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CD4⁺ T Cells Recognizing a Single Self-Peptide Expressed by APCs Induce Spontaneous Autoimmune Arthritis

Andrew L. Rankin,* Amy J. Reed,** Soyoung Oh,* Cristina Cozzo Picca,* Heath M. Guay,* Joseph Larkin III,* Laura Panarey,* Malinda K. Aitken,* Brigitte Koeberlein,† Peter E. Lipsky,§ John E. Tomaszewski,§ Ali Naji,† and Andrew J. Caton‡*

We have examined processes leading to the spontaneous development of autoimmune inflammatory arthritis in transgenic mice containing CD4⁺ T cells targeted to a nominal Ag (hemagglutinin (HA)) and coexpressing HA driven by a MHC class II promoter. Despite being subjected to multiple tolerance mechanisms, autoreactive CD4⁺ T cells accumulate in the periphery of these mice and promote systemic proinflammatory cytokine production. The majority of mice spontaneously develop inflammatory arthritis, which is accompanied by an enhanced regional immune response in lymph nodes draining major joints. Arthritis development is accompanied by systemic B cell activation; however, neither B cells nor Ab is required for arthritis development, since disease develops in a B cell-deficient background. Moreover, arthritis also develops in a recombinase activating gene-deficient background, indicating that the disease process is driven by CD4⁺ T cells recognizing the neo-self HA Ag. These findings show that autoreactive CD4⁺ T cells recognizing a single self-Ag, expressed by systemically distributed APCs, can induce arthritis via a mechanism that is independent of their ability to provide help for autoantibody production. The Journal of Immunology, 2008, 180: 833-841.

Inflammatory arthritis can develop in a variety of human autoimmune diseases (including systemic lupus erythematosus, inflammatory bowel disease, and psoriasis) and is the hallmark of rheumatoid arthritis (RA) (1, 2). RA patients develop chronic inflammatory arthritis with progressive damage to articular structures, including cartilage, bone, tendons, and ligaments and can also develop extra-articular manifestations affecting vasculature and organs such as the lungs (2). The etiology of inflammatory arthritis has been most extensively studied in RA, in which association with specific MHC II alleles and infiltration of synovial tissue by CD4⁺ T cells suggest a role for CD4⁺ T cells (2). However, a role for autoantibodies is also implied by the association between disease prognosis and the presence of autoantibodies to IgG (rheumatoid factor) and to cyclic citrullinated peptide (CCP) (2, 3). RA shows by far its strongest genetic linkages with particular HLA genes, especially those encoding specific β-chains of the HLA-DR molecule (2, 4), and recent evidence has shown that specific polymorphisms of the PTPN22 molecule (a phosphatase that controls the degree of T cell activation) can also contribute to RA susceptibility (5). These linkages suggest that some aspect of the CD4⁺ T cell response to self-peptides may promote arthritis development, although whether this relates to direct effector functions of CD4⁺ T cells or the provision of help for autoantibody-producing cells remains unclear.

Efforts to understand the etiology of inflammatory arthritis using animal models have placed varying emphasis on the relative contributions of CD4⁺ T cells and B cells to disease pathology (4). Clear evidence that Ab can mediate the effector phase of inflammatory arthritis has been obtained in both collagen-induced arthritis (CIA) and K × B/N mice (6, 7). In each case, passive transfer of Abs directed against collagen or the glycolytic enzyme glucose 6-phosphate isomerase (GPI), respectively, induces joint destruction by binding and directing immune effector responses to cartilage surfaces. CD4⁺ T cells also play a crucial role in disease pathogenesis in CIA and K × B/N mice by promoting activation of the autoreactive B cells that produce pathogenic autoantibodies (6, 7). The recently described SKG mouse develops arthritis because of a mutation in the Zap-70 gene that alters the threshold for negative selection of thymocytes (8). Adoptive transfer studies in this model showed that CD4⁺ T cells can mediate arthritis development in the absence of Ab (8), although whether this is a manifestation of autoreactive CD4⁺ T cells recognizing particular self-peptides or a consequence of polyclonal activation of autoreactive CD4⁺ T cells is not known.

In this report, we have examined how autoimmune arthritis develops in transgenic mice expressing a CD4⁺ TCR with specificity for a nominal Ag (influenza hemagglutinin (HA)) and coexpressing the HA as a neo self-Ag expressed under the control of a MHC class II promoter (TS1 × HACII mice). We show that the majority of adult TS1 × HACII mice spontaneously develop autoimmune arthritis even though HA-specific CD4⁺ T cells are subjected to multiple mechanisms of tolerance induction. Disease development is accompanied by systemic cytokine production and B cell activation, and an enhanced immune response develops in lymph nodes (LNs) draining the major joints of arthritic mice. Notably,
we also show that arthritis can develop in TS1 × HACII mice that lack B cells and/or recombinase-activating gene activity, indicating that autoantibody production is not required for disease development. These findings identify a novel pathway for the development of inflammatory arthritis, which can result from an autoreactive CD4+ T cell response to a single self-peptide expressed by systemically distributed APCs.

Materials and Methods

Mice

HACII, HA104, and TS1 mice were previously described (9–11) and backcrossed for at least 10 generations with BALB/c mice before use. JH+/− mice (12) and RAG+/− mice (13) (both BALB/c background) were obtained from Taconic Farm. Mice were housed in sterile microisolators under specific pathogen-free conditions at The Wistar Institute Animal Facility. All animal experiments were approved by The Wistar Institutional Animal Care and Use Committee.

Flow cytometry

Single-cell suspensions were analyzed using the following Abs: anti-CD4-PE (GK1.5), anti-CD8-FITC (53-6.7), anti-CD25-PC61, anti-CD45RB-PE (RB6-8C5), anti-CD11b-allophycocyanin (M1/70), and anti-I-A/FITC (AMS-32.1), anti-CD45RB-B220-PE (RA3-6B2), anti-CD11c-PE (HL3), anti-IL-17-PE (TC11-18H10), anti-IFN-γ-allophycocyanin (XMG1.2), anti-IL-10-PE (JES5-16E3), anti-IL-6-PE (MP5-20F3; all obtained from BD Pharmingen), anti-TNF-FITC (MP6-X22), and anti-IL-4-FITC (BVD6-24G2; obtained from eBioscience). 6.5-biotin (10) and anti-PR8 HA-biotin (B6-82) were detected using streptavidin-670 (Invitrogen Life Technologies). Intracellular Foxp3 expression was examined by staining with anti-Foxp3-PE (FJK-16s) according to the manufacturer’s protocol (eBioscience). Intracellular cytokine expression was examined using the BD Cytofix/Cytoperm kit according to the manufacturer’s protocol (BD Pharmingen). More than 50,000 events were collected on a FACScalibur (BD Biosciences) for each sample and analyzed using FlowJo (Tree Star) or Summit (DakoCytomation) software. Purification of cells by fluorescence-activated cell sorty was conducted at The Wistar Institute Flow Cytometry Facility using a MoFlo and Summit software (DakoCytomation) and typically yielded >95% pure cell populations.

In vitro APC stimulation

For B cell stimulation, splenocytes were cultured for 48 h in 24-well flat-bottom plates at 5 × 10^6 cells/ml in supplemented IMDM plus 10% FBS with 1 μg/ml of F(ab’)2 anti-mouse IgG (Jackson ImmunoResearch Laboratories). For stimulation of dendritic cells (DCs), splenocytes (5 × 10^6/ml) were incubated for 2 h at 37°C on 150-mm petri dishes, after which nonadherent cells were removed and the plates were incubated overnight at 37°C with fresh IMDM plus 10% FBS. Nonadherent cells were harvested the following day and examined by flow cytometry.

In vivo T cell proliferation assays

Single-cell suspensions from LNs of TS1 mice were prepared in serum-free IMDM and incubated with 5 μM CFSE (Molecular Probes) at 1 × 10^6 cells/ml for 4 min. An equal volume of PBS was then added, and cells were washed with IMDM. Adoptive transfer recipients were injected i.v. with 1 × 10^7 labeled cells and after 3 days LNs from recipient mice were harvested and analyzed by flow cytometry.

In vitro T cell proliferation assays

For analysis by flow cytometry, CD4+ T cells were purified from LNs of TS1, TS1 × HA104 or TS1 × HACII mice by staining with Abs to MHC class II, B220, and CD8 followed by MACS depletion (Miltenyi Biotec) according to the manufacturer’s protocol and were then cultured for 2 h in supplemented IMDM plus 10% FBS with or without the addition of 10 μM of the inhibitor 2 (SDFEREFIPKE) peptide (synthesized and HPLC purified by The Wistar Institute peptide synthesis facility) or 5 μM of the inhibitor 2 (10 mg/ml). In some cases, CFSE-labeled TS1 LN cells (5 × 10^6 cells) were cocultured in IMDM plus 10% FBS with graded numbers of CD4-depleted splenocytes from HACII mice mixed with CD4-depleted splenocytes from BALB/c mice (5 × 10^6 total APCs). After 72 h, cells were analyzed by flow cytometry. For analysis based on [H]thymidine uptake, 5 × 10^6 MACS-enriched CD4+ cells or unfractionated LNs cells were cocultured with 3 × 10^6 APCs with or without peptide. In some cases, plasmacytoid DCs (DC11c- B220+PDCA–CD19+), B cells (CD11c–B220+PDCA–CD19+), and myeloid DCs (DC11c+ B220+PDCA–CD19+) were purified by cell sorting from pooled LNs (popliteal, cervical, brachial, and axillary) of arthritic TS1 × HACII mice and used as APCs. For suppression assays, graded numbers of 6.5 CD4+CD25+ or 6.5 CD4+CD25– cells purified by cell sorting from TS1 or TS1 × HACII mice were added to cultures.

Autoantibody ELISAs

For quantification of serum IgG titers, U-bottom vinyl plates (Costar) were coated with goat anti-mouse IgG (H + L) (Southern Biotechnology Associates) and bound Ab was detected with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates). Purified mouse IgG (Sigma-Aldrich) was used as a standard. Plates for rheumatoid factor were coated with a purified monoclonal mouse IgG. Bound Ab was detected with goat anti-mouse IgM/AP (Southern Biotechnology Associates); positive controls were sera from mice undergoing a chronic graft-versus-host response (provided by Dr. T. Lauer, University of Pennsylvania, Philadelphia, PA). For GPI assays, plates were coated with GPI (Sigma-Aldrich) and bound Ab was detected with goat anti-mouse IgM plus IgG-AP. Anti-type II collagen and anti-CCP titers were analyzed using the Arthrogen-CIA ELISA Kit and the QUANTA Lite CCP IgG ELISA Kit (Chondrex, respectively), as per the manufacturer’s instructions.

LN cell cytokine production

Unfractionated LN cells (2.75 × 10^6 cells/ml) were cultured at 37°C for 72 h in supplemented IMDM plus 10% FBS. ELISAs were performed on culture supernatants using anti-cytokine Ab pairs and recombinant cytokine standards per the manufacturer’s instructions (BD Pharmingen).

Tissue analysis

Tissues were fixed in 10% buffered formalin (Fisher Scientific), immobilized in paraffin, and 5-μm sections were cut and stained using H&E and/or Masson’s trichrome. Sections were counterstained with hematoxilin, dehydrated, and mounted. Hematoxylin and eosin-stained slides were reviewed by an independent consultant (M. Sigal, Temple University) who was blinded to the experimental conditions.

Results

Spontaneous autoimmune arthritis in TS1 × HACII mice

TS1 mice express a TCR transgene that recognizes the major I-Ealpha-restricted T cell determinant from the PR8 HA (termed S1) (10), and HACII mice express a transgene encoding the influenza virus PR8 HA under the control of a MHC class II I-Eα promoter (9, 14). To examine how HA (acting as a surrogate self-peptide) shapes HA-specific CD4+ T cell development, we mated TS1 mice with HACII mice. As we developed these progeny, we found that most adult TS1 × HACII mice exhibited swelling of their ankle and/or wrist joints that was usually symmetrical (Fig. 1, A–C; data not shown). Swollen joints from adult TS1 × HACII mice contained a mononuclear lymphocytic infiltrate in the synovium accompanied by substantial cartilage loss, bone erosions, and inflammatory tissue over the cartilage surface (pannus) (Fig. 1D).

The majority of cells in synovial exudates obtained from arthritic TS1 × HACII mice coexpressed CD11b and GR-1 and were mostly neutrophils (with some macrophages) based on morphology (Fig. 1E; data not shown). The remainder was composed primarily of CD4+ T cells, of which ~17% expressed the clonotypic 6.5 TCR (Fig. 1E; data not shown). Lung tissue from arthritic TS1 × HACII mice contained an intense perivascular mononuclear cell infiltration that was usually accompanied by interstitial pneumonitis, and some mice also displayed evidence of lymphocytic cardiac valvulitis and myocarditis (data not shown). How- ever, there was no evidence of inflammatory pathology in kidney, liver, thyroid, salivary glands, pancreatic islets, or intestinal tissue from arthritic TS1 × HACII mice (data not shown).

As we increased the number of TS1 × HACII mice for analysis, a subset was found to develop a distinct multifocal autoimmune disease characterized by severe wasting, alopecia, and scaling of the skin (data not shown). Histologic analyses of tissues from these acutely

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diseased mice revealed cellular infiltrates present in a variety of tissues including the kidney, liver, lung, heart, and salivary gland (data not shown). In an analysis of one cohort of 38 TS1/H11003 HACII mice, 6 (15%) mice exhibited this acute multifocal disease, which arose sporadically and affected individual mice from litters in which other mice did not develop the multifocal disease. Of the remaining 32 TS1/H11003 HACII mice in this cohort, 28 (88%) went on to develop joint swelling by 11 wk of age (data not shown). Thus, although a fraction develop a severe wasting disease and autoimmunity affecting multiple major organs, the majority of TS1/H11003 HACII mice appear healthy until they are adults, when they spontaneously develop autoimmune disease with inflammatory arthritis as a prominent manifestation accompanied by inflammatory pathology affecting the lung.

**HA is a potent stimulator of CD4 T cells in HACII mice**

It was notable that TS1/H11003 HACII mice spontaneously develop inflammatory arthritis, because this disease does not develop in other lineages that use alternate promoters to drive HA expression, including five lineages of TS1/H11003 HA-transgenic mice that we have previously described (Refs. 11, 15, and 16 and unpublished observations). Nor has arthritis development been reported in additional lineages of TS1/H11003 HA-transgenic mice that use other promoters to control HA expression (17–21). To determine the extent to which expression driven by a MHC class II promoter targets HA expression to APCs, we first analyzed APCs from HACII mice for expression of cell surface HA by flow cytometry. For comparison, we also examined HA104 mice, which also express HA as a systemically distributed self-Ag, in this case driven by a SV40 early region promoter enhancer (11). B220+ and CD11c+ splenocytes from HACII mice were found to express higher cell surface HA levels than those from either BALB/c or from HA104 mice, especially following in vitro stimulation under conditions that induce APC activation and up-regulation of MHC class II (Fig. 2A).

**FIGURE 1.** The majority of adult TS1 × HACII mice develop arthritis. A, Photographs show 20-wk-old TS1 (left) vs TS1 × HACII (right) mice (left panel) with close-ups of forepaws (top right panel) and hind paws (lower right panel). B, Graph shows the largest ankle widths of TS1 (○) and TS1 × HACII (●) mice, with lines connecting the means. ***, p = 0.01; Mann-Whitney U test. C, Graph shows percentages of mice exhibiting inflammation and/or swelling affecting at least one paw based on weekly observation (n = 60 mice/time point). D, H&E-stained knee sections from 23-wk-old TS1 (left panel) and TS1 × HACII littermates (right panel); b, bone; c, cartilage; s, synovium; p, pannus; arrowhead, bone erosion. Original magnification, ×4. E, Dot plot shows expression of CD11b and GR-1 on live cells obtained from synovial fluid from arthritic TS1 × HACII mice with the mean percentage of CD11b+GR-1+ cells indicated (n = 3; left panel). Histogram shows 6.5 staining on CD4+ cells from synovial fluid with the mean percentage of 6.5+ cells indicated (n = 6; right panel).

**FIGURE 2.** APCs from HACII mice synthesize HA. A, Histograms show HA expression by B220+ and CD11c+ cells from HACII mice (black lines), BALB/c mice (gray lines), and HA104 mice (dashed lines) either directly ex vivo (upper panels) or following stimulation in vitro (lower panels). Insets, Expression of MHC class II on unstimulated or stimulated APCs. B, LN cells from TS1 mice were CFSE-labeled and transferred into BALB/c, HA104, and HACII recipients. Dot plots show expression of 6.5 vs CFSE on CD4+ splenocytes 3 days posttransfer (left panels) and histograms show expression of MHC class II and CD86 on B220+ cells from HACII (black lines) of BALB/c (gray lines) recipient mice. Data are representative of at least three experiments.
We next compared HACII and HA104 mice for their ability to present HA-derived peptides to CD4⁺ T cells in vivo, based on their ability to promote division of CD4⁺ T cells from TS1 mice (which can be detected with the mAb 6.5 (10)). 6.5⁺CD4⁺ T cells divided multiple times following transfer into both HACII and HA104 mice, although more divisions occurred in HACII mice (Fig. 2B). In addition, B220⁺ splenocytes in HACII (but not HA104 or BALB/c) mice became activated by the transferred TS1 LN cells, as evidenced by their elevated expression of MHC class II and CD86 (22) (Fig. 2B). Together, these data indicate that APCs from both HACII and HA104 mice present the HA-derived S1 peptide in vivo, but that HA is a more potent inducer of 6.5⁺CD4⁺ T cell division in HACII mice and, moreover, interactions with 6.5⁺CD4⁺ T cells can induce activation of APCs in HACII mice.

**TS1 × HACII mice expressing only the clonotypic TCR and/or lacking B cells develop arthritis**

Because 6.5⁺CD4⁺ T cells could induce B cell activation in HACII mice and because autoantibody production has been implicated in disease pathogenesis in several models of arthritis, we examined the phenotype of B cells in TS1 × HACII mice. Indeed, the spleens of arthritis TS1 × HACII mice contained greater numbers of B220⁺ cells expressing higher levels of CD86 and MHC class II than TS1 and TS1 × HA104 mice, and sera from TS1 × HACII mice also contained higher levels of serum IgG than either TS1 or TS1 × HA104 mice (Fig. 3, A and B). Although they were hyperggammaglobulinemic, sera from TS1 × HACII did not exhibit elevated reactivity with IgG (rheumatoid factor), GPI, CCP, or collagen II in ELISA (data not shown). Thus, the presence of autoactive 6.5⁺CD4⁺ T cells in TS1 × HACII mice leads to systemic B cell activation, although autoantibodies that are elevated in sera from some (but not all) RA patients were not elevated in sera from TS1 × HACII mice (4).

To directly test whether autoantibodies and/or B cells are required for arthritis development in TS1 × HACII mice, we generated TS1 × HACII.RAG mice, which lack B cells because they cannot undergo productive Ig H chain gene rearrangement (12, 13). The ankles of adult TS1 × HACII.RAG mice and TS1 × HACII.RAG⁺/− mice were significantly swollen relative to those of TS1.JH⁻/⁻ or TS1.RAG⁺/− mice (p values <0.01, Student’s 2 test; Fig. 3, C and D). Histologic examination of sections

**FIGURE 3.** B cells are not required for arthritis development. A, Histograms show CD86 and MHC class II expression on B220⁺ splenocytes from TS1 (gray line), TS1 × HA104 (dashed line), and TS1 × HACII (black line) mice. B, Bar graphs show the numbers of B220⁺ splenocytes and serum IgG levels, with circles representing individual mice. Bars indicate the averages for TS1 (□), TS1 × HA104 (□), and TS1 × HACII (■) mice. C, Graph indicates the largest ankle widths from individual TS1.JH⁻/⁻ (□) and TS1 × HACII.JH⁻/⁻ (□) mice. The mean ankle width of TS1 × HACII.JH⁻/⁻ mice is significantly greater than that of TS1.JH⁻/⁻ mice (p < 0.01, Student’s 2 test). Photomicrograph shows H&E-stained knee section from an arthritic TS1 × HACII.JH (-/-) mouse. p, Pannus; arrowhead, bone erosion. Original magnification, ×10. D, Histograms show CD86 and MHC class II expression on B220⁺ splenocytes and serum IgG levels from TS1 (gray line), TS1 × HA104 (dashed line), and TS1 × HACII (black line) mice. The mean ankle width of TS1 × HACII.RAG⁻/⁻ mice is significantly greater than that of TS1.RAG⁻/⁻ mice (p < 0.01, Student’s 2 test). Photomicrograph shows H&E-stained wrist section from an arthritic TS1 × HACII.RAG⁻/⁻ mouse. p, Pannus. Original magnification, ×10.

**FIGURE 4.** 6.5⁺CD4⁺ T cells accumulate in the periphery of TS1 × HACII mice. A, Histograms show 6.5 expression (6.5⁺ gate indicated) on CD4SP thymocytes (left panels) and CD4⁺ splenocytes (right panels) from TS1 (gray line), TS1 × HA104 (dashed line), and TS1 × HACII (black line) mice at 4 wk of age (upper panels) or ≥12 wk of age (lower panels). Graphs show numbers of thymocytes, 6.5⁺CD3SP thymocytes, CD4⁺ splenocytes, and 6.5⁺CD4⁺ splenocytes, with bars indicating the averages for TS1 (□), TS1 × HA104 (□), and TS1 × HACII (■) mice. B, Histograms show 6.5 expression on CD4⁺ splenocytes from TS1 (gray line) and TS1.RAG⁻/− (black line) mice (left panel) and from TS1 × HACII (gray line), TS1 × HACII.RAG⁻/− (black line), and TS1.RAG⁺/− (dashed line) mice (right panel).
CD69, CD44, and CD45RB expression by 6.5 levels on CD4

from the peripheral joints of both TS1 × HACII.JH−/− and TS1 × HACII.RAG−/− mice revealed intra-articular pathology including bone erosions, pannus formation, synovial hyperplasia, and cartilage loss (Fig. 3, C and D).

Thus, even though B cells are systemically activated in TS1 × HACII mice, neither Ab production, cytokine production, nor Ag presentation by B cells is required for arthritis to develop. Moreover, because disease develops in TS1 × HACII.RAG−/− mice, no other cells types that require RAG-mediated gene re-arrangement (such as γ-δ and NK T cells) are required for arthritis development. Importantly, the development of arthritis in TS1 × HACII.RAG−/− mice also shows that disease can be the result of CD4+ T cells recognizing a single self-peptide expressed by APCs, and that additional specificities generated through allelic inclusion of TCR α- or β-chains are not required.

HA-specific CD4+ T cells accumulate in arthritic TS1 × HACII mice

Because 6.5+ T cells are necessary and sufficient for arthritis development, we analyzed their development in TS1 × HACII mice relative to TS1 and TS1 × HA104 mice. We first compared the frequency and phenotype of 6.5+ CD4+ T cells in 4- and 12-wk-old TS1 × HACII mice with that in TS1 and TS1 × HA104 mice.

6.5+ CD4 single-positive (CD4SP) thymocytes were substantially reduced in the thymi and spleens of 4-wk-old TS1 × HACII and TS1 × HA104 mice, consistent with deletion of 6.5+ CD4SP thymocytes (Fig. 4A). Four week-old TS1 × HACII mice also contained fewer total CD4SP thymocytes and CD4+ T cells than TS1 × HA104 mice. By 12 wk of age, however, CD4SP thymocyte and CD4+ T cell numbers in TS1 × HACII mice had recovered to the same levels as in TS1 × HA104 mice and, strikingly, TS1 × HACII mice also contained twice as many 6.5+ CD4+ T cells as TS1 × HA104 mice. Indeed, the spleens of 12-wk-old TS1 × HACII mice contained half as many 6.5+ CD4+ T cells as TS1 mice, even though they contained many fewer 6.5+ CD4SP thymocytes (Fig. 4A). It was also noteworthy that the 6.5 TCR was expressed at lower levels on CD4+ cells in the thymi and spleens of 4-wk-old TS1 × HACII mice (Fig. 4B), which cannot be due to allelic inclusion of TCR α- or β-chains and must reflect lower TCR levels (either through TCR down-modulation or by expansion of rare cells expressing low TCR levels) (Fig. 4A). Thus, synthesis of HA by APCs induces severe deletion of 6.5+ CD4SP thymocytes in TS1 × HACII mice; however, CD4+ T cells expressing reduced levels of the 6.5 TCR nevertheless accumulate in the periphery of

FIGURE 5. Phenotypic characteristics of 6.5+ CD4+ T cells from TS1 × HACII mice. A, Histograms show CD69, CD44, and CD45RB expression by 6.5+ CD4+ (top row) and 6.5+ CD4− (bottom row) splenocytes from 12-wk-old TS1 (gray lines/outline), TS1 × HA104 (dashed black lines, plain font), and TS1 × HACII (bold black lines, bold font) mice, with the mean percentage of cells in the indicated gates shown (n = 3 mice/ger-}

otype). B, Purified CD4+ T cells from TS1 (top row), TS1 × HA104 (middle row), and TS1 × HACII (bottom row) were CFSE-labeled and cultured with or without S1 peptide in the presence or absence of exogenous IL-2 as indicated. Dot plots show 6.5 vs CFSE without S1 peptide in the presence or absence of exogenous IL-2 as indicated. D, Unfractionated LN cells isolated from adult mice were cultured in vitro for 3 days without addition of exogenous peptides or cytokines. The concentrations of the indicated cytokines in supernatants were then assessed by ELISA. C, Values from individual mice; bars represent the average for TS1 (□), TS1 × HA104 (□), and TS1 × HACII (□) mice.

D. Dot plots show intracellular cytokine staining of CD4+ (upper panels) and 6.5+ CD4+ (lower panels) LN cells from TS1 × HACII mice following stimulation immediately ex vivo either with PMA and ionomycin for 4 h (for IL-17, IFN-γ, IL-10, and IL-4) or with 3 μM S1 peptide for 4 h (for IL-6 and TNF). Cells were then stained with CD4, 6.5, and the indicated Abs and analyzed by flow cytometry. Percentages of cells in indicated gates are shown (n = 3 mice/condition).
TS1 × HACII mice. Moreover, this peripheral accumulation of HA-specific CD4+ T cells appears to be promoted by synthesis of HA by APCs, because it is enhanced in TS1 × HACII mice relative to TS1 × HA104 mice.

CD4+ T cells promote spontaneous cytokine production in TS1 × HACII mice

The 6.5 CD4+ T cells in the spleens of both TS1 × HACII and TS1 × HA104 mice expressed higher levels of CD69 and CD44 and lower levels of CD45RB than those from TS1 mice, with higher percentages in TS1 × HACII mice exhibiting these activated/Ag-experienced phenotypes (Fig. 5A). When examined for their ability to proliferate in response to S1 peptide in vitro, the 6.5 CD4+ T cells from TS1 × HACII mice were markedly hypoproliferative relative to those from either TS1 or TS1 × HA104 mice, even in the presence of excess IL-2 (Fig. 5B).

Notably, however, freshly explanted LN cells obtained from TS1 × HACII mice spontaneously secreted substantially higher amounts of IL-17, IFN-γ, TNF, IL-6, and IL-10 than were secreted by LN cells from either TS1 or TS1 × HA104 mice when cultured in vitro without the addition of any exogenous Ags or cytokines; they also secreted higher levels of IL-4, although the absolute amount of IL-4 based on ELISA measurement was modest (Fig. 5C). Intracellular cytokine staining of LN cells from TS1 × HACII mice showed that IFN-γ and IL-17-secreting CD4+ T cells mostly comprise distinct populations and that 6.5 CD4+ T cells are enriched for the production of these cytokines (Fig. 5D). 6.5 CD4+ T cells were also enriched for IL-10-secreting cells, and consistent with the ELISA data, relatively few CD4+ T cells from TS1 × HACII mice secreted IL-4 (Fig. 5D). In addition, whereas IL-6-secreting CD4+ and 6.5 CD4+ T cells were detectable in LN cells from TS1 × HACII mice, TNF-secreting CD4+ cells were rare, suggesting that other cell types are the major contributors to TNF production in these LN cultures (Fig. 5D). Thus, the 6.5 CD4+ T cells that accumulate in TS1 × HACII mice exhibit an Ag-experienced phenotype and even though they are hypoproliferative when stimulated with S1 peptide, they promote spontaneous cytokine production by LN cells from TS1 × HACII mice.

TS1 × HACII mice contain regulatory T cells

To determine whether the autoimmunity that develops in TS1 × HACII mice reflects a paucity of regulatory T cells, we examined CD4+ T cells from TS1 × HACII mice for the expression of CD25 and Foxp3 (23, 24). TS1 × HACII mice contained higher percentages of CD4+CD25+ T cells than were present in TS1 mice, and the majority expressed low levels of the clonotypic TCR and were Foxp3+ (Fig. 6, A–C). TS1 × HACII mice also contained on average half as many 6.5 CD4+Foxp3+ cells as TS1 mice (Fig. 6, A–C) and, moreover, 6.5 CD4+CD25+ T cells from TS1 × HACII mice efficiently suppressed the in vitro proliferation of CD4+ T cells from TS1 mice in response to S1 peptide (Fig. 6D). Suppression was also mediated (albeit less efficiently) by 6.5 CD4+CD25+ T cells from TS1 × HACII mice, resembling findings in mice expressing HA driven by an Ig promoter (Ref. 19 and Fig. 6D). Thus, TS1 × HACII mice develop autoimmune arthritis despite the presence of regulatory T cells, although it is possible that the reduced frequency of Ag-specific regulatory T cells contributes to disease development.

An enhanced regional immune response accompanies arthritis development

Since the autoimmune disease that develops in TS1 × HACII mice exhibits selectivity for major joints, we examined whether it was accompanied by a regional immune response in the popliteal LNs, which drain the ankles and knees. Indeed, popliteal LNs of arthritic TS1 × HACII mice contained on average six times as many cells as popliteal LNs from TS1 mice (Fig. 7A). By contrast, the cervical LNs were of similar size and the inguinal LNs were much smaller in TS1 × HACII than in TS1 mice. The increased cellularity of the popliteal LNs of TS1 × HACII mice was associated with increases in the numbers of CD11c+ cells relative to TS1 mice (Fig. 7A), and...
unfractionated popliteal LN cells from TS1 × HACII mice induced substantially higher proliferation of CD4^+ T cells from TS1 mice than was induced by other LN cells from TS1 × HACII mice or by poplitel LN cells from HACII mice (Fig. 7B). This likely reflects the enhanced representation (and/or activation status) of myeloid DCs, because these cells from TS1 × HACII mice induce CD4^+ T cell proliferation far more efficiently than either B cells or plasmacytoid DCs (Fig. 7C).

6.5^CD4^+ T cells also appear to accumulate preferentially in the poplitel LNs of TS1 × HACII mice. Thus, although the percentages of CD4^+ T cells that were 6.5^+ were similar in various LNs (and spleens) of TS1 × HACII mice (data not shown), the number of 6.5^CD4^+ T cells was significantly lower in the cervical and inguinal LNs of TS1 × HACII than in TS1 mice. By contrast, the number of 6.5^CD4^+ T cells in the poplitel LNs was similar in TS1 × HACII and TS1 mice, suggesting a relative accumulation of 6.5^CD4^+ T cells in the poplitel (vs cervical and inguinal) LNs of TS1 × HACII mice (Fig. 7A). The poplitel LNs of arthritic TS1 × HACII mice contained both IL-17- and IFN-γ-secreting CD4^+ T cells, and their levels of expression of the 6.5 TCR were similar to those found on CD4^+ T cells isolated from the synovial exudates (Figs. 1D and 7D). The poplitel LNs also contained relatively high frequencies of CD4^+CD25^Foxp3^+ T cells, although the frequency expressing the 6.5 TCR was lower than for cytokine-secreting LN cells (Fig. 7E). Thus, TS1 × HACII mice develop an enhanced autoimmune response in LNs draining major joints that is associated with increased APC activity and accrual of cytokine-producing 6.5^CD4^+ T cells.

**Discussion**

When HACII mice coexpress the HA-specific TS1 TCR transgene, the HA becomes a target for autoreactive HA-specific CD4^+ T cells that induce the development of autoimmune arthritis. It is notable that HA-specific CD4^+ T cells promote autoimmunity despite evidence that they are subjected to multiple mechanisms of tolerance induction. Thus, autoreactive 6.5^ CD4^+SP thymocytes are subjected to severe deletion by the S1 self-peptide and autoreactive CD4^+ T cells that persist display reduced TCR levels that could potentially curtail their reactivity, autoreactive CD4^+ T cells in TS1 × HACII mice also appeared anergic based on their hypoproliferative response to in vitro peptide stimulation even in the presence of exogenous IL-2 (26). Moreover, TS1 × HACII mice develop autoimmune arthritis despite the presence of regulatory T cells both systemically and in the poplitel LNs, resembling human RA patients who have similarly been found to contain CD4^+ T cells exhibiting regulatory phenotypes (e.g., CD25^brightFoxp3^+) at elevated frequencies in the inflamed joints (27–29). However, despite these various mechanisms that could potentially curtail their reactivity, autoreactive CD4^+ T cells bearing differentiated phenotypes accumulate in TS1 × HACII mice. The ability of otherwise hyporesponsive CD4^+ T cells to persist and elaborate effector functions in the context of continual antigenic stimulation has been previously described (30, 31), including a study in which hypoproliferative autoreactive CD4^+ T cells were found to induce B cell activation, hypergammaglobulinemia, and arthritis development (32). TS1 × HACII mice similarly exhibited evidence of polyclonal B cell activation and hypergammaglobulinemia. Significantly, however, we showed that neither B cell activation nor autoantibody production is required for arthritis development, since both TS1 × HACIIJH^-/- and TS1 × HACII/RAG^-/- mice become arthritic.
CD4+ T cells induce arthritis

The finding that arthritis develops spontaneously in TS1 × HACII mice, but not in numerous other lineages of TS1 × HA-transgenic mice that use other promoters to drive expression of the HA (11, 15–21), strongly suggests that the breakdown of tolerance is promoted by the synthesis of the target Ag by APCs. Synthesis of HA by APCs could promote tolerance breakdown and autoimmunity in TS1 × HACII mice by several mechanisms: First, it could be that lymphopenia secondary to the severe thymocyte deletion that occurs in TS1 × HACII mice contributes to the autoimmune process, since lymphopenia has been associated with the development of autoimmune disease in some other systems (33–35). Second, synthesis of the HA by APCs may promote disease development because it provides strong stimulatory signals that are sufficient to stimulate effector function by autoreactive CD4+ T cells, even though they have been subjected to multiple tolerance mechanisms. Strong stimulatory signals delivered by APCs might also contribute to a failure of the 6.5° CD4+CD25+Foxp3+ T cells that are present to prevent disease in TS1 × HACII mice, since strong antigenic stimulation has been shown to overcome regulatory T cell function in vitro (36). Third, synthesis of the target Ag may enhance reciprocal activation of APCs by autoreactive CD4+ T cells, and cause APCs to acquire properties that facilitate autoreactive CD4+ T cell activation. Consistent with this, we found that adaptively transferred TS1 CD4+ T cells induced B cell activation much more efficiently in HACII than HA104 mice, and that B cells in intact TS1 × HACII mice exhibit a similarly activated phenotype. This ability of autoreactive CD4+ T cells to induce APC activation is noteworthy since recent studies showed that autoreactive CD4+ T cells with genetically altered T cell signaling thresholds can induce APCs to secrete cytokines (such as IL-6) that can promote Th17 cell differentiation (34), although how the specificity of CD4+ T cells for individual self-peptides might contribute to this process has not been determined. We have shown here that spontaneous differentiation of autoreactive IL-17-secreting CD4+ T cells can similarly occur in mice with nonmutated T cell signaling pathways, when the CD4+ T cell repertoire is skewed toward reactivity with a self-peptide that is expressed by APCs. Moreover, the expansion and differentiation of HA-specific CD4+ T cells occurs much more efficiently in TS1 × HACII than in TS1 × HA104 mice, demonstrating that these processes are facilitated by expression of HA by APCs.

The development of arthritis is accompanied by an enhanced accumulation of autoreactive effector CD4+ T cells in the popliteal LNs of TS1 × HACII mice, and the popliteal LNs also possess increased numbers of DCs and an enhanced ability to stimulate HA-specific CD4+ T cells. Since the popliteal LNs drain the knees and ankles of arthritic mice, this resembles studies in other organ-specific autoimmune diseases showing that lymphocyte responses develop preferentially in LNs draining the target organ (37–39). In those studies, this was thought to reflect the high relative concentration of autoantigen presented by DCs in LNs draining the target tissue(s). However, the target peptide recognized by autoreactive HA-specific CD4+ T cells in TS1 × HACII mice is synthesized by systemically distributed APCs. The enhanced immune response that develops in the popliteal LNs therefore likely reflects the regional activation of APCs, rather than selective expression of the HA by popliteal LN cells. Previous studies have shown that proinflammatory cytokines can stimulate synovioocytes (which include DCs, macrophages, and synovial fibroblasts) to proliferate and produce cytokines and mediators that damage surrounding tissue and bone (40). Moreover, transgenic mice engineered to systemically overexpress TNF or IL-1α develop inflammatory arthritis, demonstrating that synovioocyte activation and arthritis can result from imbalanced proinflammatory cytokine production (41, 42).

Based on these findings, then, we propose that proinflammatory cytokine production by autoreactive CD4+ T cells in TS1 × HACII mice may induce intra-articular activation of APCs (including DCs) and their subsequent trafficking to the popliteal LN where they can interact with HA-specific CD4+ T cells. Biomechanical stresses acting on joints such as the knees and ankles may also enhance APC activation within the synovial tissue (43) (including HA up-regulation on APCs, which we have shown is induced by activating stimuli) and promote local stimulation of activated HA-specific CD4+ T cells that enter the synovial tissue. Enhanced APC activation caused by environmental Ags may similarly contribute to the lung pathology that develops in arthritic TS1 × HACII mice. In this model, then, arthritis appears to be the result of chronic cytokine production by autoreactive CD4+ T cells whose hyporesponsiveness may limit their ability to mediate pathology affecting major organs such as the kidney and liver, even though their target peptide is synthesized by systemically distributed APCs. However, the spontaneous differentiation of and chronic cytokine production by these autoreactive CD4+ T cells cause arthritis because it promotes activation of intra-articular APCs and precipitates a self-reinforcing cycle of interaction between HA-specific CD4+ T cells and HA-expressing APCs in LNs draining the joints. In this respect, the presence of IL-17-secreting cells in the popliteal LNs is particularly noteworthy: IL-17 has been implicated in arthritis pathogenesis in other mouse models (34, 44, 45) and can promote arthritis both by attracting neutrophils (which are abundant in the arthritic joints of TS1 × HACII mice) and by direct bone damage through induction of metalloproteases, differentiation of osteoclasts, and inhibition of proteoglycan synthesis (46, 47).

The findings in TS1 × HACII mice show that inflammatory arthritis can be the result of CD4+ T cells recognizing a single peptide that is synthesized by APCs and can develop in the absence of autoantibodies or B cells. It is important to note that these studies do not address the possibility that Abs and/or B cells can contribute to disease severity; we have shown that CD4+ T cells can be necessary and sufficient for the development of inflammatory arthritis, but it remains possible that B cells and/or autoantibodies can modify the penetrance, severity, or course with which inflammatory arthritis develops in TS1 × HACII mice. In human RA, the presence of autoantibodies, such as rheumatoid factor or Abs to CCP, is associated with more severe disease, suggesting a role for autoantibodies in disease pathogenesis (2, 3). Indeed, B cell depletion is emerging as a therapeutic strategy that can be effective in some RA patients, supporting the conclusion that B cells can at least in some cases play a role in the disease process (48). With respect to RA, the studies here may also provide clues into the basis by which particular MHC class II alleles can confer susceptibility to the disease. Because we have shown that targeting CD4+ T cells to an Ag synthesized by APCs can promote arthritis, it is possible that RA-associated MHC class II susceptibility alleles could act by facilitating the presentation of a target peptide (or peptides) synthesized by APCs themselves. Individuals that express such a susceptibility allele and that develop a high frequency of autoreactive CD4+ T cells recognizing these target peptides may then be predisposed toward development of arthritis, as occurs in TS1 × HACII mice. Further analyses in this model should provide additional insights into the etiology of inflammatory arthritis and allow evaluation of various treatment modalities.

Disclosures

The authors have no financial conflict of interest.
References


