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The Degree of CD4+ T Cell Autoreactivity Determines Cellular Pathways Underlying Inflammatory Arthritis

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Although therapies targeting distinct cellular pathways (e.g., anticytokine versus anti–B cell therapy) have been found to be an effective strategy for at least some patients with inflammatory arthritis, the mechanisms that determine which pathways promote arthritis development are poorly understood. We have used a transgenic mouse model to examine how variations in the CD4+ T cell response to a surrogate self-peptide can affect the cellular pathways that are required for arthritis development. CD4+ T cells that are highly reactive with the self-peptide induce inflammatory arthritis that affects male and female mice equally. Arthritis develops by a B cell–independent mechanism, although it can be suppressed by an anti-TNF treatment, which prevented the accumulation of effector CD4+ Th17 cells in the joints of treated mice. By contrast, arthritis develops with a significant female bias in the context of a more weakly autoreactive CD4+ T cell response, and B cells play a prominent role in disease pathogenesis. In this setting of lower CD4+ T cell autoreactivity, B cells promote the formation of autoreactive CD4+ effector T cells (including Th17 cells), and IL-17 is required for arthritis development. These studies show that the degree of CD4+ T cell reactivity for a self-peptide can play a prominent role in determining whether distinct cellular pathways can be targeted to prevent the development of inflammatory arthritis. The Journal of Immunology, 2014, 192: 3043–3056.

Inflammatory arthritis is a debilitating manifestation of a variety of autoimmune disorders (including rheumatoid arthritis [RA]) that are often grouped together because disease develops in the context of systemic immune activation (1, 2). A common feature of these diseases is that susceptibility is strongly linked to certain MHC class II alleles, implying an important role for CD4+ T cells in disease pathogenesis (1–3). However, the extent to which CD4+ T cells participate in arthritis development through the promotion of proinflammatory cytokine production (either derived from T cells or from additional populations such as macrophages), and/or through the support of autoantibody production (such as rheumatoid factor or Abs to citrullinated proteins) remains unclear (1, 2). Moreover, in distinct mouse models of inflammatory arthritis, dysregulated cytokine production and autoantibody production have each been shown to drive disease pathology (4–8), and whether these differences in disease pathogenesis are caused by variations in the autoreactive CD4+ T cell response is currently not known. Mutations in CD4+ TCR signaling molecules have been found to alter the spectrum of disease manifestations that can arise in mouse models of autoimmunity (9, 10). However, the extent to which differences in TCR recognition of self-peptides by autoreactive CD4+ T cells might affect the cellular pathways that are required for arthritis development is not understood.

Extensive studies in human patients support the conclusion that CD4+ T cells can promote arthritis development via both cytokine- and B cell–dependent effector mechanisms. For example, anti-TNF reagents, which were the first biologic therapies developed for RA, have high response rates in RA patients (11, 12), and antagonists targeting other proinflammatory cytokines (including IL-1, IL-6, and IL-17) are also being evaluated for therapeutic efficacy (13–15). More recently, studies evaluating anti-B cell agents (such as rituximab) have demonstrated efficacy in some patients (16–18). Anti–B cell therapy might affect arthritis development by reducing the levels of arthritogenic autoantibodies (16–19), but B cells can also act as an APC population for effector CD4+ T cells (20–25). Whether B cells can play an important role in supporting CD4+ T cell differentiation in inflammatory arthritis is not well understood (23–25). It is also unclear why therapies targeting particular pathways (e.g., cytokines versus B cells) might exhibit different efficacies in arthritis patients. A simple explanation could be that distinct autoantigens are targeted by the immune system in patients that respond to different therapeutic strategies. However, an alternative explanation is that qualitative and/or quantitative differences in the autoreactive CD4+ T cell response that drives the disease process can determine which cellular pathways are required for disease pathogenesis. This latter possibility is difficult to assess in human patients because the self-Ags that are recognized by autoreactive CD4+ T cells remain poorly characterized (26, 27).

We have addressed these questions using a transgenic mouse model in which autoreactive CD4+ T cells with defined specificity for a surrogate self-peptide drive the spontaneous development of inflammatory arthritis (28–30). By varying the reactivity of the CD4+ T cell response to a single self-peptide, we show that B cells are not required for arthritis to develop in the context of a strongly autoreactive CD4+ T cell response (although proinflammatory cytokines such as TNF are required). By contrast, eliminating B cells significantly suppresses disease development in the context of a weakly autoreactive CD4+ T cell response, and the requirement for B cells appears to reflect a role for these cells in sup-

The online version of this article contains supplemental material.

Abbreviations used in this article: CCP, cyclic citrullinated peptide; HA, hemagglutinin; LN, lymph node; RA, rheumatoid arthritis; Treg, regulatory T cell.
porting autoreactive effector CD4+ T cell formation. Additional pathways appear to also be required to support arthritis development in the context of lower CD4+ T cell autoreactivity, because the disease displays a pronounced female gender bias in this setting. These studies demonstrate that the degree of CD4+ T cell reactivity for self-peptide(s) can play a prominent role in determining the cellular pathways that participate in the development of inflammatory arthritis.

Materials and Methods

**Mice**

TS1, TS1(SW), HACII, TS1×HACII, and TS1×HACII.JH−/− mice were previously described (28–36) and have been backcrossed with BALB/c mice for at least 10 generations. BALB/c mice were purchased from Charles River Laboratories. TS1(SW)×HACII mice were generated by mating TS1(SW) mice with HACII mice. To generate TS1(SW)×HACII.JH−/− mice, both TS1(SW) and HACII mice were first bred to JH−/− mice (37) on the BALB/c background, and then TS1(SW)×JH−/− mice were mated with HACII.JH−/− mice. All JH−/− mice were screened for the absence of B cells by flow cytometry. Arthritic mice and aged-matched control mice were analyzed between 15 and 24 wk of age. All mice were housed in The Wistar Institute Animal Facility under specific pathogen-free conditions. All experiments were performed according to protocols approved by The Wistar Institutional Animal Care and Use Committee.

**Assessment of arthritis**

Mice were assessed weekly for signs of arthritis. All four paws were analyzed for swelling by a blinded examiner, and each paw was assigned a score, as follows: 0, no visible swelling or discoloration; 1, visible swelling with/w/o discoloration; 2, severe swelling accompanied by skin discoloration. The minimum score per mouse is 0, and the maximum score per mouse is 8 (combined score of all four limbs scoring a 2).

**Histology**

Limbs and lungs (perfused with formalin) were fixed in 10% formalin (Globe Scientific), and limbs were decalcified. Tissues were then embedded in paraffin and cut at ∼5 μm to generate sections, which were stained with H&E, and blinded pathological scoring was performed. Grading scale is as follows: 0, not present; 1, minimal; 2, mild; 3, moderate; 4, marked.

**Flow cytometry and cell sorting**

Single-cell suspensions of joint-draining lymph nodes (LN)s; spleens, thymi, or joints were stained with the Live/Dead Fixable Aqua Dead Cell Stain Kit from Invitrogen (except when sorting) and then for surface markers at 4°C for 30 min. The following Abs were purchased from eBioscience or BD Pharmingen: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD20 (310), anti-Vβ10 (B21.5), anti-CD5 (50-F11), anti-Foxp3 (FJK-16s), anti–IL-17 (eBio17B7), and anti-Vβ10 (B21.5). 6.5 biotin (31) and anti-Vα8.3 biotin (KT50; BD Pharmingen) were detected with streptavidin-alkaline phosphatase. Anti-type II collagen, anti–cyclic citrullinated peptide (CCP), and anti-dsDNA titers were determined using the anti-type-mouse type II collagen IgG (Chondrex), QUANTA Lite CCP3 IgG (INOVA Diagnostics), or mouse anti-dsDNA total Ig (s Diagnostic Interna
tional) ELISA kits, respectively, according to the manufacturer’s instructions.

**Serum transfers**

Blood from donor mice was collected by heart puncture postmortem and was allowed to coagulate for at least 1 h at room temperature before centrifugation to isolate the serum fraction of the blood. Each recipient mouse was injected i.p. with 150 μL donor serum on day 0 and was boosted with 100 μL donor serum on day 3. Recipient mice were monitored for arthritis development every 3–4 d for 3 wk.

**In vitro suppression assay**

Regulatory T cells (Tregs) were purified by sorting CD4+CD25high cells from spleens and cocultured in varying numbers with 5 × 10^5 CFSE (Invitrogen)-labeled or CellTrace Violet (Invitrogen)–labeled CD4+CD25 responder T cells (also isolated from spleens) and with 2 × 10^5 CD3+ splenocytes from BALB/c mice (as APCs) in supplemented IMDM plus 10% FBS in 96-well U-bottom plates. To stimulate both effector cells and Tregs, anti-CD3 was added at 0.15 μg/mL. After 3 d of culture, cells were analyzed by flow cytometry for CFSE dilution. Luminex assays for serum cytokines

Serum samples were analyzed on MILLIPLEX MAP mouse cytokine/che
mokine luminex assay kits (Millipore) by the University of Pennsylvania Human Immunology Core.

**Intracellular cytokine staining**

Cells were stimulated in IMDM plus 10% FBS with 50 ng/mL PMA (Sigma-Aldrich), 1 μM ionomycin (Sigma-Aldrich), and a 1:1000 dilution of brefeldin A (eBioscience) for 4 h at 37°C. Following staining for surface markers, cells were fixed and permeabilized with the Foxp3 Buffer Set (eBioscience), according to the manufacturer’s protocol, and then intracellular cytokine staining was performed.

**In vivo Ab treatments**

For anti-IL-17R treatment, mice were injected i.p. with 0.5 mg of either rat anti-mouse IL-17R blocking Ab (M751; provided by Amgen) or an isotype control Ab (MOPC-21; BioXcell) weekly from 5–14 wk of age. For anti-CD20 treatment, mice were injected i.v. with 0.25 mg of either anti-mouse CD20-depleting Ab (18B12; provided by Biogen Idec) or an isotype control Ab (2B8, anti-human CD20 with no cross-reactivity to mouse CD20; provided by Biogen Idec) once every 3 wk from 5–14 wk of age (23). For anti-TNF treatment, mice were injected i.p. with 0.5 mg of either rat anti-mouse TNF-α-neutralizing Ab (XT3.11; BioXcell) or an isotype control Ab (HRPN; BioXcell) weekly from 5–14 wk of age.

**ELISAs for Abs**

Concentrations of total IgG in the serum were determined using U-bottom vinyl plates (Costar) that were coated with goat anti-mouse Ig (H+L; Southern Biotechno
tic), and bound Abs were detected with goat anti-mouse IgG alkaline phosphatase (Southern Biotech). Purified mouse IgG (Southern Biotech) was used as a standard. To detect rheumatoid factor, plates were coated with purified mouse IgG1, IgG2a, and IgG2b (BD Pharmingen), and bound Abs were detected with rat anti-mouse κ L chain-biotin (Southern Biotech), followed by streptavidin-alkaline phosphatase. Anti-type II collagen, anti–cyclical citrullinated peptide (CCP), and anti-dsDNA titers were determined using the anti-type-mouse type II collagen IgG (Chondrex), QUANTA Lite CCP3 IgG (INOVA Diagnostics), or mouse anti-dsDNA total Ig (s Diagnostic Interna
tional) ELISA kits, respectively, according to the manufacturer’s instructions.

**Statistics**

Statistical analyses were performed using Fisher’s exact test, Mann-
Whitney U test, or one-way ANOVA with Tukey posttest, as appropriate, with GraphPad Prism software (GraphPad). The p values <.05 were considered significant.
Results

Autoreactive TCR affinity influences penetrance and gender bias of inflammatory arthritis

TS1 × HACII mice express a transgenic TCR (which can be detected with the anti-clonotypic mAb 6.5) that recognizes the I-Ek-restricted S1 determinant of the influenza virus PR8 hemagglutinin (HA) (31), and coexpress the PR8 HA as a surrogate self-Ag under the control of a MHC class II promoter, which directs expression of HA to MHC class II+ APCs (Fig. 1A) (32). As previously reported, the majority of TS1 × HACII mice (but not mice expressing either the TS1 or HA transgenes alone) spontaneously develop inflammatory arthritis, as evidenced by overt joint inflammation and swelling that can affect both front and rear paws (Fig. 1B) (28–30). Joint inflammation first becomes evident between 6 and 8 wk of age, and by 14 wk almost all TS1 × HACII mice have developed at least one inflamed paw (Fig. 1B) (28–30). Arthritis penetrance (determined by the presence of at least one inflamed paw) and kinetics were similar in male and female TS1 × HACII mice, and the combined scores of arthritis severity in all limbs also did not differ significantly between males and females.

A notable feature of TS1 × HACII mice is that the TS1 TCR recognizes the S1 self-peptide as a high-affinity, cognate Ag (31, 33, and we were interested in investigating whether an autoreactive TCR with a lower affinity for the S1 self-peptide would be able to drive arthritis development. To this end, we used TS1(SW) mice, which express a transgenic Vα3.3/Vβ10 TCR that was raised against a variant influenza virus containing an analog of the S1 determinant [termed S1(SW)] that differs from S1 by 2 aa residues (Fig. 1E) (34–36). Thus, whereas CD4+ T cells from TS1 mice underwent robust proliferation in response to micromolar amounts of S1 peptide (as measured by CFSE dilution), CD4+ T cells from TS1(SW) mice proliferated weakly in response to high concentrations (3 μM) of S1 peptide and appeared unresponsive when incubated with lower S1 peptide concentrations (Fig. 1F). This is a reflection of a low intrinsic affinity of the TS1(SW) TCR for the S1 peptide, because these CD4+ T cells were able to proliferate robustly in response to submicromolar concentrations of their cognate peptide, S1(SW). When we mated TS1(SW) mice with HACII mice (Fig. 1G), we again found that adult TS1(SW) × HACII mice developed overt joint swelling that could affect both front and hind paws (Fig. 1H), similar to what had been observed in TS1 × HACII mice. Notably, however, in this case, there was a pronounced sex bias, because male TS1(SW) × HACII mice exhibited significantly lower penetrance and a significant delay in disease onset relative to female TS1(SW) × HACII mice (Fig. 1I, 1J).

We also performed histopathological examinations of joints and tissues from mice that had been designated as either arthritic or nonarthritic based on overt joint swelling. Sections taken from the swollen joints of female TS1 × HACII and TS1(SW) × HACII mice exhibited high degrees of synovitis and articular degeneration and generated significantly higher severity scores than did sections obtained from control TCR-only mice, supporting their designation as arthritic (Fig. 2). By contrast, when we examined sections that had been obtained from TS1(SW) × HACII mice (both male and female) that did not exhibit overt joint swelling, we found that these sections did not differ from control mice with respect to

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**FIGURE 1.** CD4+ T cell reactivity for a self-peptide influences the development of inflammatory arthritis. (A) Schematic depicting the generation of TS1 × HACII mice through mating of TS1 mice and HACII mice. (B) Photographs show front and rear paws from representative control TS1 and arthritic TS1 × HACII mice. (C) Graph shows the mean percentages of male (n = 15) and female (n = 14) TS1 × HACII mice that developed at least one arthritic paw over time. (D) Arthritis scores for individual female (F) and male (M) TS1 × HACII mice at 14 wk of age. (E) Schematic shows relative affinity of the CD4+ T cells from TS1 and TS1(SW) mice for the S1 and S1(SW) peptide analogs. (F) Histograms show CFSE levels of CD4+ T cells from TS1 and TS1(SW) mice following incubation with indicated concentrations of S1 (red histograms) and S1(SW) (blue histograms) peptide. (G) Schematic depicting the generation of TS1(SW) × HACII mice through mating of TS1(SW) mice and HACII mice. (H) Photographs show front and rear paws from representative control TS1(SW) and arthritic TS1(SW) × HACII mice. (I) Graph shows the mean percentages of male (n = 12) and female (n = 14) TS1(SW) × HACII mice that developed at least one arthritic paw over time; *p < 0.05, Fisher’s exact test. (J) Arthritis scores for individual female (F) and male (M) TS1(SW) × HACII mice at 14 wk of age. *p < 0.05, Mann–Whitney test.
synovitis or articular degeneration, supporting their designation as nonarthritic. Moreover, the synovitis and articular degeneration scores generated from arthritic male TS1(SW)3HACII mice were significantly lower than those from arthritic female TS13HACII and TS1(SW)3HACII mice, indicating that, in addition to lower disease penetrance, the severity of arthritis was lower in arthritic male versus arthritic female mice. We also examined extra-articular tissues for evidence of inflammation, and, as previously reported, we found extensive perivascular infiltrates in the lungs of arthritic TS13HACII mice (Supplemental Fig. 1) (28). By contrast, no differences were observed in the extent of perivascular infiltration in the lungs of either male or female TS1(SW)3HACII mice versus control mice, irrespective of arthritis development. Moreover, whereas mild inflammatory processes were observed in the hearts and kidneys of some arthritic TS13HACII mice, these were either not observed or only rarely found in arthritic TS1(SW)3HACII mice (data not shown).

Thus, autoreactive CD4+ T cells with either a high or a low affinity for the S1 self-peptide can drive the development of spontaneous inflammatory arthritis in TS13HACII and TS1(SW)3HACII mice. However, arthritis develops with a significantly lower penetrance and severity in male versus female TS1(SW)3HACII mice, reflecting the lower affinity of the TS1(SW) TCR for the S1 self-peptide. This lower affinity is also associated with a reduction in TS1(SW)3HACII mice of the extra-articular inflammation that affects the lungs of TS13HACII mice.

**Autoreactive CD4+ T cell development in TS13HACII and TS1(SW)3HACII mice**

To begin to examine how the introduction of the TS1 and TS1(SW) TCR transgenes can precipitate inflammatory arthritis development in TS13HACII and TS1(SW)3HACII mice, we first examined thymocytes and peripheral lymphoid organ cells for the frequencies of CD4+ T cells expressing the clonotypic TCRs in young adult, prearthritic mice. As previously reported, HA-specific 6.5+CD4+CD8- single-positive thymocytes are subjected to severe deletion in TS13HACII mice (Fig. 3A) (28). Nevertheless, a subset of 6.5+CD4+ T cells evades deletion and can be found to accumulate in the spleens and LNs of TS13HACII mice (Fig. 3B and

![Figure 2. Histopathological analysis of joints from TS1×HACII and TS1(SW)×HACII mice.](http://www.jimmunol.org/Downloadedfrom)
FIGURE 3. Autoreactive CD4+ T cell development in TS1×HACII and TS1(SW)×HACII mice. (A) Dot plots show CD8 versus CD4 staining of thymocytes from 4- to 6-wk-old TS1, TS1×HACII, TS1(SW), and TS1(SW)×HACII mice. Histograms show staining of CD4+CD8+ cells for the transgene-encoded clonotypic TCR (6.5 for TS1 and TS1×HACII mice; Vα8.3 for TS1(SW) and TS1(SW)×HACII mice). Percentages of cells in indicated gates are shown. Bar graphs show thymic cellularity and numbers of clonotypic CD4+CD8+ thymocytes as means ± SEM in indicated mice (n = 4–7). **p < 0.01, Mann–Whitney U test. (B) As for (A), except spleens of indicated mice are shown. **p < 0.01, Mann–Whitney U test. (C) Dot plots show Foxp3 versus CD25 staining of CD4+CD8+ cells, and histograms show clonotype staining on CD4+Foxp3+ cells isolated from thymi of mice described in (A). Percentages of cells in indicated gates are shown. Bar graphs show numbers of CD4+Foxp3+ and clonotypic CD4+Foxp3+ cells as means ± SEM in indicated mice. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey posttest. (D) As for (C), except spleens of indicated mice are shown. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey posttest. (E) Histograms on left show CFSE levels of CFSE-labeled (Figure legend continues)
data not shown). HA-specific Vα8.3/CD4+CD8- thymocytes were also subjected to deletion by the S1 self-peptide in TS1(SW)×HACII mice, and we could again find clonotypic CD4+ T cells (expressing Vα8.3) in their spleens and LNs (Fig. 3A, 3B and data not shown).

We also examined the expression of the transcription factor Foxp3, which confers regulatory function in CD4+ T cells (38). There were modest increases in the percentages of CD4+CD8- Foxp3+ thymocytes and of CD4+Foxp3+ splenocytes in both TS1×HACII and TS1(SW)×HACII mice relative to TCR-only controls (Fig. 3C, 3D); however, the numbers of these cells that expressed the clonotypic TS1 or TS1(SW) TCRs were substantially reduced relative to TCR-only mice, reflecting the severe deletion of clonotype-expressing thymocytes in both TS1×HACII and TS1(SW)×HACII mice. We also purified CD4+CD25bright Treg cells from the different strains and examined their ability to suppress proliferation of responder cells in vitro following stimulation with anti-CD3. The Tregs from TS1×HACII and TS1(SW)×HACII mice were each able to suppress proliferation of responder cells as efficiently as Tregs from BALB/c mice (Fig. 3E). Thus, in line with our previous observations in TS1×HACII mice (29), inflammatory arthritis develops in both TS1×HACII and TS1(SW)×HACII mice, despite substantial deletion of autoreactive CD4+ T cells, and also despite the formation of Foxp3+ Tregs, including small subsets expressing TCRs that confer specificity for the S1 self-peptide. TCR affinity affects the extent of systemic immune activation in arthritic mice

In diseases such as RA, inflammation develops in the context of not only local but also systemic immune activation (2, 11, 39–41), and we wanted to determine whether the reactivity of CD4+ T cells toward the S1 self-peptide was affecting systemic inflammatory processes. We first examined CD4+ T cells from arthritic female TS1×HACII and TS1(SW)×HACII mice for their abilities to proliferate in vitro in response to autologous APCs. CD4+ T cells from TS1×HACII mice underwent division in response to APCs from TS1×HACII mice, but those from TS1(SW)×HACII mice underwent little or no proliferation in response to APCs from TS1(SW)×HACII mice (Fig. 4A). This difference in responsiveness is a reflection of the differing reactivities of CD4+ T cells for S1 peptide, because CD4+ T cells from both strains underwent little proliferation in response to BALB/c APCs (which do not express the S1 peptide) but proliferated robustly in response to anti-CD3 cross-linking. We also found that the mean levels of IL-6 were substantially higher and that those of other proinflammatory cytokines were elevated in serum from arthritic TS1×HACII mice relative to TCR single-transgenic control mice, whereas lower levels were found in arthritic TS1(SW)×HACII mice (Fig. 4B). Notably, despite the low relative reactivity of CD4+ T cells from TS1(SW)×HACII mice, IL-17 was present at significantly higher levels in the serum of arthritic TS1(SW)×HACII mice than in control TS1(SW) mice. Intracellular cytokine staining also revealed increased frequencies of IL-17–secreting CD4+ T cells in the joint-draining LNs and spleens of arthritic TS1×HACII and TS1(SW)×HACII mice (Fig. 4C, 4D and data not shown). These findings suggested that IL-17 might be an important contributor to arthritis development in TS1(SW)×HACII mice, resembling our previous studies showing that IL-17 is required for arthritis development in TS1×HACII mice (29). Indeed, treatment with an anti–IL-17R mAb abrogated arthritis development in female TS1(SW)×HACII mice (Fig. 4E). Arthritic TS1×HACII mice also contained a significantly higher level of serum IgG than control mice expressing only the TCR transgene, and, whereas serum IgG was also elevated in arthritic TS1(SW)×HACII mice relative to controls, it was significantly lower than in TS1×HACII mice (Fig. 4F).

To evaluate whether the decreased arthritis penetrance and severity observed in male TS1(SW)×HACII mice are associated with differences in systemic immune activation, we compared male and female TS1(SW)×HACII mice, and we distinguished between mice from both genders that had or had not developed overt inflammatory arthritis. Serum cytokine levels and IgG titers were generally highest in female TS1(SW)×HACII mice that had developed arthritis, whereas those from both arthritic and nonarthritic male TS1(SW)×HACII mice were lower (Fig. 4G, 4H). Notably, nonarthritic female TS1(SW)×HACII mice contained lower levels of IL-17 relative to females that were arthritic, whereas IgG titers were not significantly different. Consistent with the more severe inflammatory processes affecting the joints of female versus male TS1(SW)×HACII mice, the joint-draining LNs of arthritic female TS1(SW)×HACII mice were significantly larger than was the case for either arthritic or nonarthritic male TS1(SW)×HACII mice (Supplemental Fig. 2). However, no significant differences were found in the percentages of CD4+ T cells (either total or of those expressing the clonotypic TCR), of CD4+CD25bright Foxp3+ T cells, of CD4+IL-17+ cells, or of B cells in the joint-draining LNs among the different mice.

Collectively, these data demonstrate that inflammatory arthritis can develop by an IL-17–dependent mechanism in both TS1×HACII and TS1(SW)×HACII mice, and that the extent of systemic immune activation can be affected by the affinity with which autoreactive CD4+ T cells recognize a target self-Ag.

B cells promote arthritis development in TS1(SW)×HACII mice

To examine whether differences in the affinity of the autoreactive CD4+ T cell response can influence the requirement for B cells in the development of inflammatory arthritis, we generated cohorts of female TS1×HACII and TS1(SW)×HACII mice that congenitally lacked B cells by mating with JH−/− mice (37). As previously reported, disease penetrance and severity were similar in TS1×HACII and in B cell–deficient TS1×HACII.JH−/− mice, and arthritis may develop more quickly in TS1×HACII.JH−/− mice (Fig. 5A) (28). In contrast to the findings with TS1×HACII mice, TS1(SW)×HACII.JH−/− mice exhibited a significant impairment in arthritis development relative to B cell–sufficient TS1(SW)×HACII mice, indicating that B cells make a significant contribution to arthritis development in the context of a lower-affinity autoreactive CD4+ T cell response (Fig. 5B).

Because B cells make an important contribution to arthritis development in TS1(SW)×HACII mice but not in TS1×HACII mice, we compared sera from the two strains for the presence of Ab specificities that are associated with arthritis development in other settings (2, 42, 43). However, there were no differences in the levels of rheumatoid factor, Abs to type II collagen, or anti-dsDNA Abs in the two strains, and sera from TS1×HACII mice actually had higher levels of anti-CCP Abs than were found in arthritic TS1(SW)×HACII mice (Supplemental Fig. 3). We also transferred sera from arthritic TS1(SW)×HACII mice into various
recipient mice and found no evidence of arthritis development, whereas sera from K/BxN mice (in which Abs are a principal effector mechanism) (7) did cause arthritis (Supplemental Fig. 3). Thus, the ability of B cells to promote arthritis development in TS1(SW)HACII mice does not appear to correlate with the presence in the serum of higher levels of arthritogenic Ab specificities than are present in TS1HACII mice.

We also evaluated whether B cells contribute to arthritis development in TS1(SW)HACII mice by supporting the development or differentiation of CD4+ T cells. Consistent with an absence of B cells, there was a sizable decrease in the overall cellularity of the spleens and joint-draining LNs of TS1(SW)HACII+/- mice, and, although the percentage of CD4+ T cells increased, the representation of CD4+ Vα8.3+Vβ10+ clonotypic TCR did not appear to differ between TS1(SW)HACII mice that did or did not have B cells (Fig. 6A, 6B). Notably, however, there were significant decreases in the percentages of IL-17-- and IFN-γ-secreting Vα8.3+Vβ10+ CD4+ T cells in the joint-draining LNs of nonarthritic TS1(SW)HACII+/- mice and similar differences in the spleens. Moreover, in the subset of

FIGURE 4. Systemic immune activation in TS1×HACII and TS1(SW)×HACII mice. (A) Histograms show levels of CFSE in CFSE-labeled CD4+ T cells from TS1×HACII and TS1(SW)×HACII mice following incubation for 3 d with autologous splenocytes as APCs, with BALB/c splenocytes alone, or with BALB/c splenocytes and anti-CD3. Percentages of divided cells are indicated. (B) Graphs show mean concentrations ± SEM of indicated cytokines in the serum of control female TCR single-transgenic [TS1 and TS1(SW)] mice, arthritic female TS1×HACII mice, and arthritic female TS1(SW)×HACII mice (n = 17–30). *p < 0.05, **p < 0.01, ***p < 0.001 in a one-way ANOVA with Tukey posttest. (C) Dot plots show IFN-γ versus IL-17 staining of CD4+ cells (upper panels) and of 6.5+CD4+ cells (lower panels) isolated from the joint-draining LNs (jdlN) of TS1 and arthritic female TS1×HACII mice. Percentages of cells in indicated gates are shown. Graphs indicate the mean percentages ± SEM of CD4+ (upper) and of CD4+6.5+ (lower) cells that secrete IFN-γ or IL-17 (n = 9–11). *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U test. (D) As for (C), except that cells were obtained from TS1(SW) and from arthritic female TS1(SW)×HACII mice, and CD4+Vα8.3+ cells are shown. (E) Graph shows the mean concentrations ± SEM and individual levels of serum IgG in control female TCR single-transgenic, arthritic female TS1×HACII, and arthritic female TS1(SW)×HACII mice that developed at least one arthritic paw over time. *p < 0.05, **p < 0.01, Fisher’s exact test. (F) Graph shows the mean concentration ± SEM and individual levels of serum IgG in control female TCR single-transgenic, arthritic female TS1×HACII, and arthritic female TS1(SW)×HACII mice (n = 8–14). ***p < 0.001, one-way ANOVA with Tukey posttest. (G) Graphs show mean concentrations ± SEM of indicated cytokines in the serum of male (M) and female (F) TS1(SW)×HACII mice with and without arthritis (arthritic female, n = 17; non-arthritic female, n = 7; arthritic male, n = 8; non-arthritic male, n = 8). *p < 0.05, **p < 0.01, one-way ANOVA with Tukey posttest. (H) Graph shows the mean concentration ± SEM and individual levels of serum IgG in male (M) and female (F) TS1(SW)×HACII mice with and without arthritis (n = 5–8).

*p < 0.05, **p < 0.01, one-way ANOVA with Tukey posttest.
TS1(SW) × HACII J−/− mice that developed arthritis despite the absence of B cells, the frequencies of IFN-γ–secreting Vo8.3+ Vβ10+CD4+ T cells were significantly reduced relative to arthritic TS1(SW) × HACII mice, but the frequencies of IL-17–secreting Vo8.3+ Vβ10+CD4+ T cells were not. To evaluate whether the reductions in clonotypic effector cells were due to an absence of B cells or instead secondary to a failure for arthritis to have developed in the nonarthritic TS1(SW) × HACII J−/− mice, we compared nonarthritic TS1(SW) × HACII J−/− mice with nonarthritic TS1(SW) × HACII mice, and again found significantly lower frequencies of clonotypic, but not of total, IFN-γ– and IL-17–producing CD4+ T cells in the B cell–deficient mice (Supplemental Fig. 4). We also examined whether B cells might be required to support Foxp3+ Treg formation, and, whereas TS1(SW) × HACII mice contained higher frequencies of CD4+Foxp3+ cells than were found in TS1(SW) mice, TS1(SW) × HACII and TS1(SW) × HACII J−/− mice did not significantly differ in the percentages of CD4+ T cells that were Foxp3+ in the spleens or in the joint-draining LNs (Fig. 6C). Because the preceding results showed that anti–IL-17R blockade can also prevent arthritis, these studies suggest that B cells support arthritis development in TS1(SW) × HACII mice at least partly by supporting the formation of IL-17–secreting effecter CD4+ T cells expressing the autoreactive Vo8.3+Vβ10 TCR.

**Anti–B cell treatment prevents arthritis development in TS1(SW) × HACII mice**

It was possible that the reduced frequencies of clonotypic cytokine-producing CD4+ T cells observed in TS1(SW) × HACII J−/− mice were a consequence of alterations in the immune system’s development that arose due to the congenital lack of B cells. We therefore examined the effects of eliminating B cells from mice in which B cell development had been allowed to occur by treating 5- to 6-wk-old TS1(SW) × HACII mice with an anti–CD20 mAb (23). Following 8 wk of anti–CD20 treatment, the majority (10 of 13) of TS1(SW) × HACII mice contained few or no splenic B cells (based on CD19 staining), and levels of serum IgG were greatly reduced relative to isotype control-treated mice (Supplemental Fig. 4). We also examined the effects of eliminating B cells from mice in which B cell development had been allowed to occur by treating 5- to 6-wk-old TS1(SW) × HACII mice with an anti–CD20 mAb (23). Following 8 wk of anti–CD20 treatment, the majority (10 of 13) of TS1(SW) × HACII mice contained few or no splenic B cells (based on CD19 staining), and levels of serum IgG were greatly reduced relative to isotype control-treated mice (Supplemental Fig. 4). We also examined the effects of eliminating B cells from mice in which B cell development had been allowed to occur by treating 5- to 6-wk-old TS1(SW) × HACII mice with an anti–CD20 mAb (23). Following 8 wk of anti–CD20 treatment, the majority (10 of 13) of TS1(SW) × HACII mice contained few or no splenic B cells (based on CD19 staining), and levels of serum IgG were greatly reduced relative to isotype control-treated mice (Supplemental Fig. 4).
TS1×HACII mice that had received anti-TNF– or isotype control-treated TS1×HACII mice, irrespective of disease status. In addition, CD4+CD25high T cells purified from the spleens of anti-TNF–treated TS1×HACII mice were no better at suppressing the proliferation of effector T cells in vitro than those from isotype control-treated TS1×HACII mice, or those from untreated BALB/c mice (Fig. 8C). Collectively, these observations argue against the possibility that anti-TNF treatment modulates arthritis development in TS1×HACII mice through its ability to increase the representation or activity of CD4+Foxp3+ Tregs.

We also examined whether anti-TNF treatment might prevent arthritis development by altering the generation or accumulation of cytokine-secreting effector CD4+ T cells in peripheral lymphoid organs, or in the joints themselves. The overall cellularity of the joint-draining LNs (but not of the spleens) was reduced in anti-TNF-treated TS1×HACII mice that did not develop arthritis, but there were no significant differences in the frequencies of CD4+ T cells in the joint-draining LNs or spleens of anti-TNF– or isotype control-treated TS1×HACII mice (Fig. 9A and data not shown). Although there was a significant decrease in the frequency of 6.5+CD4+ T cells in the joint-draining LNs of nonarthritic anti-TNF–treated mice, the representations of IL-17– and IFN-γ–secreting cells within total and clonotypic CD4+ T cell populations were unaffected by anti-TNF treatment. Notably, however, there was a significant decrease in the accumulation of IL-17–secreting CD4+ T cells (but not of IFN-γ–secreting CD4+ T cells) in the joints of anti-TNF–treated TS1×HACII mice that did not develop arthritis, and the accumulation of CD11b+ cells (primarily neutrophils; data not shown) was also significantly reduced (Fig. 9B). Notably, no such decrease in the accumulation of
IL-17–secreting CD4+ T cells or of CD11b+ cells was found in TS13HACII mice that had developed arthritis despite receiving anti-TNF Ab treatment. Thus, the ability of anti-TNF treatment to prevent arthritis development in TS13HACII mice was associated with a reduced accumulation of Th17 cells in the joints, whereas the representation of Th17 cells in the spleens and the joint-draining LNs was relatively unaffected.

Discussion

The studies in this work demonstrate that the overall reactivity of the CD4+ T cell response to a ubiquitously expressed self-peptide can play a prominent role in determining the cellular pathways that participate in the development of inflammatory arthritis. In TS1×HACII mice, an autoreactive CD4+ T cell response to the S1 self-peptide was measurable in an autologous MLR, and serum contained elevated levels of both proinflammatory cytokines and Ig. Although arthritis development could be prevented by anti-TNF treatment, it was not affected by B cell elimination. By contrast, serum cytokine levels were lower in TS1(SW)3HACII mice in which the peripheral CD4+ T cell repertoire was less responsive to the S1 self-peptide, and, in this case, elimination of B cells significantly suppressed arthritis development. Notably, both anti-TNF treatment of TS1×HACII mice and B cell elimination in TS1(SW)3HACII mice appeared to prevent arthritis development at least in part by disrupting Th17 cell activity, albeit by distinct mechanisms.

The presence of elevated levels of serum cytokines and the lack of requirement for B cells suggested that cytokines may play a prominent role in promoting arthritis development in TS1×HACII mice, and we found that administration of an anti-TNF mAb can signif-
significantly reduce arthritis development. TNF is a pleiotropic cytokine that could promote arthritis development by modulating cytokine networks, by inducing apoptosis, or by increasing the expression of bone-destructive enzymes such as metalloproteases, among other possible effects (11, 44). Although anti-TNF treatment can restore the representation and/or activity of Foxp3+ Tregs in human RA patients (45, 46), we did not obtain evidence that anti-TNF treatment alters the representation of Tregs in the peripheral lymphoid organs and/or activity of Tregs in the spleens of TS1×HACII mice. Anti-TNF treatment did, however, lead to a decreased representation of Th17 cells in the joints of TS1×HACII mice in which arthritis was prevented, which is noteworthy because we have previously shown that treatment with an anti–IL-17 mAb can also ameliorate arthritis development in TS1×HACII mice (29). Notably, anti-TNF treatment did not appear to exert systemic effects on Th17 cell development, because the frequencies of Th17 cells did not differ in the spleens of anti-TNF or isotype control-treated TS1×HACII mice, irrespective of disease status. Similarly, whereas there were fewer CD4+ T cells expressing the clonotypic TCR in the joint-draining LNs of anti-TNF–treated TS1×HACII mice in the absence of disease, the percentages of these cells that could secrete IL-17 did not differ. Instead, anti-TNF treatment appeared to selectively prevent the accumulation of Th17 cells in the joints of TS1×HACII mice. Indeed, whereas the joints of anti-TNF–treated mice that did not develop disease contained reduced percentages of IL-17–secreting CD4+ T cells compared with arthritic, isotype control-treated mice, there were no differences in the overall percentages of CD4+ T cells, and the percentages of IFN-γ–secreting CD4+ T cells were the same or higher. Moreover, no such effects on Th17 cell accumulation were found in TS1×HACII mice that developed arthritis despite anti-TNF treatment. It is possible that anti-TNF treatment can suppress the migration of Th17 cells to the joints of TS1×HACII mice, most likely by inhibiting the production of chemokines such as CCL20 that can be produced by synovocytes in response to TNF that can attract CCL6-expressing Th17 cells (47). Alternatively, anti-TNF treatment may inhibit the ability of APCs (such as macrophages) to support the local proliferation of Th17 cells in the joints, thereby preventing their accumulation (48). We also found that anti-TNF treatment inhibited the accumulation of CD11b+ cells in the joints, resembling studies in RA patients showing that anti-TNF treatment led to a reduced retention of radiolabeled neutrophils in arthritic joints (44).

In contrast to TS1×HACII mice, arthritis development was significantly reduced in TS1(SW)×HACII mice either by congenital B cell ablation, or by B cell depletion with an anti-CD20 mAb. Unlike some other models of inflammatory arthritis (7, 8, 49), the requirement for B cells did not appear to reflect a prominent requirement for the production of arthritogenic Abs; Ab specificities associated with arthritis development (2, 42) were no more abundant in sera from TS1(SW)×HACII mice than from TS1×HACII mice (in which B cells are not required for disease), and we were also unable to induce arthritis by transferring serum from arthritic TS1(SW)×HACII mice into naive mice. The frequencies of IL-17– and IFN-γ–secreting CD4+ T cells expressing the clonotypic TCR were, however, significantly lower in B cell–deficient TS1(SW)×HACII mice that failed to develop disease, which is notable because anti–IL-17R treatment of TS1(SW)×HACII mice was also able to prevent arthritis development. B cells have previously been shown to support the formation of Ag-specific effector CD4+ T cells in response to both foreign Ags and autoantigens, including

![Graph showing arthritis scores](http://www.jimmunol.org/)

**FIGURE 8.** Anti-TNF treatment prevents arthritis development but does not augment Treg representation or function in TS1×HACII mice. (A) Left graph shows mean percentage of anti-TNF–treated (n = 13) and isotype control-treated (n = 11) TS1×HACII mice that developed at least one arthritic paw over time. *p < 0.05, **p < 0.01, ***p < 0.001, Fisher’s exact test. Right graph shows arthritis scores at 14 wk of age. ****p < 0.001, Mann–Whitney U test. (B) Dot plots show Foxp3 versus CD25 staining on CD4+ cells isolated from the spleens (top row) or joint-draining LNs (iDLN; bottom row) of female TS1 mice (n = 4), arthritic isotype control-treated female TS1×HACII mice (n = 8), arthritic anti-TNF–treated female TS1×HACII mice (n = 4), and nonarthritic anti-TNF–treated TS1×HACII mice (n = 6). Percentages of cells in respective gates are shown, and mean percentages of Foxp3+ cells ± SEM are shown in graphs on the right. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey posttest. (C) Graph shows inhibition of in vitro proliferation of anti-CD3–stimulated effector CD4+CD25+ T cells caused by addition of differing ratios of CD4+CD25high cells isolated from BALB/c mice, from arthritic TS1×HACII mice that received isotype control Ab, or from nonarthritic TS1×HACII mice that received anti-TNF mAb. Data obtained from three independent experiments are shown with means indicated. A, arthritic; NA, nonarthritic.
studies in an Ag-induced model of inflammatory arthritis (21–25). Accordingly, B cells appear to promote arthritis development in TS1(SW)3HACII mice at least in part through their ability to support the development of Th17 cells expressing the clonotypic TCR, which accumulated in higher numbers in the joint-draining LNs of arthritic TS1(SW)3HACII mice than was the case for either IFN-γ–secreting CD4+ T cells in TS1(SW)×HACII mice, or IL-17–secreting CD4+ T cells in TS1×HACII mice. Although the selective accumulation of Th17 cells in the joint-draining LNs of TS1(SW)×HACII mice is likely a consequence of the chemokine CCL20 produced by the inflamed joint (47), it is noteworthy that the elimination of B cells from TS1(SW)3HACII mice led to the systemic disruption of Ag-specific effector CD4+ T cell formation that could be observed in the spleens of B cell–depleted mice. This contrasts the findings in anti-TNF–treated TS1×HACII mice, in which there was little effect of anti-TNF treatment on the frequencies of Th17 cells in the spleens or the joint-draining LNs, but Th17 cells did not accumulate in the joints themselves. The conclusion that B cells support effector CD4+ T cell differentiation in TS1(SW)×HACII mice is also noteworthy because there were subsets of TS1(SW)×HACILJH−/− mice, and of anti-CD20–treated TS1(SW)×HACII mice, that exhibited efficient Ab depletion but nevertheless developed arthritis. Based on these observations, it will be of interest to determine whether B cells may also promote arthritis development in human patients at least in part by supporting effector CD4+ T cell formation.

Although there has been recent success in the use of biological therapeutics to modulate the immune system in patients with inflammatory arthritis, the factors that determine which cellular pathways are required for disease development in individual patients remain poorly understood. One reason that the therapeutic targeting of distinct cellular pathways (e.g., anti-TNF versus anti-CD20 treatment) may be effective in different individuals could be that the cellular Ags being recognized in those individuals are different. This possibility is difficult to assess in human patients, not least because the Ags that are recognized by autoreactive lymphocytes in inflammatory arthritis (such as can occur in RA and systemic lupus erythematosus) remain poorly understood (26, 27). Our studies in this work have used a system in which the identity of an eliciting surrogate self-peptide is known and have shown that the overall reactivity of the autoreactive CD4+ T cell response can determine whether B cells are required for arthritis development. Whereas our data do
not preclude a role for Abs in affecting the development of arthritis in TSI(SW)×HACII mice, B cells were clearly required to support the formation of effector CD4+ T cells that recognize the nominal self-Ag in arthritic mice, under conditions of low T cell reactivity for the self-peptide. The observation that there is a female gender bias in arthritis development in TSI(SW)×HACII mice, but not in TSI×HACII mice, suggests that additional pathways (e.g., estrogen-induced immune activation) (50, 51) are also necessary for arthritis development in the context of a relatively weak autoreactive CD4+ T cell response. As Abs that are recognized in human patients become better characterized, it will be of interest to determine whether the level of autoreactive CD4+ T cell reactivity can predict the response. As Ags that are recognized in human patients become modifiers.

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Disclosures

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References


