150 µg anti–IL-17 Ab (clone M210; provided by Amgen) or rat IgG2a isotype control Ab.

Statistical analysis

The numbers of arthritic limbs among cohorts were analyzed using the Mann–Whitney U test. Other analyses were conducted using the unpaired two-tailed Student t test.

Results

Arthritic TS1 × HACII mice contain CD4⁺CD25⁺Foxp3⁺ Tregs

TS1 × HACII mice express a TCR transgene (detected by the mAb 6.5) that recognizes the major I-Ek-restricted T cell determin-ant (termed S1) from the influenza virus PR8 hemagglutinin (HA) (27), and they coexpress PR8 HA as a self Ag under the control of an MHC class II I-Ek promoter (25). We previously reported that thymocytes expressing the clonotypic 6.5⁺ TCR undergo extensive deletion in TS1 × HACII mice, and that despite this deletion, a population of 6.5⁺CD4⁺ T cells expressing activated phenotypes can be found in the spleens and LNs (Fig. 1A) (22). Moreover, most TS1 × HACII mice develop spontaneous inflammatory arthritis with overt articular manifestations becoming apparent in 10- to 12-wk-old mice (Fig. 1B, 1C and Ref. 22). Importantly, we also showed that arthritis develops in TS1 × HACII.JH⁻⁻ mice (which congenitally lack B cells), indicating that neither B cells nor Ab is required (22). Arthritis also develops despite the presence of CD4⁺CD25⁺Foxp3⁺ Tregs, which comprise a similar or greater proportion of CD4⁺ T cells as are present in control TS1 mice (Fig. 1D), and are found in similar pro- portions in young (prarthritic) and older (arthritic) TS1 × HACII mice (Fig. 1E). The CD4⁺CD25⁺Foxp3⁺ T cells in arthritic TS1 × HACII mice included a population that expresses the 6.5 TCR, although the proportion of CD4⁺Foxp3⁺ cells expressing the 6.5 TCR was substantially lower than in TS1 mice (Fig. 1F). Thus, TS1 × HACII mice spontaneously develop inflammatory arthritis despite the presence of CD4⁺CD25⁺Foxp3⁺ Tregs, although Tregs expressing the 6.5 TCR (which confers specificity for the surrogate autoantigen S1) appear to be relatively underrepresented.

Polyclonal Tregs prevent arthritis development in TS1 × HACII mice

Because TS1 × HACII mice develop autoimmune disease and appear to have limited representation of Treg cells expressing the clonotypic 6.5 TCR, we wondered whether increasing the repre-sentation of Tregs in prarthritic mice could prevent disease develop-ment. We first examined whether transfer of CD4⁺CD25⁺ T cells that are enriched in specificity for the S1 peptide could modulate arthritis development, because S1 is the target peptide that is recognized by autoreactive 6.5⁺CD4⁺ T cells in TS1 × HACII mice. As a source of S1-specific Tregs we used TS1 × HA28 mice, which also coexpress the HA self-Ag (at lower levels than in TS1 × HACII mice), and which contain a large population of 6.5⁺CD4⁺CD25⁺Foxp3⁺ T cells that develop intrathymically in response to the S1 peptide (24). CD4⁺CD25⁺ T cells from TS1 × HA28 mice are heavily enriched for 6.5⁺Foxp3⁺ cells and can exert regulatory function in response to S1 peptide both in vitro (Supplemental Fig. 1) and in vivo (30, 31). Accordingly, 1 × 10⁶ CD4⁺CD25⁺ T cells from TS1 × HA28 mice were transferred into 5- to 6-wk-old prarthritic TS1 × HACII mice; however, no effects on arthritis development were observed, since the average number of arthritic limbs per mouse did not differ from unma-nipulated TS1 × HACII mice at any of the examined time points, and in both cohorts most mice developed arthritis (Fig. 2A).

We also examined whether the administration of polyclonal CD4⁺CD25⁺ Tregs (i.e., Tregs that are not enriched in specificity for the S1 peptide) to prarthritic TS1 × HACII mice might modify arthritis development. We obtained these cells from two
sources: HACII mice, which express the same HA transgene as TS1HACII mice in the context of an otherwise unmanipulated T cell repertoire (and which are isogenic with respect to the self-peptides that are present in TS1HACII mice), and BALB/c mice. Strikingly, TS1HACII mice that had received $1 \times 10^6$ polyclonal CD4+CD25+ T cells from either HACII or BALB/c mice possessed significantly fewer arthritic limbs per mouse, and a significantly lower fraction of these mice developed arthritis than was the case for unmanipulated TS1HACII mice (Fig. 2B, 2C). Collectively, these data demonstrate that arthritis development in TS1HACII mice can be prevented by administration of exogenous polyclonal CD4+CD25+ T cells, including those from BALB/c mice that cannot have undergone any form of repertoire selection in response to S1 as a self-peptide. Moreover, similar numbers of CD4+CD25+ T cells enriched in specificity for the S1 Ag were ineffective.

**Polyclonal Tregs inhibit an arthritogenic Th17 response**

To examine how polyclonal CD4+CD25+ Tregs modulate arthritis development in TS1 × HACII mice, we first compared the number and phenotype of CD4+ T cells from unmanipulated arthritic TS1 × HACII mice with those from TS1 × HACII mice that had received 1 $\times$ 10^6 polyclonal CD4+CD25+ T cells from either HACII or BALB/c mice possessed significantly fewer arthritic limbs per mouse, and a significantly lower fraction of these mice developed arthritis than was the case for unmanipulated TS1 × HACII mice (Fig. 2B, 2C). Collectively, these data demonstrate that arthritis development in TS1 × HACII mice can be prevented by administration of exogenous polyclonal CD4+CD25+ T cells, including those from BALB/c mice that cannot have undergone any form of repertoire selection in response to S1 as a self-peptide. Moreover, similar numbers of CD4+CD25+ T cells enriched in specificity for the S1 Ag were ineffective.

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**S1-specific Tregs fail to suppress arthritis development in TS1 × HACII mice**

Because CD25 is also expressed on activated T cells, we crossed TS1 × HA28 mice with Foxp3EGFP mice (28) and used GFP expression as an additional parameter to more stringently purify CD4+CD25+Foxp3+ Tregs. Administration of CD4+CD25+GFP+ T cells from TS1 × HA28.Foxp3EGFP mice (half of which express the 6.5 TCR) to 5- to 6-wk-old prearthritic TS1 × HACII mice affected neither the incidence nor the severity of arthritis, as had been seen in mice that received CD4+CD25+ T cells from TS1 ×
HA28 mice (Fig. 4A). Moreover, GFP-expressing CD4\(^+\)Foxp3\(^+\) T cells were present in the LNs and spleens of the recipient mice 9–10 wk after the initial transfer, indicating that the donor Tregs had engrafted the recipient TS1 × HACII mice (Fig. 4A). We next examined whether administering 6.5\(^{+}\)CD4\(^+\)CD25\(^+\)GFP\(^{+}\) T cells from TS1 × HACII mice to 2-d-old neonatal TS1 × HACII mice might affect arthritis development. By purifying cells based on 6.5 expression, we ensured that the Tregs were heavily...
enriched for specificity for the S1 peptide; however, here, too, the provision of Tregs failed to affect arthritis development, and the degree of engraftment with GFP-expressing CD4+Foxp3+ cells 15 wk after Treg transfer was similar to that seen in 5- to 6-wk-old recipient TS1 × HACII mice (Fig. 4B). Lastly, we generated HACII.Foxp3EGFP mice and found that administration of polyclonal CD4+CD25+GFP+ Tregs from these mice to 5- to 6-wk-old recipient TS1 × HACII mice (Fig. 4B). As for (A), except that 1 × 10^6 CD4+CD25+GFP+ cells from HACII.Foxp3EGFP mice were transferred into 5- to 6-wk-old recipient TS1 × HACII mice. (C) As for (A), except that 1 × 10^6 CD4+CD25+GFP+ Tregs from HACII.Foxp3EGFP mice were transferred into 5- to 6-wk-old recipient TS1 × HACII mice.

FIGURE 4. Polyclonal and Ag-specific Foxp3+ Tregs engraft TS1 × HACII mice but have differing abilities to prevent arthritis development. (A) CD4+CD25+GFP+ T cells (1 × 10^6) from TS1 × HA28.Foxp3EGFP mice were transferred into 5- to 6-wk-old prearthritic TS1 × HACII mice. Graph shows the arthritis score per mouse; lines represent means. Dot plots show GFP expression versus anti-Foxp3 mAb staining (numbers indicate percentages) by CD4+ cells in recipient mice and in a TS1 × HACII mouse that did not receive GFP+ cells. Average percentages (±SEM) of GFP+Foxp3+ cells are shown in graph. (B) As for (A), except that 0.5 × 10^6 6.5+ CD4+CD25+GFP+ T cells from TS1 × HA28, Foxp3EGFP mice were transferred into 2-d-old neonatal TS1 × HACII mice. (C) As for (A), except that 1 × 10^6 CD4+CD25+GFP+ Tregs from HACII.Foxp3EGFP mice were transferred into 5- to 6-wk-old prearthritic TS1 × HACII mice.

S1-specific Tregs can mediate Ag-specific suppression in vivo CD4+CD25+ T cells from TS1 × HA28 mice are enriched in specificity for the S1 peptide, and it was unexpected that these cells would be unable to suppress arthritis development in TS1 × HACII mice. One reason could be that these cells are for some
reason incapable of modulating CD4+ T cell responses to the S1 self peptide that is expressed in HACII mice, even though they express Foxp3 and can suppress S1-specific T cell responses in vitro (Supplemental Fig. 1) and in other settings in vivo (30, 31). To examine this, we analyzed their ability to suppress the proliferation and differentiation of S1-specific effector CD4+ T cells (obtained from TS1.Tcrα/β Foxp3EGFP mice) following transfer into HACII.Tcrα/β−/− mice (which do not contain T cells). CD4+ CD45+CD25+GFP+ T cells from TS1.Tcrα/β Foxp3EGFP mice expressed high levels of the cell proliferation marker Ki-67 1 wk after transfer into HACII.Tcrα/β−/− mice (but not in BALB/c.Tcrα/β−/− mice), and most of these proliferating responder cells produced IFN-γ upon restimulation (Fig. 5A). When these responder cells were mixed with equal numbers of CD4+CD25+GFP+ T cells from TS1×HA28.Foxp3EGFP mice prior to transfer, there were substantial decreases in both the level of Ki-67 expression and in the production of IFN-γ by the effector cells in response to the S1 self-peptide in HACII.Tcrα/β−/− mice (Fig. 5A). Although there appeared to be a modest increase in the percentages of effector cells that could produce IL-17 (without coproducing IFN-γ) in the mice that received Tregs, the absolute number of these cells was quite small (because of reduced proliferation; data not shown). Thus, the Tregs from TS1×HA28 mice are capable of modulating the in vivo proliferation and differentiation of effector CD4+ T cells in response to the S1 self-peptide that is expressed in HACII mice.

**Limited acquisition of cytokine production by S1-specific Tregs in TS1 × HACII mice**

Tregs can under certain conditions acquire the ability to produce Th cell-associated cytokines, including IL-17 (35–37), so we examined whether the Tregs from TS1 × HACII mice might fail to suppress arthritis because they undergo differentiation to become cytokine-producing effector cells in response to S1 peptide in vivo. Substantially higher numbers of CD4+GFP+Foxp3+ T cells were recovered 1 wk after transfer of CD4+CD25+GFP+ Tregs from TS1×HA28.Foxp3EGFP into HACII.Tcrα/β−/− mice than from BALB/c.Tcrα/β−/− mice, reflecting expansion of these cells in response to the S1 peptide (Fig. 5B). Notably, the GFP+ Foxp3+ Tregs isolated from HACII.Tcrα/β−/− mice, but not those obtained from BALB/c.Tcrα/β−/− recipients, produced both IL-17 and IFN-γ following stimulation, with preferential production of IL-17 (Fig. 5B). This acquisition of IL-17 production appears to be a propensity of CD4+Foxp3+ T cells, because conventional CD4+ T cells isolated from TS1 mice (and also expressing the 6.5 TCR) were strongly polarized to produce IFN-γ, rather than IL-17, upon transfer into HACII.Tcrα/β−/− mice (Fig. 5A).

**FIGURE 5.** Limited differentiation of autoantigen-specific CD4+CD25+ Tregs in TS1 × HACII mice. (A) CD4+CD45+CD25+ GFP+ responder cells (1 × 10⁶) from TS1.Tcrα/β Foxp3EGFP mice were transferred with or without 1 × 10⁶ CD4+CD25+GFP+ Tregs from TS1 × HA28.Foxp3EGFP mice into HACII.Tcrα/β−/− or BALB/c.Tcrα/β−/− mice. Histogram shows Ki67 staining of CD4+ GFP+ responder T cells transferred alone or in the presence of GFP+ Tregs 1 wk after transfer. Contour plots show intracellular IL-17 and IFN-γ staining (numbers indicate percentages) among responder CD4+GFP+ T cells transferred without or with GFP+ Tregs into HACII.Tcrα/β−/− mice. (B) 6.5+CD4+CD25+GFP+ cells (1 × 10⁶) from TS1×HA28.Foxp3EGFP mice were transferred into HACII.Tcrα/β−/− or BALB/c.Tcrα/β−/− mice. Graph shows the number of GFP+Foxp3+ cells in the spleens 1 wk after transfer. Contour plots show IL-17 and IFN-γ production (numbers indicate percentages) by GFP+Foxp3+ cells. (C) CD4+CD25+GFP+ cells (1 × 10⁶) from TS1 × HA28.Foxp3EGFP mice were transferred into TS1 × HACII and BALB/c mice. Contour plots show GFP and Foxp3 Ab expression in CD4+ splenocytes 2 wk after transfer, and IL-17 and IFN-γ production by CD4+GFP+Foxp3+ cells (numbers indicate percentages). All results are representative of three independent experiments.
Because we had obtained evidence that S1-specific Tregs from TS1 × HA28.Foxp3EGFP mice are capable of becoming IL-17–producing CD4+Foxp3+ T cells in response to S1 peptide in vivo, we examined whether a similar process could be occurring when these cells are transferred into TS1 × HACII mice. Accordingly, CD4+CD25GFPP+ T cells from TS1 × HA28.Foxp3EGFP mice were transferred into prearthritic TS1 × HACII mice; 2 wk later, higher numbers of donor-derived GFP+Foxp3+ T cells were found in TS1 × HACII mice than in BALB/c controls, again reflecting expansion in response to the S1 peptide (Fig. 5C). In this case, however, a much smaller fraction of the GFP+Foxp3+ cells had acquired the ability to produce cytokines than had been observed in the HACII.Tcra−/− recipients, and of those that were cytokine producers, a greater proportion produced IFN-γ than IL-17 (contrasting the bias toward IL-17 production observed in HACII.Tcra−/− recipients) (Fig. 5A, 5C). Indeed, the frequency of cytokine-producing Foxp3+ T cells that were isolated from TS1 × HACII mice was only modestly higher than could be found in Foxp3+ cells from TS1 × HA28 mice, before transfer into the recipient mice (data not shown). Thus, despite a capacity to differentiate into IL-17–secreting T cells in response to S1 peptide that was observed in HACII.Tcra−/− recipients, there appeared to be only limited formation of Foxp3+ cytokine-secreting cells when CD4+CD25GFPP+ T cells from TS1×HA28.Foxp3EGFP mice as neonates (see Fig. 4B) with unmanipulated controls. The mice that had received Tregs as neonates contained increased frequencies of 6.5+CD4+Foxp3+ cells than were present in unmanipulated mice (Fig. 6A), consistent with the preceding studies showing that GFP+ cells could be recovered from the recipient mice (see Fig. 4B). There was also very little acquisition of cytokine production by 6.5+CD4+Foxp3+ T cells in TS1 × HACII mice that had received S1-specific Tregs as neonates (data not shown), consistent with the limited acquisition of cytokine production observed 1 wk after transfer into TS1 × HACII mice (see Fig. 5B). There was a smaller increase in the representation of 6.5+CD4+Foxp3− effector T cells in the LNs of mice that had received the S1-specific Tregs, and notably, the frequency of IL-17–secreting cells in this subset was substantially reduced relative to mice that had not received Treg cells, whereas the frequency of IFN-γ secretors was increased (Fig. 6B). Interestingly, this effect of the S1-specific Tregs was only observed among the CD4+Foxp3+ cells expressing the clonotypic 6.5 TCR; when we examined the nonclonotypic 6.5+CD4+Foxp3+ cells (which comprise ~75% of the CD4+ cells in the popliteal LNs of arthritic mice; Fig. 6A), no differences were seen in the frequencies of IFN-γ– or IL-17–secreting cells between mice that had or had not received Tregs (Fig. 6C).

Collectively, these data argue against the possibility that the 6.5+CD4+CD25GFPP+ T cells from TS1 × HA28.Foxp3EGFP mice fail to suppress arthritis because they undergo abundant differentiation into CD4+Foxp3+ IL-17+ effectors. Indeed, the representation of 6.5+CD4+Foxp3− IL-17+ T cells was significantly reduced in mice that had received 6.5+CD4+CD25GFPP+ T cells from TS1 × HA28.Foxp3EGFP mice, indicating that the formation of Th17 cells expressing the clonotypic 6.5 TCR had been suppressed by the 6.5+CD4+CD25GFPP+ T cells from TS1 × HA28.Foxp3EGFP mice. Notably, however, these Tregs did not prevent non–6.5-expressing CD4+IL-17+ T cells from accumulating in the joint draining LNs of arthritic TS1 × HACII mice.

**Discussion**

Most TS1 × HACII mice spontaneously develop an IL-17–driven inflammatory arthritis. Because arthritis develops despite the presence of CD4+CD25Foxp3+ Tregs, it was possible that some aspect of the inflammatory environment in TS1 × HACII mice prevents Foxp3+ Tregs from suppressing this disease. Using adoptive transfer approaches, we showed that Tregs can modulate immune responses in this environment; however, the specificity of the Tregs was critical, since polyclonal Tregs could prevent arthritis development, whereas Tregs enriched in specificity for the S1 self-peptide were ineffective, even when administered to neonatal mice.

The failure of Tregs from TS1 × HA28 mice to suppress arthritis development despite their enrichment in specificity for the S1 peptide is notable in several respects. First, the S1 peptide is a defined target Ag that is recognized by a major population of autoreactive CD4+ T cells in arthritic mice, since despite extensive thymic deletion, ~7% of the CD4+ T cells in TS1 × HACII mice express the 6.5 TCR (which confers specificity for the S1 peptide), and many of these cells are capable of cytokine production. Interestingly, although 6.5+CD4+ T cells in the popliteal LNs of TS1 × HACII mice that had received TS1 × HA28 Tregs as neonates produced significantly lower levels of the arthritogenic cytokine IL-17 compared with 6.5+CD4+ T cells in untreated mice (indicating that the TS1 × HA28 Tregs did indeed modulate the effector 6.5+CD4+ T cell response), there was no effect on arthritis development. In contrast, the presence of TS1 × HA28 Tregs had no effect on cytokine production by CD4+ T cells expressing allelically included (i.e., 6.5+) TCRs in TS1 × HACII mice. Second, the failure of Tregs from TS1 × HA28 mice to suppress arthritis development did not appear to be due to their differentiation into IL-17–secreting effector T cells. CD4+CD25Foxp3+ Tregs from TS1 × HA28 mice produced limited amounts of IL-17 following transfer into TS1 × HACII mice, and, as noted above, the popliteal LNs of TS1 × HACII mice that had received TS1 × HA28 Tregs actually contained significantly fewer 6.5+CD4+IL-17+ effector T cells than did untreated controls. Interestingly, 6.5+CD4+CD25Foxp3+ Tregs from TS1 × HA28 mice were capable of producing IL-17 following transfer into HACII.Tcra−/− mice, consistent with reports indicating that Tregs can under certain conditions acquire effector properties (38–40). Because a low frequency of IL-17–producing cells is present among the CD4+CD25GFPP+ population from TS1 × HA28.Foxp3EGFP mice when analyzed directly ex vivo (data not shown), it remains possible that these cells underwent preferential expansion, rather than being generated de novo following transfer into HACII.Tcra−/− mice. Whatever the basis, however, this process did not occur to any great extent when these cells were transferred into intact TS1 × HACII mice, perhaps because resources necessary for driving such an accumulation were unavailable or limiting. Third, because the S1 self-peptide (whose synthesis is under control of an I-E promoter) is expressed directly by MHC class II+ APCs (including B cells and dendritic cells), the Tregs from TS1 × HA28 mice should be readily able to interact with APCs in TS1 × HACII mice. Although the mechanisms by which Tregs can suppress immune responses in vivo remain poorly understood, there is evidence that they might do so at least in part by bystander mechanisms, such as modulating APC function (11, 41, 42).
However, the administration of Tregs that are enriched in specificity for a self-Ag that is abundantly expressed by MHC class II+ cells was insufficient to prevent arthritis from developing in this model, arguing against the possibility that Tregs act via their ability to modulate APC function in this system.

Because Tregs from TS13HACII mice were ineffective, why then were polyclonal Tregs from HACII and/or BALB/c mice able to suppress arthritis development in TS13HACII mice? It may be that the recognition of the systemically expressed S1 peptide by 6.5+CD4+ T cells initiates the autoimmune response, but that the effector CD4+ T cells that cause joint inflammation primarily use non-6.5 TCRs. Notably, the large majority of IL-17–producing 6.5+CD4+Foxp3+ cells in unmanipulated and Treg recipient TS13HACII mice (bars indicate mean).

Because Tregs from TS13HA28 mice were ineffective, why then were polyclonal Tregs from HACII and/or BALB/c mice able to suppress arthritis development in TS13HACII mice? It may be that the recognition of the systemically expressed S1 peptide by 6.5+CD4+ T cells initiates the autoimmune response, but that the effector CD4+ T cells that cause joint inflammation primarily use non-6.5 TCRs. Notably, the large majority of IL-17–producing 6.5+CD4+Foxp3+ cells in unmanipulated and Treg recipient TS13HACII mice (bars indicate mean).

In this regard, the endogenous Treg repertoire that develops in TS13HACII mice, and which fails to prevent arthritis development, may be qualitatively insufficient because it lacks certain requisite specificities that are necessary to suppress the formation of these IL-17–secreting effector CD4+ T cells. The endogenous Treg repertoire in TS13HACII mice is necessarily generated using non–transgene-encoded TCRs that arise through allelic inclusion principally of the alpha-chain locus (data not shown).

Because there is also substantial thymocyte deletion in young TS13HACII mice, one can imagine that the range of specificities that is present in Tregs from TS13HA28 mice is also likely limited in the range of specificities that are produced, because we have previously shown that non-S1-specific Tregs in TS13HA28 mice are generated through use of allelically included TCR alpha-chains paired

**FIGURE 6.** S1-specific Tregs do not suppress the formation of nonclonotypic Th17 cells in TS1×HACII mice. (A) 6.5 and Foxp3 staining of CD4+ cells (numbers indicate percentages) from popliteal LNs of 15-wk-old TS1×HACII mice that were unmanipulated (n = 11) or had received 5×10^6 6.5+CD4+CD25+GFP+ Tregs from TS1×HA28.Foxp3EGFP mice at 2 d age (n = 15) (from experiment in Fig. 4B). Graphs show the percentages of 6.5+CD4+Foxp3+ and 6.5+CD4+Foxp3− cells in unmanipulated and Treg recipient TS1×HACII mice (bars indicate mean). (B) Dot plots show IFN-γ and IL-17 production (numbers indicate percentages) by 6.5+CD4+Foxp3+ popliteal LN cells from unmanipulated and Treg recipient TS1×HACII mice. Graphs show the percentages of IFN-γ− and IL-17−producing 6.5+CD4+Foxp3+ cells (bars indicate mean).
with the transgenic Vβ8.2 chain (26). Thus, even though approximately half of the CD4+Foxp3+ T cells from TS1 × HA28 mice do not express the clonotypic 6.5 TCR, these nonclonotypic Tregs are likely to possess much less TCR sequence diversity than is the case for the polyclonal Tregs from BALB/c or HACII mice because they all use the same TCR Vβ-chain. We further note that we have not examined how administration of varying doses of Tregs from the different sources might affect their ability to suppress arthritis development, which may be significant if, for example, the different populations can exert regulatory effects by different mechanisms. However, it is clear that polyclonal Tregs from BALB/c or HACII mice are more effective than are Tregs from TS1 × HA28 mice when administered in similar numbers to prearthritic TS1 × HACII mice. These polyclonal Tregs may be protective because they provide a more diverse repertoire of TCR specificities than are present among Tregs from either TS1 × HACII or TS1 × HA28 mice, and because they are able to suppress disease by recognizing a variety of target peptides that are also recognized by the effector T cells.

The conclusion that TS1 × HACII mice develop arthritis at least partly because their Treg repertoire contains limited specificities is notable because CD4+IL-17+ T cells in the popliteal LNs of arthritic mice also appear to have been generated predominantly through use of allelically included TCR chains. Thus, under conditions in which both the effector and Treg populations were constrained, the effector population appeared to retain sufficient specificities to cause disease, and it was necessary to augment the Treg population with cells derived from unmanipulated mice to prevent arthritis development. A similar situation arises in mice that receive a d-3 thymectomy (d3Tx) and develop a systemic autoimmune disease that can be rescued by provision of polyclonal Tregs from unmanipulated mice (43). The frequencies of CD4+CD25+ and CD4+CD25− T cells are reduced ~10-fold in d3Tx mice relative to controls, and here, too, the TCR repertoires are likely constrained because they must be generated from only those cells that had populated the periphery in the first days of life (43). In this case also then, restriction of both the effector and Treg repertoires allows for the spontaneous development of autoimmunity, implying that conditions that limit the formation of both effector and Treg specificities can lead to an imbalance that favors autoimmunity.

An alternative explanation for the ability of polyclonal Tregs to protect against arthritis is that they contain a greater phenotypic heterogeneity than is found in the Tregs from TS1 × HA28 mice. There is increasing evidence that Foxp3+ T cells can exhibit distinct phenotypes (e.g., through expression of transcription factors and/or chemokine receptors) (44), and the polyclonal Tregs from HACII and BALB/c mice might contain populations expressing phenotypic characteristics that make them better able to populate TS1 × HACII mice and/or suppress the development of effector CD4+ T cells. In support of this, the polyclonal Tregs appeared to be better able to engraft the LNs (but not the spleens) of recipient TS1 × HACII mice than was the case for Tregs from TS1 × HA28 mice. If this is the case, however, the formation of these putative Treg populations appears to be at least partly linked to TCR specificity, because Tregs from TS1 × HA28 mice (in which the TCR repertoire has been manipulated to produce elevated numbers of 6.5 Tregs) must lack the appropriate phenotypic heterogeneity, since they fail to prevent arthritis.

As has been observed in other models of inflammatory arthritis (32–34), IL-17 is a critical effector cytokine in TS1 × HACII mice. Treatment with an anti–IL-17A mAb could prevent arthritis development, and, moreover, there were significant reductions in IL-17–producing CD4+ T cells in the LNs of mice that were protected from arthritis with polyclonal Tregs. However, how the specificity of IL-17–secreting effector cells contributes to arthritis development is less clear. IL-17–secreting 6.5 CD4+ T cells appeared to be underrepresented in the popliteal LNs, and Tregs from TS1 × HA28 mice could inhibit the accumulation of such cells without preventing disease. A process of “epitope spreading” has been described in a number of autoimmune settings (45), and it is possible that IL-17–secreting 6.5 CD4+ T cells cause disease because they use endogenous TCRs that recognize joint-specific self-peptides, including cryptic self-peptides that might be presented by unconventional APCs such as synoviocytes that become MHC class II in the inflamed joint (46). However, a recent study suggested that any Th17 cells that are able to traffic to the joints (regardless of Ag specificity) can induce arthritis development by acting as a local source of IL-17, and that direct recognition of joint Ags may not be required for arthritis to develop in such a setting (47). In this case, then, the key role of the 6.5 TCR in disease development could be in establishing conditions that favor Th17 cell formation; these could include the lymphopenic environment that develops in young TS1 × HACII mice as a consequence of severe thymocyte deletion (22), since lymphopenic environments have been found to contribute to Th17 cell formation in the SKG model of arthritis (34). In either event, the findings in this study extend studies showing that specificity for defined autoantigens can be a critical parameter in determining Treg function in autoimmune settings. Notably, however, whereas studies in diabetes, experimental autoimmune encephalitis, and autoimmune ovarian disease demonstrated that targeting a single autoantigen can result in disease suppression (7–10), our findings suggest that there can be disease settings, such as inflammatory arthritis, in which a diverse set of autoantigens may be involved in pathogenesis. In such a setting, suppressing the immune response to a single major autoantigen may not be sufficient to prevent arthritis development; rather, targeting a wide range of autoantigens may be required for disease suppression.

Disclosures
The authors have no financial conflicts of interest.

References


Figure S1  CD4⁺CD25⁺ cells from TS1×HA28 mice suppress in vitro T cell responses to S1 peptide. CFSE-labeled purified CD4⁺ T cells from TS1 mice were cultured with 1 μM of S1 peptide (SFERFEIFPKE) and irradiated BALB/c splenocytes, in the absence or presence of purified CD4⁺CD25⁺ or 6.5⁺CD4⁺CD25⁺ T cells from BALB/c, TS1 or TS1×HA28 mice at a 1:2 Treg:responder cell ratio. After 3 d of culture at 37° C, responder CD4⁺ T cell proliferation and cytokine production were analyzed by flow cytometry. (A) Dot plots show Foxp3 versus 6.5 expression on CD4⁺ LN cells from BALB/c, TS1 and TS1×HA28 mice. (B) Left column shows CD25 versus Foxp3 expression on CD4⁺ LN cells from BALB/c, TS1 and TS1×HA28 mice, and on 6.5⁺CD4⁺ LN cells from TS1 and TS1×HA28 mice. (C) Dot plots show CFSE versus 6.5 levels on responder CD4⁺ T cells from TS1 mice cultured for 3 d with APCs and 1 μM (left column) or 0.3 μM (right column) S1 peptide either alone, or with CD4⁺CD25⁺ or 6.5⁺CD4⁺CD25⁺ T cells from BALB/c, TS1 or TS1×HA28 mice as indicated by arrows. Numbers indicate the percentages of divided 6.5⁺CD4⁺ cells. n ≥ 3 for all experiments.