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$CD4^+CD25^+$ regulatory T cells in autoimmune arthritis

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Summary

$CD4^+CD25^+$ regulatory T (Treg) cells can play a critical role in the prevention of autoimmunity, as evidenced by the cataclysmic autoimmune disease that develops in mice and humans lacking the key transcription factor forkhead box protein 3 (Foxp3). At present, however, how and whether Treg cells participate in the development of rheumatoid arthritis (RA), which has both systemic manifestations and a joint-targeted pathology that characterizes the disease, remains unclear. In this review, we describe work that has been carried out aimed at determining the role of Treg cells in disease development in RA patients and in mouse models of inflammatory arthritis. We also describe studies in a new model of spontaneous autoimmune arthritis (TS1×HACII mice), in which disease is caused by $CD4^+$ T cells recognizing a neo-self-antigen expressed by systemically distributed antigen-presenting cells. We show that TS1×HACII mice develop arthritis despite the presence of $CD4^+CD25^+Foxp3^+$ Treg cells that recognize this target autoantigen, and we outline steps in the development of arthritis at which Treg cells might potentially act, or fail to act, in the development of inflammatory arthritis.

Keywords

autoimmunity; tolerance/suppression/anergy; transgenic/knockout mice

Introduction

To anticipate infections by viruses and other invading microorganisms, the immune system uses gene rearrangement mechanisms to produce an enormous diversity of T-cell receptors (TCRs) and immunoglobulins (Igs) with distinct antigenic specificities (1,2). Recognition of a foreign antigen leads to the activation of antigen-specific T and B cells from the pre-immune repertoire, and both $CD4^+$ T and B cells undergo further differentiation following primary activation; $CD4^+$ T cells can differentiate into distinct phenotypes that secrete characteristic patterns of cytokines and promote the development of qualitatively distinct immune responses (such as cell-mediated versus humoral immunity) (3). Superimposed on these processes is a requirement that those TCRs and Igs that can react with self-antigens be prevented from mediating autoimmune damage to the host's own cells and tissues. This can be achieved via elimination or inactivation of self-reactive T and B cells (4), and in recent years it has become apparent that regulatory T (Treg) cells, including $CD4^+CD25^+$forkhead box protein 3 $(Foxp3)^+$ T cells, can also play a role in preventing autoimmunity (5). However, despite the many advances that have been made in defining mechanisms of tolerance induction, how these processes fail in the two to five percent of the population that develops autoimmune disease remains poorly understood.

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It is clear that genetic factors play an important role in determining susceptibility to autoimmune diseases. The risk of developing an autoimmune disease increases when there is an affected family member, and many autoimmune diseases exhibit strong associations with susceptibility alleles (6). The most common and strongest genetic linkages with autoimmune diseases are with major histocompatibility complex (MHC) class II alleles (6); in rheumatoid arthritis (RA), alleles of the HLA-DR molecule that share a common motif in the third hypervariable region of the β-chain are associated with disease (6). Since MHC class II alleles primarily present foreign and self-peptides for recognition by $CD4^+$ T cells, these linkages point to an important role for $CD4^+$ T cells in these diseases. MHC alleles that confer susceptibility to autoimmune diseases could exert their effects because they impact $CD4^+$ T-cell tolerance induction; for example, they may fail to present a self-peptide(s) in a way that effectively purges autoreactive thymocytes during their development in the thymus, or may for some reason fail to promote the development of an appropriate population of $CD4^+CD25^+$ Treg cells. As noted above, there is now convincing evidence that $CD4^+CD25^+Foxp3^+$ Treg cells can be formed intrathymically, as an alternative to deletion, when thymocytes bearing an autoreactive TCR encounter self-peptides (7). The impact that Treg cells can exert in preventing autoimmunity is most apparent in the catastrophic multisystem autoimmune disease that develops abruptly in mice and humans that cannot express a functional Foxp3 molecule (8-10). These observations suggest that some failure to generate appropriate Treg cell populations, which might be linked to certain MHC alleles, could contribute to autoimmune diseases such as RA, which has systemic manifestations but is more typically characterized by the chronic inflammatory disease that affects joints and surrounding tissue. Understanding the role that Treg cells may play in the development of RA is therefore an important priority.

The strong linkages between MHC class II alleles and RA may also be a reflection of the central role that $CD4^+$ T cells play in promoting and controlling distinct aspects of autoimmune responses. Studies in murine models have revealed the ability of $CD4^+$ T cells to initiate inflammatory arthritis via diverse mechanisms. For example, $CD4^+$ T cells specific for the systemically distributed self-protein glucose-6-phosphoisomerase (GPI) can initiate joint pathology by activating GPI-specific B cells, leading to the production of pathogenic autoantibody(s) that bind to articular surfaces and recruit immune effector responses locally to the joint (11). Similarly, in collagen-induced arthritis, joint pathology is initiated by $CD4^+$ T cells that activate B-cell autoantibody production in response to the joint protein, type-II collagen (12,13). However, there are also models of inflammatory arthritis in which $CD4^+$ T cells themselves appear to act as the principal instigators of joint destruction. The recently described SKG mouse develops arthritis because of a mutation in the ZAP-70 (ζ-associated protein of 70 kDa) gene that alters the threshold for negative selection of thymocytes (14). Adoptive transfer studies in this model showed that $CD4^+$ T cells can mediate arthritis development in the absence of antibody, although the specificity of the TCR(s) that mediates arthritis and the nature of the peptide(s) that they recognize are unknown. Subsequent studies showed that the development of arthritis in SKG mice depends on signals received from environmental antigens, because the disease does not develop in mice housed under specific pathogen-free conditions (15). Arthritis can also develop in mice engineered to systemically overexpress tumor necrosis factor-α (TNF-α) or interleukin-1α (IL-1α,) and in mice bearing a mutated IL-6 receptor, suggesting that dysregulated systemic cytokine networks can also promote arthritis development (16-18). More recently, we described a transgenic mouse model (TS1×HACII mice) in which an autoreactive $CD4^+$ T-cell response to a self-peptide that is expressed by ubiquitously distributed antigen-presenting cells (APCs) causes the spontaneous development of autoimmune arthritis, in a process that requires the direct activity of $CD4^+$ T cells, since it can develop in mice that congenitally lack B cells (19).

This review describes studies aimed at understanding the role of Treg cells in the development of RA in humans and in mouse models of inflammatory arthritis. It describes our recent studies in TS1×HACII mice, including the finding that these mice develop inflammatory arthritis despite the presence of $CD4^+CD25^+Foxp3^+$ Treg cells that are specific for the target antigen that is recognized by autoreactive $CD4^+$ T cells in arthritic mice. It concludes with speculations regarding possible targets of Treg cell activity in autoimmune arthritis as exemplified by findings in TS1×HACII mice, along with consideration of how the specificity that Treg cells possess for self-peptides might contribute to the disease process.

$CD4^+CD25^+$ Treg cells in human arthritis

In light of the compelling evidence that $Foxp3^+$ Treg cells play an active role in preventing the spontaneous development of systemic autoimmunity, many recent studies have aimed at determining whether some deficits in Treg cell activity might contribute to the development of autoimmune diseases such as RA. Interestingly, many of these studies have reached the seemingly paradoxical conclusion that autoimmune arthritis can develop despite the presence of $CD4^+CD25^+$ Treg cells. It appears that Treg cells can be enriched in arthritic patients, since increased frequencies of $CD4^+CD25^+$ T cells have been found in synovial fluid (i.e. the primary disease site) (20-24) and in some cases also systemically in the peripheral blood of arthritic patients (25). Indeed, an enhanced representation of Treg cells in the joints and synovial fluid of affected individuals has been observed in patients with RA, with juvenile idiopathic arthritis (JIA), and with other rheumatic diseases in which arthritis is a secondary manifestation of disease (20-25). Identifying Treg cells based only on CD25 expression is limiting, however, in that it may also detect activated $CD4^+$ T cells that have upregulated the IL-2Rα chain. However, analysis of Foxp3 mRNA and protein expression supported the conclusion that the $CD4^+CD25^{bright}$ population isolated from RA patients were indeed enriched for Treg cells (20,22,23). A potential explanation for the enrichment of $CD4^+CD25^+$ Treg cells in arthritic joints is that the expression of specific patterns of chemokine receptors leads to preferential trafficking of Treg cells to the disease site(s). Studies of human peripheral blood Treg cells have shown that they express certain chemokine receptors, such as CCR4, and studies of mouse Treg cells indicated that there are many different subsets of chemokine receptor expression on Treg cells that could promote trafficking to specific locations (27,28). A comparison of $CD4^+CD25^+$ T cells from the synovial fluid and peripheral blood of patients with active RA showed a significant enrichment in the synovial fluid of Treg cells expressing the chemokine receptors CCR4, CCR5, and CXCR4, which are associated with migration to sites of inflammation (26). Additionally, comparison of the chemokine profiles of dendritic cells and synovial tissue from RA patients and healthy individuals indicated that certain chemokines are enriched during RA, potentially resulting in the preferential recruitment of a variety of immune system cells, including Treg cells (28,29). Thus in RA patients, disease develops not only despite the presence of $CD4^+CD25^+$ Treg cells but also in spite of an enrichment of the Treg cells at a primary site of autoimmune pathology.

These observations raise the question of whether the $CD4^+CD25^+$ Treg cells that are present in arthritic patients are perhaps dysfunctional, or are functional and are either unable to prevent disease, or are modifying it in some manner. There is evidence for both effective and dysfunctional Treg cell activity in disease settings. Support for the beneficial effects of Treg cells that localize in arthritic joints arose in studies of patients with JIA, where greater numbers of $CD4^+CD25^+$ T cells were found in patients with persistent oligoarticular JIA (which is a relatively mild form of the disease) than in patients with the more severe extended oligoarticular JIA (20). Additionally, Treg cells isolated from patients with the milder form of JIA expressed higher levels of Foxp3 mRNA, which have been correlated

with better suppressor function, than did Treg cells from patients with more severe disease (20). Even within individual JIA patients, there appeared to be a divergence of $CD4^+CD25^+$ Treg cell function based on the location from which the Treg cells were isolated. Results of *in vitro* suppression assays indicated that $CD4^+CD25^+$ T cells from the synovial fluid of JIA patients were more effective suppressors than those isolated from the peripheral blood, suggesting that the Treg cells at the primary disease site possessed more potent regulatory function (20,22). From a clinical standpoint, it has also been reported that the duration of remission following corticosteroid treatment in JIA patients showed a positive correlation with the number of $CD4^+CD25^+$ Treg cells present in the synovial fluid (20). Thus, in JIA patients there seemed to be a correlation between an increased frequency of Treg cells and a reduction in disease severity, with the possibility that more effective Treg cells localize to the joints and synovial fluid.

The alternative concept of dysfunctional $CD4^+CD25^+$ Treg cells in RA has been supported by findings that Treg cells isolated from RA patients exhibit reduced suppressor function (30,31). Much of this work has examined the possible effects of the inflammatory environment in RA on $CD4^+CD25^+$ Treg cell function. Several groups have shown that Treg cells isolated from RA patients post-infliximab (anti-TNF-α) treatment show improved regulatory activity in *in vitro* suppression assays (30-32). $CD4^+CD25^+$ T cells isolated from patients with active RA, pre-infliximab treatment, were able to suppress the *in vitro* proliferation but not cytokine production of responder $CD4^+$ T cells. However, after infliximab treatment, Treg cells originating from RA patients acquired the ability to suppress responder cytokine production (30). The improved suppressive activity of the $CD4^+CD25^+$ Treg cells also correlated with increased levels of Foxp3 mRNA, and correspondingly, it has been shown that treatment of healthy donor Treg cells with TNF-α leads to a reduction in Foxp3 expression and loss of suppressor function (31). Other *in vitro* work has shown that addition of cytokines such as IL-2, IL-7, and IL-15 to suppression assays can abrogate $CD4^+CD25^+$ Treg cell function, suggesting that multiple cytokines that may be elevated in RA patients can negatively affect Treg cell function (22,31,33).

There is also work suggesting that anti-TNF-α treatment may lead to the induction of peripheral Treg cells rather than an improvement in the function of pre-existing Treg cells (32). After infliximab treatment, an increased percentage of $CD4^+Foxp3^+$ cells was observed in the peripheral blood of active RA patients. Corresponding *in vitro* studies showed that upon culture with infliximab, a subset of $CD4^+CD25^-$ T cells from RA patients expressed Foxp3, which could be prevented by TGF-β blockade. Interestingly, this increase in Foxp3-expressing cells was not observed when $CD4^+CD25^-$ T cells from healthy donors were cultured with infliximab (32). The lack of Foxp3 induction in conventional $CD4^+$ T cells from healthy individuals upon infliximab treatment suggests that not only Treg cells but also effector $CD4^+$ T cells from RA patients exhibit phenotypic changes in response to the inflammatory environment. Indeed, there is work suggesting that conventional $CD4^+$ T cells isolated from the synovial fluid of RA patients are refractory to suppression by $CD4^+CD25^+$ Treg cells (20,33). While these studies of $CD4^+CD25^+$ T cells in RA have predominantly focused on the possibility of detrimental effects of the inflammatory environment on Treg cell function, more recent work has shown that Treg cells from RA patients can exhibit deficiencies in cytotoxic T-lymphocyte antigen-4 (CTLA-4) regulation that may also affect their suppressor capabilities (34). It has also been shown that higher percentages of $CD4^+CD25^+Foxp3^+$ T cells and monocytes from RA patients express glucocorticoid induced TNF receptor (GITR) and GITR-L, respectively, than in healthy donors (25). Ligation of GITR has been linked to abrogation of Treg cell function (35,36), suggesting another possible mechanism by which Treg cells might be rendered dysfunctional in RA patients.

$CD4^+CD25^+$ Treg cells in mouse models of arthritis

Studies in multiple mouse models of inflammatory arthritis have indicated that $CD4^+CD25^+$ Treg cells are capable of modifying disease, and the role of Treg cells has been most extensively studied in the collagen-induced and K/BxN arthritis models. As seen in human arthritis, $CD4^+CD25^+$ Treg cells can be found in the synovial fluid, joints, and draining lymph nodes of arthritic mice (37-39). $CD4^+CD25^+$ T cells isolated from arthritic animals are capable of exerting suppressor function in *in vitro* assays (40,41), although in some situations they have been found to be less functional than their naive counterparts (42). $CD4^+CD25^+$ T cells from interferon-γ (IFN-γ) receptor knockout mice, which develop accelerated and more severe collagen-induced arthritis (43,44), exhibit less potent suppressive activity *in vitro*, and express lower levels of Foxp3 mRNA, akin to the effects of TNF-α on Treg cells seen in RA patients (42). Additional work suggested that in the absence of IFN-γ, other cytokines such as IL-17 are unchecked and contribute to exacerbated disease (45), suggesting that the altered and perhaps more severe inflammatory environment in the IFN-γ receptor knockout mice is affecting the phenotype and function of Treg cells.

Work examining the impact of $CD4^+CD25^+$ Treg cell deficiency on arthritis development, achieved by genetic means or antibody depletion, has provided evidence that Treg cells indeed modulate the autoimmune response in inflammatory arthritis. K/BxN mice, which develop spontaneous inflammatory arthritis initiated by a $CD4^+$ T-cell response to a GPI peptide, have been crossed to Foxp3-*scurfy* (33) mice to determine how a congenital absence of $CD4^+CD25^+Foxp3^+$ Treg cells affects disease development. K/BxN.Foxp3-*sf* mice were found to develop an accelerated and more severe disease than K/BxN mice containing $CD4^+CD25^+$ Treg cells, suggesting that while the Treg cells do not ultimately prevent arthritis, their activity is affecting disease pathogenesis and severity (39). These mice did not suffer from the multi-organ autoimmunity associated with Foxp3-*sf* mice, presumably because the TCR repertoire was restricted by expression of the transgenic TCR. In contrast to the effects of a genetic deficiency in $CD4^+CD25^+$ Treg cells, $CD4^+CD25^+$ T-cell depletion by antibody treatment did not appear to affect arthritis development in K/BxN mice, as neither disease onset nor severity were affected (40). However, $CD4^+CD25^+$ T-cell-depleted K/BxN mice exhibited more extensive lymphocyte infiltration into other organs and also an increase in serum anti-double stranded (ds) DNA antibody levels, indicating that the endogenous Treg cells are able to modulate other aspects of an autoimmune response even as arthritis develops (40). In the collagen-induced arthritis model, depletion of $CD4^+CD25^+$ T cells by antibody treatment did lead to increased disease severity (42,46). The effect on arthritis development could be reversed by transferring $CD4^+CD25^+$ T cells into Treg cell-depleted mice, suggesting that while the Treg cells are not able to prevent disease development, they are reducing severity (46).

Complementary studies involving the transfer of exogenous $CD4^+CD25^+$ T cells into pre-arthritic mice also suggest that Treg cells are capable of modifying arthritis development. Transferring pre-activated $CD4^+CD25^+$ T cells from healthy mice or Foxp3-transduced $CD4^+$ T cells ameliorated disease in the collagen-induced arthritis model (37,47,48). However, there appeared to be a limited time frame in which transferred Treg cells could modulate disease severity. $CD4^+Foxp3^+$ T cells were best at ameliorating collagen-induced arthritis when they were transferred prior to the primary immunization with collagen. At later time points, such as after a secondary immunization, a greater number of transferred Treg cells was required to achieve a comparable reduction in disease severity, and if transferred after the booster immunization, the Treg cells had no effect on arthritis development (48). It has been shown that as soon as one day post transfer, $CD4^+CD25^+$ T cells that have been injected systemically can be found in the draining lymph node, synovial fluid, and synovial tissue, indicating that the cells are trafficking to the disease site (37).

$CD4^+CD25^+$ T-cell transfers were more effective at modifying disease severity when the cells were injected systemically rather than directly into the joints, raising questions about how and where the Treg cells are acting to modulate disease (48). Together, the results of depletion and addition of Treg cells in multiple mouse models of inflammatory arthritis suggest that functional Treg cells are present and able to modulate disease severity and other aspects of the immune response (such as lymphocyte infiltration into different tissues and the generation of autoantibodies) but that their activity can fail to prevent the development of autoimmune arthritis.

There are conflicting data as to whether $CD4^+CD25^+$ Treg cells are modifying the immune response in an antigen-specific manner, and the exact mechanisms by which Treg cells affect arthritis development remain unclear. In K/BxN.Foxp3-*sf* mice, there is an accelerated accumulation of anti-GPI antibodies in the absence of $CD4^+CD25^+$ Treg cells (39). There is additional evidence for antigen-specific Treg cell-mediated changes in the collagen-induced arthritis model, with observations of increased collagen-specific T and B-cell responses after Treg cell depletion, as well as a reduction in collagen-specific antibodies after transfer of exogenous Treg cells (46,48). However, there are also data showing no alterations in the collagen-specific immune response upon manipulation of $CD4^+CD25^+$ Treg cells in collagen-induced arthritis (37,47). The same study did however find decreased amounts of TNF-α and IL-6 in the serum of treated mice, which are two cytokines that can contribute to arthritis development and have been shown to interfere with Treg cell function (16,18,30-32,49,50). Thus another potential mechanism by which $CD4^+CD25^+$ Treg cells may affect arthritis development is by altering the inflammatory environment. Indeed it appears that the balance of cytokines can play an important role in arthritis development and pathogenesis. Recently, IL-35 has been identified as an effector cytokine of $CD4^+CD25^+$ Treg cells, and separate work has shown that IL-35 treatment reduces disease severity in collagen-induced arthritis, possibly by reducing IL-17 levels (51,52). Thus, IL-35 production by Treg cells may be able to modulate arthritis development by altering the inflammatory environment. Other work also suggests that $CD4^+CD25^+$ Treg cells may induce changes in the cytokine environment that lead to inhibition of osteoclastogenesis. In an *in vitro* culture system, high levels of cytokines that can inhibit osteoclastogenesis were found and accompanied by reduced osteoclast formation when Treg cells were present (47). Given that endogenous Treg cells are present in the joints, and exogenously administered Treg cells can traffic to the disease site one to two days post transfer, it is possible then that Treg cells may reduce osteoclastogenesis in the joints and thereby modify disease severity.

The TS1×HACII model of inflammatory arthritis

Our laboratory has had a long-standing interest in determining how $CD4^+$ T-cell tolerance to self-peptides is established and has developed several lineages of transgenic mice that express the influenza virus PR8 hemagglutinin (HA) as a neo-self-antigen using a variety of promoters to drive HA expression (HA Tg mice). To increase the frequency of autoreactive $CD4^+$ T cells we have mated these mice with TS1 mice, which express a transgene-encoded TCR that recognizes the major I-E^d-restricted T-cell determinant from the PR8 HA (termed S1) and can be recognized with the anti-clonotypic monoclonal antibody 6.5 (53). Using this approach, we have found that HA-specific $CD4^+$ T cells can undergo deletion and/or become $CD4^+CD25^+Foxp3^+$ Treg cells to varying degrees when they encounter the self-S1 peptide and that these different outcomes are dictated by the amount and/or cell type in which the HA is expressed as a self-antigen (54,55). While pursuing these studies, we wanted to examine how $CD4^+$ T-cell tolerance may be established when the self-HA molecule is expressed at high levels by MHC class II^+ APCs, such as dendritic cells and B cells. Accordingly we generated HACII mice, which express a transgene encoding the PR8 HA under the control of a MHC class II I-Eα promoter to target expression to APCs (19,56).

Cell surface HA can be detected at low levels on $B220^+$ and $CD11c^+$ cells from HACII mice when analyzed directly *ex vivo* and at higher levels following *in vitro* activation (Fig. 1A). By contrast, HA cannot be detected on $B220^+$ or $CD11c^+$ cells either from BALB/c mice or from HA104 mice, which also express PR8 HA as a neo-self antigen but unlike HACII mice use an SV40 early region promoter/enhancer to drive expression of the neo-self PR8 HA molecule (Fig. 1A). To examine how these different modes of antigen expression influence the immunogenicity of the neo-self HA, carboxyfluoresein succinimidyl ester (CFSE)-labeled $CD4^+$ T cells from TS1 mice were analyzed for the extent of their division following transfer into HACII, HA104, or BALB/c mice (Fig. 1B). Substantially more division was induced by the self-S1 peptide in HACII than in HA104 mice, and notably, $B220^+$ splenocytes in HACII (but not HA104 or BALB/c) mice also became activated by the transferred TS1 lymph node cells, as evidenced by their elevated expression of MHC class II and CD86 (57) (Fig. 1B). Thus, in HACII mice, HA is synthesized by APCs themselves, and both potently stimulates HA-specific $CD4^+$ T cells and promotes $CD4^+$ T-cell-mediated activation of APCs.

To examine mechanisms by which the highly immunogenic self-HA molecule might induce $CD4^+$ T-cell tolerance, we mated HACII mice with TS1 mice (19). As we developed these matings, we unexpectedly found that although they appear otherwise healthy, most adult TS1×HACII mice exhibited swelling of their ankle and/or wrist joints that was almost always symmetrical and did not resolve with age (Fig. 2A,B). The swollen joints of 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. 2C). The majority of the cells (~98%) present in synovial exudates from arthritic TS1×HACII mice co-expressed CD11b and GR-1, and were mostly neutrophils (with some macrophages) based on morphology, with the remainder composed primarily of $CD4^+$ T cells, approximately 17% of which expressed the 6.5 TCR (data not shown). Lung tissue from arthritic TS1×HACII mice also contained an intense perivascular mononuclear cell infiltration that was usually accompanied by interstitial pneumonitis, and some arthritic TS1×HACII mice also displayed evidence of lymphocytic cardiac valvulitis and myocarditis (authors' unpublished data). However, there was no evidence of inflammatory pathology observed in kidney, liver, thyroid, salivary glands, pancreatic islets, or intestinal tissue from arthritic TS1×HACII mice (authors' unpublished data). Thus, the majority of TS1×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. Notably, this disease develops in mice housed in a specific pathogen-free facility, unlike the SKG model of spontaneous arthritis that depends on activation of the innate immune system by microbial antigens (15). Moreover, its development appears to be critically dependent on synthesis of the HA by APCs, because it does not develop in five other lineages of TS1xHA transgenic mice that we have developed using alternate promoters to drive HA expression (54,55,58) and has not been reported in additional lineages of TS1xHA transgenic mice generated by other groups that use other promoters to control HA expression (59-63).

HA-specific $CD4^+$ T cells drive arthritis development in TS1×HACII mice

Because 6.5^+CD4^+ T cells could induce B-cell activation in HACII mice (Fig. 1) and autoantibody production has been implicated in disease pathogenesis in several models of arthritis (11-13), we examined the phenotype of B cells in TS1×HACII mice (19). The spleens from arthritic 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. 3A,B). Although they were hypergammaglobulinemic, sera from

TS1×HACII mice did not exhibit elevated reactivity with IgG (rheumatoid factor), GPI, cyclic citrullinated peptides, or collagen II in enzyme-linked immunosorbent assays (authors' unpublished data). Thus, the presence of autoreactive 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.

To determine whether B cells and/or antibody production are required for the development of arthritis in TS1×HACII mice, we generated TS1×HACII.$JH^{-/-}$ mice [which lack mature circulating B cells (64)]. The ankles of adult TS1×HACII.$JH^{-/-}$ mice were significantly larger than those of TS1.$JH^{-/-}$ mice, and histologic examination of swollen joints revealed bone erosions, pannus formation, synovial hyperplasia, and cartilage loss (Fig. 3C). Thus, even though B cells are systemically activated in TS1×HACII mice, neither antibody production, cytokine production, nor antigen presentation by B cells is required for arthritis to develop.

We also generated TS1×HACII.$RAG^{-/-}$ mice, which likewise do not contain B cells and in which $CD4^+$ T cells can only express the 6.5 TCR. Arthritis also developed in TS1×HACII.$RAG^{-/-}$ mice (Fig. 4A,B), indicating that no other cells types that require RAG-mediated gene rearrangement (such as γδ and NK T cells) are required for arthritis development. Importantly, the development of arthritis in TS1×HACII.$RAG^{-/-}$ mice 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.

Autoimmune arthritis develops despite thymic deletion of autoreactive $CD4^+$ T cells in TS1×HACII mice

To understand how failures of tolerance induction can allow HA-specific $CD4^+$ T cells to induce the development of autoimmune inflammatory arthritis, we examined the frequency and phenotype of 6.5^+CD4^+ T cells in TS1×HACII mice at four and 12 weeks of age in comparison to TS1 mice and to TS1×HA104 mice (which co-express 6.5 and HA transgenes but do not develop arthritis) (19). The frequency and absolute number of 6.5^+CD4^+ single positive (CD4SP) thymocytes was substantially reduced in both four- and 12-week-old TS1×HACII mice and TS1×HA104 mice relative to TS1 mice, consistent with deletion of 6.5^+CD4SP thymocytes in response to the S1 self-peptide (Fig. 5A). Notably, however, the spleens of 12-week-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. 5B). We also examined $CD4^+$ T cells from TS1×HACII.$RAG^{-/-}$ mice and found that they expressed lower levels of the 6.5 TCR than were present on $CD4^+$ T cells from TS1.$RAG^{-/-}$ mice (Fig. 5C). Since these reduced levels of clonotype cannot be due to allelic inclusion of additional TCR Vα or Vβ chains, they suggest that TCR downmodulation may play a role in the ability of 6.5^+CD4^+ T cells to evade deletion by the S1 peptide in HACII mice. Together, these studies showed that HA-specific thymocytes are subjected to severe deletion in TS1×HACII mice but that despite thymocyte deletion, 6.5^+CD4^+ T cells accumulate in the periphery. Moreover, this process is enhanced by the synthesis of the HA by APCs, because spleens from TS1×HACII mice contained four times as many 6.5^+CD4^+ T cells as were present in TS1×HA104 mice (Fig. 5B).

Spontaneous cytokine production by autoreactive $CD4^+$ T cells in TS1×HACII mice

We also analyzed the $CD4^+$ T cells that accumulated in TS1×HACII mice for their functional capabilities (19). When stimulated via their TCR, the 6.5^+CD4^+ T cells from

TS1×HACII mice were hypoproliferative in response to S1 peptide *in vitro* relative to their counterparts from TS1 and TS1×HA104 mice, even when provided with exogenous IL-2 (Fig. 6A). To determine whether these hypoproliferative $CD4^+$ T cells are nevertheless able to elicit cytokine secretion, we assayed cultures of unfractionated lymph node cells for evidence of cytokine production (without addition of exogenous S1 peptide). Lymph node cells from autoimmune TS1×HACII mice spontaneously secreted substantially higher amounts of IFN-γ, IL-17, TNF-α, IL-6, and IL-10 than were secreted by lymph node cells from TS1 or TS1×HA104 mice; they also secreted higher levels of IL-4, although the absolute amount was modest (Fig. 6B). Intracellular cytokine staining of lymph node 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. 6C). Staining was observed with an anti-IL17A antibody but not with an anti-IL-17F antibody (data not shown). 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. 6C). In addition, whereas IL-6-secreting $CD4^+$ and 6.5^+CD4^+ T cells were detectable in lymph node cells from TS1×HACII mice, TNF-α-secreting $CD4^+$ cells were rare, suggesting that other cell types are the major contributors to TNF-α production in these lymph node cultures (Fig. 6C). Thus, the autoreactive 6.5^+CD4^+ T cells that evade thymic deletion and accumulate in the periphery of TS1×HACII mice also spontaneously differentiate and promote the production of pro-inflammatory cytokines, including IL-17.

Autoimmune arthritis develops in the presence of $CD4^+CD25^+$ Treg cells in TS1×HACII mice

Since the autoreactive $CD4^+$ T cells that evade deletion in TS1×HACII mice are promoting spontaneous cytokine production and arthritis development, it was evident that peripheral tolerance mechanisms must also be failing in these arthritic mice. To begin to examine how this could be occurring, we analyzed peripheral $CD4^+$ T cells from TS1×HACII mice for the presence of $CD4^+CD25^+Foxp3^+$ T cells. Arthritic TS1×HACII mice were found to contain $CD4^+CD25^+Foxp3^+$ T cells at frequencies that were equal to or greater than are found in non-arthritic TS1 mice (Fig. 7A). Using the clonotypic monoclonal antibody 6.5, we also examined whether the $CD4^+CD25^+Foxp3^+$ Treg cells that are present in TS1×HACII mice are specific for the target autoantigen that we have shown drives disease (since arthritis can develop in TS1×HACII.$Rag^{-/-}$ mice, which can only express the 6.5 TCR that confers specificity for the S1 self-peptide). TS1×HACII mice indeed contain HA-specific $6.5^+CD4^+CD25^+Foxp3^+$ T cells, as approximately 4% of total 6.5-specific $CD4^+$ T cells are also $Foxp3^+CD25^+$ (Fig. 7B). Even though we could identify $CD4^+CD25^+Foxp3^+$ T cells in adult arthritic mice, it seemed possible that the development of autoimmune disease could be at least partly a result of a paucity of these cells at earlier developmental stages. Accordingly, we have examined TS1×HACII mice of different ages and found that $CD4^+CD25^+Foxp3^+$ T cells are present at normal frequencies both prior to and at disease onset (Fig. 7C).

To further examine the phenotype of the Treg cells in TS1×HACII mice, we have examined the expression of GITR and CD103 on the $CD4^+CD25^+Foxp3^+$ T cells. GITR ligation has been reported to abrogate $CD4^+CD25^+$ T-cell suppressor function (35,36), while CD103 has been identified as a marker for antigen-experienced Treg cells as well as for a subset of more effective suppressors (65,66). Compared to those from BALB/c mice, the $CD4^+CD25^+Foxp3^+$ T cells from TS1×HACII mice possessed a greater frequency of cells co-expressing high levels of GITR and CD103 (Fig. 7D). Furthermore, within the $CD4^+CD25^+Foxp3^+$ T-cell pool from TS1×HACII mice, those that are HA-specific were more enriched for GITR and CD103 bright cells (Fig. 7D), suggesting that these Treg cells

may be experiencing stimulation by the target autoantigen (that is expressed by ubiquitously distributed APCs) in ways that are modifying their phenotypic characteristics relative to the other non-autoantigen-specific Treg cells.

We determined the functional capabilities of the $CD4^+CD25^+$ T cells in TS1×HACII mice by analyzing their ability to suppress $CD4^+$ T cell responses *in vitro*, using either anti-CD3 or S1-peptide to stimulate the Treg cells. This approach allowed us to assess the regulatory activity of both the total Treg cell pool and also to specifically examine the ability of Treg cells to become activated by the target autoantigen in TS1×HACII mice. We cultured CFSE-labeled $CD4^+$ T cells in the presence or absence of $CD4^+CD25^+$ T cells from TS1×HACII mice with soluble anti-CD3 and irradiated splenocytes and found that the Treg cells were able to limit the proliferation of the responder $CD4^+$ T cells (Fig. 8A). We also cultured varying numbers of purified $6.5^+CD4^+CD25^+$ T cells from TS1×HACII mice with responder $CD4^+$ T cells from TS1 mice and S1-peptide and assessed cell proliferation by thymidine incorporation. We found that the HA-specific Treg cells from TS1×HACII mice were able to suppress responder $CD4^+$ T-cell proliferation when stimulated with S1-peptide, as evidenced by reduced thymidine incorporation in the presence of the Treg cells (Fig. 8B). When cultured with anti-CD3, Treg cells from TS1×HACII mice were also able to suppress IFN-γ production by responder $CD4^+$ T cells as determined by intracellular cytokine staining (Fig. 8C). Thus, TS1×HACII mice develop arthritis despite the presence of a normal percentage of $CD4^+CD25^+Foxp3^+$ Treg cells, including a set of Treg cells that recognize the target autoantigen that drives disease. As seen in other mouse models of inflammatory arthritis, the Treg cells from TS1×HACII mice are functional *in vitro* and are able to suppress both $CD4^+$ T-cell proliferation and cytokine production. We have further shown that Treg cells from TS1×HACII mice are capable of suppressing *in vitro* responder $CD4^+$ T-cell responses when activated by the target autoantigen that is recognized by autoreactive $CD4^+$ T cells in arthritic mice.

Future directions

The findings outlined here from both human studies and mouse models seem to provide conflicting data regarding the role of $CD4^+CD25^+$ Treg cells in arthritis development. On the one hand, there is support for beneficial effects of Treg cells, as seen in JIA patients where the presence of $CD4^+CD25^+$ Treg cells inversely correlated with disease severity and from data in mouse models where depletion of Treg cells could lead to accelerated arthritis development and increased disease severity. Likewise, the transfer of exogenous $CD4^+CD25^+$ Treg cells has been shown to reduce disease severity in the collagen-induced arthritis model. However, there is also evidence that Treg cells are dysfunctional in arthritis and unable to suppress disease. $CD4^+CD25^+$ Treg cells isolated from RA patients after infliximab treatment showed improved regulatory activity, and culturing Treg cells in the presence of a variety of inflammatory cytokines has been shown to negatively affect their suppressor function. Much remains then to be uncovered regarding the role of Treg cells in arthritis development and the processes by which they are or are not able to modify disease.

The unique features of TS1×HACII mice as a model of spontaneous autoimmune inflammatory arthritis may allow us to more thoroughly understand how $CD4^+CD25^+$ Treg cell formation and activity can contribute to the development of inflammatory arthritis. One can envision a number of steps in the disease process where the impact of both endogenous and exogenous Treg cells can be examined in this model (Fig. 9). In step one, how the presentation of the S1 self-peptide is shaping Treg cell formation among HA-specific $CD4^+$ thymocytes is unclear. Even though thymocytes expressing the 6.5 TCR are subjected to severe deletion in response to HA expression by APCs, this process is incomplete, and 6.5^+CD4^+ T cells (including $6.5^+CD4^+Foxp3^+$ T cells) are present in the periphery of

TS1×HACII mice. In this context, how the highly immunogenic S1 peptide leads to HA-specific Treg cell formation remains unclear. Since TCR levels are lower on $CD4^+$ T cells in TS1×HACII.$RAG^{-/-}$ than in TS1.$RAG^{-/-}$ mice, TCR downmodulation may play a role in disease development by facilitating escape from central tolerance and may also contribute to Treg cell formation. It is also possible that because of the severe deletion of 6.5^+CD4^+ thymocytes, the $6.5^+CD4^+Foxp3^+$ T cells that are present in TS1×HACII mice develop primarily by extrathymic rather than thymic processes. Unraveling how Treg cell formation occurs in TS1×HACII mice may therefore help elucidate the extent to which MHC susceptibility alleles could affect the development of autoimmune arthritis through their effects on Treg cell formation.

In step two, the lymphopenia that develops secondary to the severe deletion of HA-specific thymocytes in TS1×HACII mice may play a role in promoting disease development, since lymphopenia-induced homeostatic proliferation has been shown to contribute to the development of autoimmune disease in other systems (67,68). In this regard it is noteworthy that the ability of Treg cells to modify autoimmune disease activity has been demonstrated most convincingly in conditions of lymphopenia, and whether disease development is a reflection of a failure of the $6.5^+CD4^+Foxp3^+$ Treg cells in TS1×HACII mice to regulate this lymphopenic expansion is unclear. Conversely, whether disease development might be abrogated by inhibiting lymphopenia-induced proliferation via administration of exogenous Treg cells remains to be determined.

Step three denotes the interaction of HA-specific $CD4^+$ T cells with APCs in which HA expression can be induced by activating signals (Fig. 1), which may promote the reciprocal activation of autoreactive $CD4^+$ T cells and APCs. Expression of the self-HA molecule by APCs appears to be key to the disease process, since none of the other lineages of TS1×HA Tg mice we have generated develop arthritis even though some (e.g. TS1×HA104 mice) mediate efficient thymic deletion of 6.5^+CD4SP thymocytes. We are currently examining whether particular APC subsets (e.g. macrophages, dendritic cells) promote these reciprocal interactions, and whether Treg cells can inhibit these activities. Along similar lines, step four represents the systemic activation of B cells in TS1×HACII mice. Although B cells and antibody production are not necessary for arthritis development in this model, they may contribute to disease severity, and autoreactive B-cell activation certainly contributes to disease development in other models. It remains unclear why the $6.5^+CD4^+Foxp3^+$ T cells that are present in TS1×HACII mice fail to prevent this B-cell activation, particularly since HA-specific Treg cells were able to suppress $CD4^+$ T cell-mediated activation of anergic anti-dsDNA B cells in a murine model of systemic lupus erythematosus (69).

Step five represents the differentiation of HA-specific $CD4^+$ T cells and secretion of pro-inflammatory cytokines in TS1×HACII mice. Although a hallmark of $CD4^+CD25^+Foxp3^+$ Treg cells is their ability to suppress both proliferation and cytokine production by $CD4^+$ T cells *in vitro*, their ability to protect against autoimmune disease through direct suppression of effector $CD4^+$ T-cell activity *in vivo* is less well understood. In this respect, TS1×HACII mice provide an opportunity to examine this possibility because the disease is clearly driven by $CD4^+$ T cells. Moreover, recent work in the Treg cell field has demonstrated the plasticity of Treg cell populations, as $CD4^+CD25^+$ Treg cells have been shown to adopt characteristics reflecting their surroundings and even develop functions traditionally attributed to effector $CD4^+$ T cells (70-72). We are currently examining the possibility that the $6.5^+CD4^+Foxp3^+$ T cells that are present in TS1×HACII mice either have or are capable of developing effector activities that make them active participants in the disease process. Finally, step six represents the development of a regional autoimmune response in lymph nodes draining the joints that accompanies arthritis. Notably, however, the target peptide recognized by autoreactive HA-specific $CD4^+$ T cells in TS1×HACII mice is synthesized by

systemically distributed APCs; yet, a regional immune response develops that includes enhanced activation of APCs in the popliteal lymph nodes (which drain the joints) (19). One possibility is that the environment of the inflamed joint (which may include high local levels of inflammatory cytokines and/or enhanced antigen presentation) promotes disease by causing the regional Treg cells to lose function, as has been suggested in human studies, and can be explored in more detail in this model system. This scheme of arthritis development in TS1×HACII mice will allow us to analyze the effects of $CD4^+CD25^+$ Treg cell function at specific steps involved in the initiating and effector stages of disease. Furthermore, this model will provide an opportunity to study the ontogeny of the autoantigen-specific Treg cells and to examine how their development may contribute to disease progression.

A final aspect of this system that is worth noting is that we will examine not only the role of $CD4^+CD25^+$ Treg cells in general but also address how the antigen specificity of Treg cells affects their phenotype and function in an autoimmune disease setting. As outlined above, TS1×HACII mice do contain HA-specific Treg cells, but despite their ability to suppress HA-specific $CD4^+$ T cells *in vitro*, these HA-specific Treg cells are clearly unable to suppress the *in vivo* HA-specific immune response of conventional 6.5^+CD4^+ T cells that is a necessary disease trigger in TS1×HACII mice. An interesting possibility is that the HA-specific Treg cells in TS1×HACII mice are undergoing phenotypic and functional changes in response to the disease setting that are affecting their ability to modulate disease. We have already found that the HA-specific $CD4^+CD25^+Foxp3^+$ T cells present in TS1×HACII mice are more enriched for GITR and CD103 bright cells than the non-HA-specific Treg cells, indicating that there is some disparity between the endogenous antigen-specific and non-specific Treg cells, and in future work it will be of interest to determine whether antigen-specific Treg cells are required for disease prevention or alternatively might be adapting to the arthritic environment and perhaps acquiring functions similar to those that conventional $CD4^+$ T cells exhibit during disease. There is much current interest in understanding how Treg cells modulate immune responses in general and autoimmune diseases such as RA in particular; yet, how the specificity of these cells for self-peptides dictates their development and activity is barely understood and is likely a prerequisite for understanding how their activity can impact human diseases. We expect that ongoing studies in TS1×HACII mice and in other model systems will allow us to better understand these questions and provide insights that may enhance the ability of Treg cells to be exploited either diagnostically or therapeutically.

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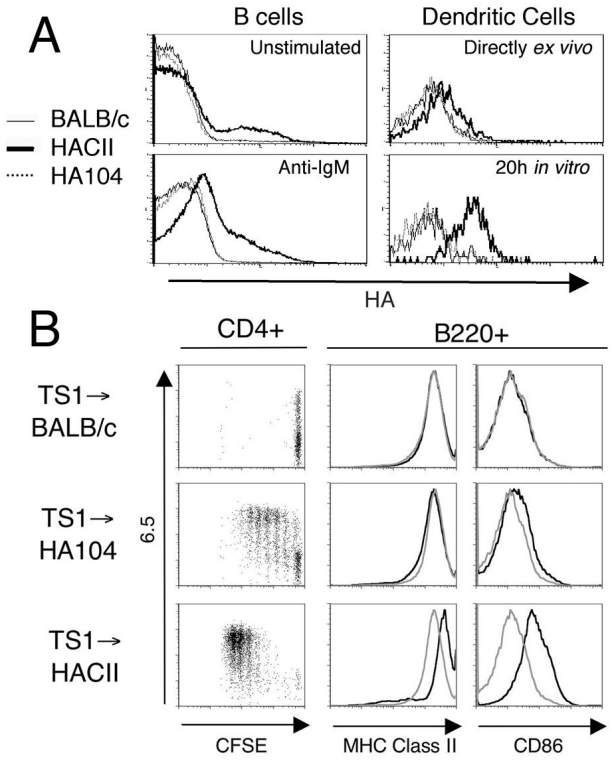

Fig. 1. HA is synthesized and presented by APCs in TS1×HACII mice

(A) Cell-surface expression of PR8 HA by $B220^+$ and $CD11c^+$ cells from BALB/c, HA104, and HACII mice analyzed directly *ex vivo* or following *in vitro* activation (anti-IgM for B cells, 20 h on plastic for DCs). (B) CFSE-labeled $CD4^+$ T cells from TS1 mice were transferred into BALB/c, HA104, or HACII mice, and after 48 h, LN cells were isolated and analyzed by flow cytometry. Left panels show staining with 6.5 versus CFSE on $CD4^+$ T cells, right panels show expression of MHC class II or CD86 on $B220^+$ cells.

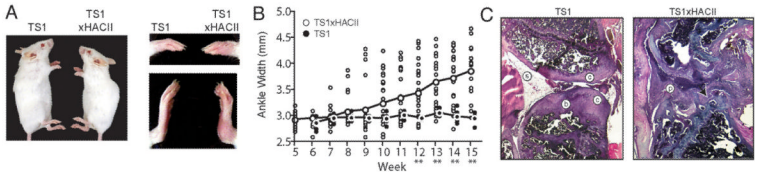

Fig. 2. TS1×HACII mice develop spontaneous autoimmune inflammatory arthritis

(A) Pictures show 20-week-old TS1 and TS1×HACII littermates, with panels showing front and rear paws. (B) Graph shows ankle widths of TS1 and TS1×HACII mice at different ages, in weeks. **P≤0.01. (C) Hematoxylin and eosin (H&E) stained knee sections from 23-week-old TS1 and TS1×HACII littermates; b, bone; c, cartilage; s, synovium; p, pannus; arrowhead, bone erosion.

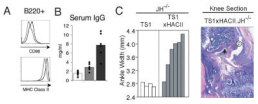

Fig. 3. B cells are not required for arthritis development in TS1×HACII mice

(A) Histograms show CD86 and MHC class II expression on $B220^+$ splenocytes from TS1 (grey lines), TS1×HA104 (dashed lines), and TS1×HACII (black lines) mice. (B) Bar graphs show the numbers of $B220^+$ splenocytes and serum IgG with circles representing individual mice. Bars indicate the averages for TS1 (white bars), TS1×HA104 (light grey bars), and TS1xHACII (dark grey bars) mice. (C) Graph indicates the largest ankle widths from individual $TS1.JH^{-/-}$ (open bars) and $TS1{\times}HACII.JH^{-/-}$ (filled bars) mice. The mean ankle width of $TS1{\times}HACII.JH^{-/-}$ mice is significantly greater than that of $TS1.JH^{-/-}$ mice ($P<0.01$, Student's t-test). Mice used in this analysis ranged in ages from 6 to 23 weeks. Picture shows H&E stained knee section from an arthritic $TS1{\times}HACII.JH^{-/-}$ mouse. p, pannus; arrowhead, bone erosion.

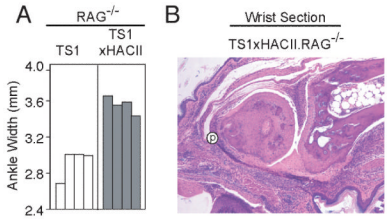

Fig. 4. Recognition of HA by 6.5^{+}CD4^{+} T cells drives arthritis in TS1×HACII mice

(A) Graph indicates the largest ankle widths from individual 9 to 13 week-old TS1.RAG$^{-/-}$ (open bars) and TS1×HACII.RAG$^{-/-}$ (filled bars) mice. The mean ankle width of TS1×HACII.RAG$^{-/-}$ mice is significantly greater than that of TS1.RAG$^{-/-}$ mice ($P<0.01$). (B) Picture shows H&E stained wrist section from an arthritic TS1xHACII.RAG$^{-/-}$ mouse. p, pannus.

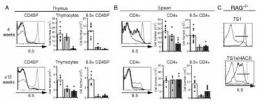

Fig. 5. Development of $6.5^{+}CD4^{+}$ T cells in TS1×HACII mice

(A) 6.5 expression and frequencies of thymocytes and 6.5^{+}CD4SP thymocytes from 4 and >12 wk-old TS1 (light lines, open bars), TS1×HA104 (dashed lines, grey bars) and TS1×HACII (dark lines, closed bars) mice. (B) 6.5 expression and frequencies of splenocytes and $6.5^{+}CD4^{+}$ splenocytes from 4 and >12 wk-old TS1 mice. TS1 (light lines, open bars), TS1×HA104 (dashed lines, grey bars), and TS1×HACII (dark lines, closed bars). (C) Histograms show 6.5 expression on $CD4^{+}$ splenocytes from TS1 (grey line) and TS1.$RAG^{-/-}$ (black line) mice (upper panel), and from TS1×HACII (grey line), TS1×HACII.$RAG^{-/-}$ (black line), and TS1.$RAG^{-/-}$ (dashed line) mice (lower panel).

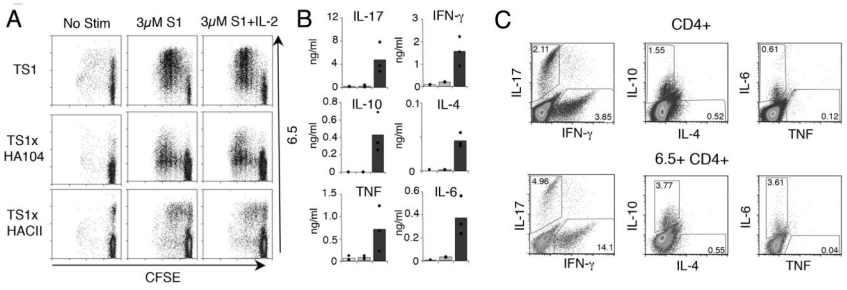

Fig. 6. Autoreactive CD4$^+$ T cells in TS1×HACII mice are activated and produce cytokines

(A) Expression of 6.5 versus CFSE on purified CD4$^+$ T cells from TS1, TS1×HA104, and TS1×HACII mice following 72 h of culture with or without S1 peptide and/or IL-2. (B) Amounts of indicated cytokines in culture supernatants obtained following incubation of unfractionated LN cells from individual TS1 (open bars), TS1×HA104 (grey bars), and TS1×HACII (filled bars) mice for 3 days in the absence of exogenous peptide or cytokines. Bars indicate means. (C) Dot plots show intracellular cytokine staining of CD4$^+$ (upper panels) and 6.5$^+$CD4$^+$ (lower panels) lymph node 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 (for IL-6 and TNF-α). Percentages of cells in indicated gates are shown.

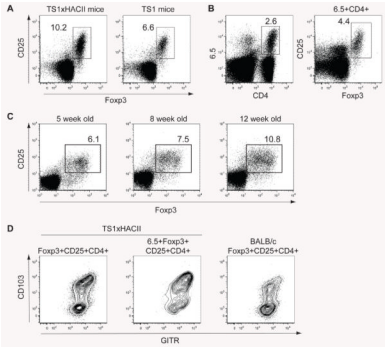

Fig. 7. TS1×HACII mice contain CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, including a population that recognizes the target autoantigen

(A) Lymph node cells from TS1×HACII and TS1 mice were stained with CD4, CD25, and Foxp3. (B) Lymph node cells from a TS1×HACII mouse were stained with 6.5 to identify HA-specific cells, CD4, CD25, and Foxp3. (C) Lymph node cells from five, eight, and 12-week-old TS1×HACII mice were stained with CD4, CD25, and Foxp3. (D) Lymph node cells from TS1×HACII and BALB/c mice were stained with CD4, CD25, Foxp3, GITR, and CD103. A 6.5 stain was also included for TS1×HACII mice.

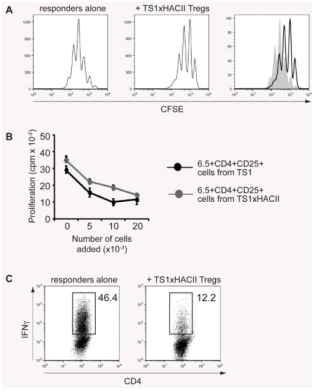

Fig. 8. $CD4^+CD25^+$ Treg cells from TS1×HACII mice suppress responder $CD4^+$ T cells *in vitro*

(A) CFSE-labeled MACS-purified $CD4^+$ T cells from TS1 mice were cultured with 0.1 μg/ml anti-CD3 and irradiated BALB/c splenocytes in the presence or absence of FACS-purified $CD4^+CD25^+$ cells from TS1×HACII mice. Cells were cultured at a Treg:responder cell ratio of 1:2. Responder $CD4^+$ T-cell proliferation was analyzed via flow cytometry at day three of culture. (B) $6.5^+CD4^+CD25^+$ T cells were FACS-purified from TS1 (black circles) or TS1×HACII (gray circles) mice. Graded numbers of Treg cells were cultured with lymph nodes cells from TS1 mice and S1 peptide. Proliferation was assessed at day three of culture by [^{3}H]-thymidine incorporation. (C) MACS-purified $CD4^+$ T cells from TS1 mice were cultured with 0.1 μg/ml anti-CD3 and irradiated BALB/c splenocytes in the presence or absence of FACS-purified $CD4^+CD25^+$ cells from TS1×HACII mice. At day four of culture, IFN-γ production by the responder $CD4^+$ T cells was determined by intracellular cytokine staining.

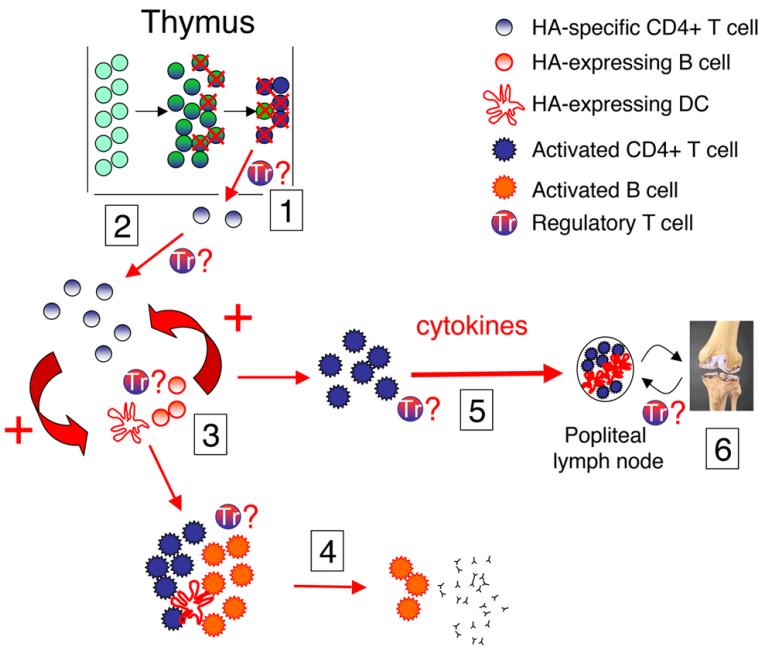

Fig. 9. Steps where regulatory T-cell activity may shape arthritis development in TS1×HACII mice

Step 1, Deletion/Treg cell formation among autoreactive $6.5^{+}CD4^{+}$ thymocytes. Step 2, Lymphopenia-induced proliferation of $6.5^{+}CD4^{+}$ T cells secondary to severe thymocyte deletion. Step 3, reciprocal activation of HA-specific $CD4^{+}$ T cells by HA-expressing antigen presenting cells. Step 4, activation of B cells by HA-specific $CD4^{+}$ T cells. Step 5, spontaneous differentiation and cytokine production by autoreactive HA-specific $CD4^{+}$ T cells. Step 6, development of a regional immune response in the lymph nodes that drain the major joints.