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Identifying the Cells Breaching Self-Tolerance in Autoimmunity

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Activation of auto-reactive T cells by activated dendritic cells (DCs) presenting self-Ag is widely assumed to be the precipitating event in the development of autoimmune disease. However, despite such widely held preconceptions, supporting data are scarce and subjective, particularly in experimental arthropathy. We have adapted a novel murine model of breach of self-tolerance allowing evaluation of the contribution of endogenous DCs to the development of autoimmune responses and disease. For the first time, we reveal the critical role played by conventional DCs, and the timing and location of this process. We further demonstrate the importance of this finding by clinically relevant, therapeutic manipulation of conventional DC function, resulting in decreased autoimmune phenotype and disease severity. *The Journal of Immunology*, 2010, 184: 6378–6385.

Rheumatoid arthritis (RA) is an autoimmune mediated disorder characterized by painful articular inflammation and erosion. The systemic nature of this aberrant immune response is highlighted by additional involvement of skin, lungs and vasculature manifesting as rheumatoid nodules, diffuse inflammation/fibrosis, and increased risk of atherosclerosis (1–5). Despite successful identification of biological targets and their therapeutic translation (anti-TNF, anti-CD20, CTLA-4-Ig, anti-IL-1, anti-IL-6) many patients remain refractory to intervention (6). As with all autoimmune disorders, the ultimate goal should be re-establishment of self-tolerance. However, patient studies have been unable to dissect the critical events mediating the induction of self-reactivity as this event likely occurs many years prior to RA diagnosis. Conversely, most animal models of RA rely on aggressive and artificial self-Ag immunization protocols (7), which do not permit analysis of the very early breach of self-tolerance and prearticular stages of disease. Understanding these early events will be critical if the immune system is to be re-educated and self-tolerance reinstated, rather than simply treating symptoms. Although approaches, such as the case control consortium, have established a number of predictive genetic variations associated with the development of RA (8), they do not

inform when to intervene for a given pathway. Understanding the events surrounding the breach of self-tolerance associated with RA will therefore reveal markers associated with the onset of preclinical disease and signal a window of early intervention.

Dendritic cells (DCs) are considered the main initiators of naive T cell responses (9), but their contribution to RA remains unclear. Phenotypic studies have identified the subsets and activation states of DCs in RA (10, 11), and current therapeutic regimes can alter their maturation/activation states (12), yet direct evidence implicating a particular DC subset in breach of self-tolerance leading to RA pathogenesis is lacking. Despite an incomplete understanding of how DCs are involved in RA pathology, tolerogenic DC populations are being developed as potential therapeutic tools, with some success being demonstrated in murine models (13–15). Indeed, clinical trials have been initiated in the United Kingdom (<http://news.bbc.co.uk/1/hi/health/7560535.stm>) and Australia (www.uq.edu.au/news/?article=13128). Although DC therapy appears an attractive proposition, care must be taken as the pivotal role of these cells in directing adaptive immune responses also endows them with the potential to initiate or exacerbate autoimmunity. For example, transfer of exogenous type II collagen (CII) pulsed, myeloid DCs can incite joint pathology in disease susceptible mice (16). As such, it will be critical to define the APC populations that regulate the breach of self-tolerance underlying the induction of RA. Our laboratory has developed a novel murine model of preclinical experimental arthritis allowing delineation of events surrounding loss of self-tolerance (17–19). Transfer of Th1 polarized OVA-specific-TCR transgenic T cells induces synovial hyperplasia and cartilage erosion proximal to joints challenged with heat aggregated OVA (HAO), thus circumventing issues associated with immunization with self-Ags in powerful, non-physiological adjuvant (20, 21). These histological pathologies are also manifest clinically (16–18). These are mild and localized to the affected joint, unlike the aggressive polyarthritis seen in collagen-induced arthritis being more akin to advanced human disease. Joint inflammation in this system is associated with elevated titers of autoantibodies, including anti-CII, IgG2a-rheumatoid factor and anticyclic citrullinated peptides (17–19). Further dysregulation of self-tolerance is evidenced by CII specific T cell proliferative responses on in vitro restimulation. Nonspecific inflammation can recapitulate many of the histological signs of pathology in this model, however, development of autoreactivity is

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Abbreviations used in this paper: cDC, conventional DC; CII, type II collagen; DC, dendritic cell; DTX, diphtheria toxin; Etan, Etanercept; HAO, heat aggregated OVA; hIgG, human IgG; iso, isotype; LN, lymph node; pDC, plasmacytoid DC; pLN, popliteal LN; RA, rheumatoid arthritis.

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exquisitely dependent on elicitation of an irrelevant Ag-specific T cell response localized in the joint (18). As such the model represents a model of early stage arthritis, reflecting patients with mild clinical presentation but strong evidence of autoreactivity, such as rheumatoid factor. Using this model, we have recently demonstrated that plasmacytoid DCs (pDCs) can function to limit self-reactivity and consequent pathology (19). Crucially, the cell population responsible for inciting autoreactive T cell responses remains to be identified.

In this study, we demonstrate conventional DC (cDC) maturation and Ag presentation immediately prior to breach of self-tolerance, the ability of these cells to transfer autoreactivity, a potential action of existing biological therapeutics on them and an essential requirement for cDCs in the development of self-reactive T and B cell responses and RA pathogenesis.

Materials and Methods

Animals

DO11.10 BALB/c TCR, OT-II C57BL/6 TCR, and CD11cDTR transgenic mice (22), 6- to 8-wk-old female BALB/c, C57BL/6 (Harlan, Bicester, U.K.) were housed in the University of Strathclyde and procedures were performed according to the United Kingdom Home Office regulations.

Induction of arthritis

Recipient mice received 2×10^6 *in vitro* Th1-polarized DO11.10 TCR transgenic CD4⁺ T cells *i.v.* (17). Transferred cells were *in vivo* expanded by *s.c.* immunization with OVA/CFA the following day. Ten days later, mice were footpad challenged with HAO, as described by Maffia *et al.* (17). Th1 V α 2⁺V β 5⁺ OT-II T cells were used for C57BL/6 recipients in E α GFP and CD11cDTR experiments. E α GFP (100 μ g) was administered with or without HAO (23) for APC identification experiments. Development of arthritis was followed by paw thickness, using a dial caliper (Kroepelin, Munich, Germany), and by histological assessment at day 7 after HAO challenge (17). Disease scoring, based on cell infiltration (0, no cells; 1, <50 cells; 2, 50–200 cells; and 3, >200 cells), synovial hyperplasia (0, no proliferation; 2, >3 layers; and 3, severe), and joint erosion (0, no erosion; 1, very mild; 2, marginal; and 3, complete) was performed on three joints for each of five mice per group. Average paw scores for each parameter were calculated across all three joints, and a total score for each paw calculated by adding individual parameter scores.

Depletion of cDCs *in vivo*

cDCs were depleted in CD11cDTR mice (22) *in vivo* by *s.c.* footpad injection of 20 ng diphtheria toxin (DTx) (Sigma-Aldrich, St. Louis, MO) 1 d before *s.c.* injection of HAO. Control CD11cDTR transgenic and C57BL/6 mice received matched injection of 50 μ l PBS or DTx, respectively.

Isolation and culture of bone marrow DCs

DCs were generated from the bone marrow of C57BL/6 mice by culture in GM-CSF conditioned medium as previously described (16). Routine flow cytometric analysis of DC cultures revealed yields of >80% for cells staining both CD11c and MHC class II positive. DCs were cultured for 6 h with OVA (100 μ g/ml), bovine CII (50 μ g/ml, Sigma-Aldrich), or both, before addition of LPS (1 μ g/ml, *Escherichia coli* 055:B5 (Sigma-Aldrich) and maturation overnight. Footpad challenge consisted of 2×10^5 DCs *s.c.* (a total of 4×10^5 DCs were transferred in the mixed DC group, 2×10^5 OVA and 2×10^5 CII-pulsed DCs).

Flow cytometry

Single-cell suspensions were prepared from axillary, inguinal, cervical, and mesenteric lymph nodes (LNs) from TCR transgenic mice. Popliteal LN (pLN) single-cell suspensions were stained with anti-CD11c, anti-PDCA1, anti-B220, anti-I-A/E (all BD Biosciences, Oxford, U.K.), and YAE (specific for I-E α 52-68 of I-E^d presented on I-A^b) (eBioscience, San Diego, CA) for APC identification. cDCs and pDCs were defined as CD11c⁺PDCA1⁻B220⁻ and CD11c⁺PDCA1⁺B220⁻, respectively.

In vitro restimulation assays

pLN cells were cultured with either medium, 1 mg/ml OVA, or 50 μ g/ml CII. Proliferation was analyzed at 96 h by flow cytometric staining for EdU incorporation (Invitrogen, Molecular Probes, U.K.).

ELISA

Anti-OVA and anti-collagen Abs were detected by ELISA as previously described (17). An adapted IFN- γ ELISA protocol (16, 17) was used for detection of IL-17 using anti-IL-17 clones TC11-18H10 and biotinylated TC11-8H4 (BD Biosciences). Standard curves for IL-17 ELISA were generated using recombinant mouse IL-17A (eBioscience).

Anti-TNF- α treatment

sTNFR-Fc (0.5 mg/kg) (24) (Etanercept, Wyeth Pharmaceuticals, Taplow, U.K.) was given *s.c.* 1 d prior to, and 1, 3, and 5 d after footpad challenge. Control mice received 0.5 mg/kg hIgG.

Statistical analysis

Results are expressed as mean \pm SD, $n = 5$. Significance was determined by Student *t* test, values of $p \leq 0.05$ were considered significant.

Results

Increased numbers of MHC class II high cDCs are observed in draining LNs of arthritic mice and are associated with anti-CII responses

Using a recently developed model of experimental arthritis (17), we sought to delineate the critical events surrounding the development of autoreactive T and B cell responses and ensuing joint pathology, identifying contributing APCs. Detailed kinetic analysis of DC markers and numbers in the draining pLN after HAO challenge revealed that although the proportion of cDCs or pDCs was not significantly altered in HAO versus PBS challenged mice (Fig. 1A, 1B, Supplemental Fig. 1), the total numbers of both DC populations increased after HAO challenge (Fig. 1C, 1D). cDC expression of MHC class II expression was significantly higher in arthritic mice than control animals by day 2 after HAO challenge (Fig. 1E) and was subsequently maintained (Fig. 1E). pDCs expressed lower levels of MHC class II than cDCs, particularly on days 2 and 3 after HAO challenge (Fig. 1F), returning to levels comparable to PBS challenge by day 4. There were no significant differences in CD80, CD86, or CD40 expression in either group (data not shown).

Elevated cDC number and MHC class II expression preceded detectable changes in the articular environment, with the first signs of synovial thickening and tissue infiltrate evident 4–5 d after HAO challenge (Fig. 1G–N, Supplemental Fig. 1), increasing along with signs of cartilage erosion, and peaking by day 7. This led us to hypothesize that breach of T cell self-tolerance may occur at these early time points after stimulation of the OVA response. Increased MHC class II expression by cDCs indicated maturation of these cells and suggested they could be the main cells responsible for Ag presentation associated with footpad challenge.

Increased numbers of Ag-presenting cDCs are observed in the pLN prior to spontaneously arising autoreactive T cell and B cell responses

To address the role of cDCs in Ag presentation directly, we used the model Ag E α GFP and the mAb YAE (23) to identify *in vivo* the major APC populations associated with the development of autoimmunity and pathology in RA. We focused on day 2 postchallenge as the earliest time point at which alterations in APC populations were detected (Fig. 1). Coadministration of E α GFP with PBS or HAO revealed significantly more cDCs staining positively with the peptide E α /MHC class II-specific mAb, YAE, in HAO-challenged mice versus PBS challenged mice (Fig. 2A, Supplemental Fig. 2). The amount of Ag presented by individual cDCs was not significantly altered in arthritic mice, with no change in YAE mean fluorescent intensity

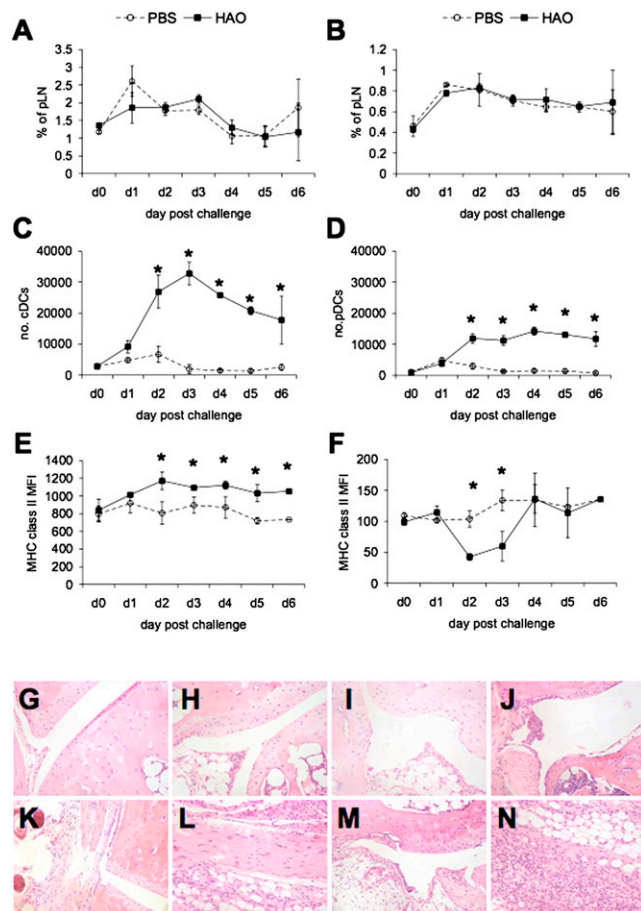


FIGURE 1. Joint inflammation induced by activated T cells of an irrelevant specificity is associated with increased numbers of cDCs and their expression of MHC class II. Draining pLNs were removed at the indicated days postchallenge (1–6 d) and FACs analysis performed to identify cDCs ($CD11c^+B220^-PDCA1^-$) and pDCs ($CD11c^+B220^+PDCA1^+$) (Supplemental Fig. 1). Percentages of cDCs and pDCs (A and B, respectively) in pLN in PBS versus HAO challenged mice were derived as a function of total LN cellularity. Absolute numbers of cDCs and pDCs were then calculated (C and D, respectively), and MHC class II expression by both populations (E, F). Ankle joints from HAO challenged mice were collected on days 1, 2, 3, 4, 5, and 6 (G–I). Some synovial thickening could be observed on day 4 with increasing s.c. infiltrate (J, K). Tissue sections from day 5 (L, M) to 6 (N) revealed increased infiltrate through joint proximal tissue and beginnings of synovial infiltrate and hyperplasia. Original magnification $\times 10$. The mean of $n = 5$ is shown \pm SD in the mean. $**p < 0.01$.

in the HAO/E α GFP versus the PBS/E α GFP nonarthritic group (Fig. 2B). The small shifts in YAc staining after in vivo E α GFP administration likely reflect the low frequency at which a single peptide/MHC class II complex is presented on the APC surface. No significant presentation of peptide E α /MHC class II was observed on pDCs (Fig. 2A), YAc staining levels were comparable between groups with or without E α GFP, regardless of inflammatory status (Fig. 2B). Thus, the profile of cells presenting the model Ag did not differ between nonarthritic and arthritic animals, being confined to the cDC subset 2 d after challenge. Despite this, the number of cDCs presenting E α peptide was elevated after HAO challenge. No detectable anti-CII–serum Ab was detected at this time (Fig. 2C), but breach of self-tolerance was apparent by day 7, with HAO-challenged mice subsequently developing autoreactive B and T cell responses (Fig. 2C, 2D).

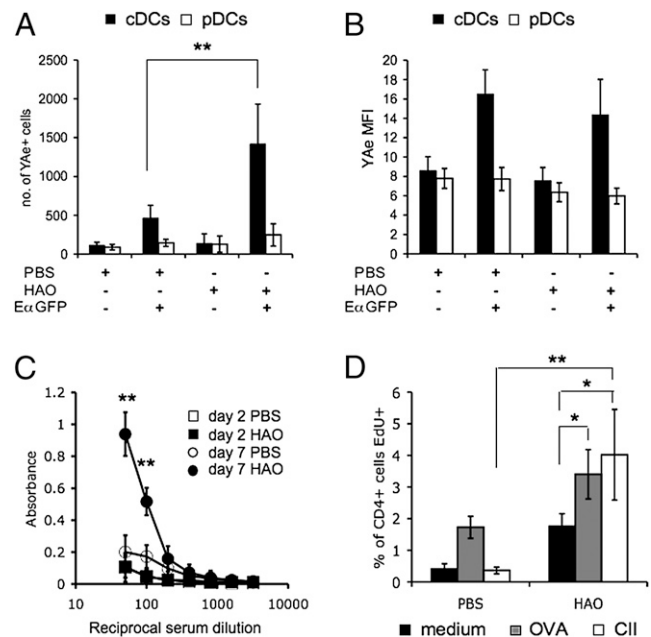


FIGURE 2. Elevated numbers of cDCs present model Ag in RA mice. C57BL/6 mice received Th1 cells and were immunized with OVA/CFA as before. Mice were footpad challenged with PBS/E α GFP or HAO/E α GFP. Control groups without E α GFP were also included. YAc staining was then used to assess presentation of E α peptide/I-A b in pLN 2 d after challenge. Absolute numbers of YAc positive cDCs and pDCs (A) were determined in addition to intensity of staining (B). Anticollagen responses were determined by ELISA on day 2 and 7 serum samples (C) after PBS or HAO footpad challenge. In vitro restimulation of day 7 HAO challenged mice was used to demonstrate successful induction of CII-specific T cells (D). The mean of $n = 5$ is shown \pm SD in the mean. $*p < 0.05$; $**p < 0.01$.

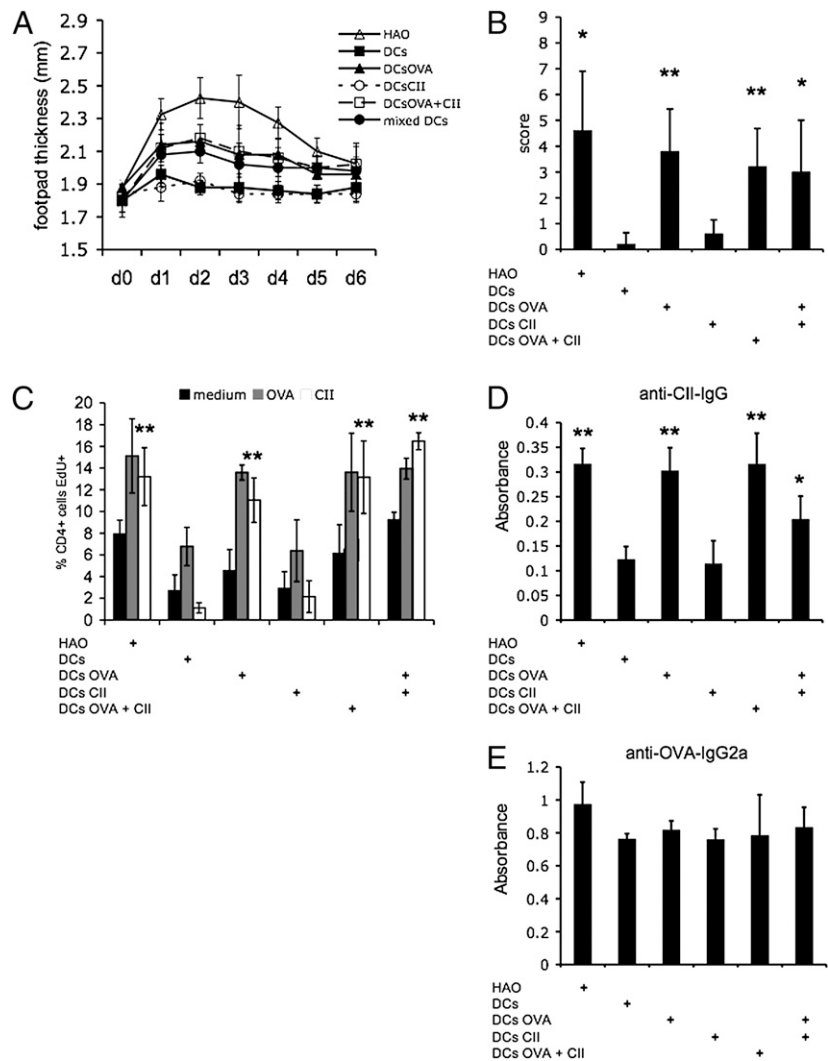
OVA-pulsed DCs induce histological signs of pathology and are sufficient to induce anti-CII T and B cell responses in recipient mice

Having identified cDCs as a major presenter of model Ag prior to development of autoreactive arthritis, we hypothesized that they were the most likely candidates to be presenting tissue-derived Ag in our model and consequently the APC population driving breach of self-tolerance. Indeed, a model of RA in which self-Ag presented by activated DCs stimulates autoreactive T cells that have escaped thymic selection has been proposed (25, 26). In support of this hypothesis, adoptive transfer of self-Ag-bearing DCs can incite autoimmunity underlying diabetes, experimental autoimmune encephalitis, and erosive arthritis (16, 27, 28).

To determine whether DCs were sufficient to drive this breach of self-tolerance and drive autoreactive arthritis, Ag-pulsed bone marrow-derived DCs were used in substitute of HAO. DCs alone, pulsed with one (OVA or CII), or both Ags (OVA and CII) were tested for their ability to induce footpad inflammation, joint pathology, and anti-CII-specific immune responses. We also included a group in which DCs were pulsed separately with OVA and CII, then mixed prior to challenge, allowing us to address the requirement for presentation of Ags by a single DC. One hypothesis for the breach of self-tolerance in our model was that the “conditioning” of DCs bearing self-peptide/MHC class II complexes by activated OVA-specific T cells could facilitate stimulation of autoreactive T cells, occurring via direct interaction if the self-Ag-bearing DCs were also presenting OVA peptides.

DCs pulsed with OVA, OVA and CII, and mixed DCs induced comparable levels of footpad swelling that was significantly greater than that observed with unpulsed or CII-pulsed DCs (Fig. 3A).

FIGURE 3. Challenge with Ag-pulsed DCs induces articular inflammation, pathology and autoreactivity. After adoptive transfer of Th1 OT-II cells and OVA/CFA immunization, recipient mice were footpad challenged with either HAO or bone marrow-derived DCs. Challenges used either unpulsed DCs or cells pulsed with OVA, CII, OVA and CII, or an equal mixture of OVA-pulsed and CII-pulsed DCs. Footpad swelling was monitored throughout the experiment using dial calipers (A). Mice were sacrificed 7 d postchallenge for histological analysis of joint tissue (B). The mean of 5 is shown \pm SD in the mean. pLN and serum were harvested 7 d after footpad challenge for in vitro restimulation and serum Ab ELISAs. pLN were stimulated with medium, OVA, or CII and proliferation of CD4⁺ T cells determined by flow cytometric analysis of EdU incorporation (C). ELISA was used for detection of serum anti-CII-IgG (D) and anti-OVA-IgG2a (E), serum was titered by doubling dilution as in the other experiments; however, values shown are of 1 in 50 for anti-CII-IgG, and 1 in 100 for anti-OVA-IgG for ease of presentation. The mean of $n = 5$ is shown \pm SD in the mean, representative of two experiment. * $p < 0.05$; ** $p < 0.01$.



Consistently, HAO footpad challenge induced the greatest swelling. OVA, OVA and CII- pulsed DCs, mixed DCs, and HAO-challenged groups exhibited similar histological scores for day 7 tissues, which were significantly higher than both the unpulsed and CII- pulsed DC groups (Fig. 3B). DCs presenting both the irrelevant Ag (OVA) and the pathophysiologically relevant joint Ag (CII) were sufficient to drive autoreactivity, demonstrated by significant CD4⁺ T cell proliferative response to CII (Fig. 3C) and the presence of CII-specific serum IgG (Fig. 3D). Mixed DCs were also able to generate CII-specific T and B cell responses (Fig. 3C, 3D), however, because DCs pulsed with OVA alone were capable of inciting autoreactivity we were unable to determine whether presentation of both Ags by a single DC was required to break CII tolerance. Activation of OVA-specific T cells by DCs was a prerequisite for, and could support activation of, collagen-specific autoreactive T cells in the absence of exogenously added autoantigen (CII) presentation, with DCs pulsed only with OVA driving anti-CII-specific T and B cell responses. Previous studies have demonstrated that adoptive transfer of arthritogenic T cells into naive recipients is sufficient to induce disease, suggesting constitutive presentation of articular Ags (29). These data support the hypothesis that both Ags do not need to be presented on the same DC, with endogenous APCs potentially acting as a source of CII peptide/MHC class II complexes. CII-pulsed DC recipients did not demonstrate significantly different proliferative or Ab responses to CII compared with DCs alone, highlighting the requirement for the OVA challenge in establishing breach of self-

tolerance in this model (Fig. 3C, 3D). This likely reflects key differences in the prior context in which CII reactive T cells have seen their specific Ag. In the erosive arthritis model by Leung et al. (16), CII-pulsed DCs reactivated CII-specific T cells in mice previously immunized with CII. In our study, CII-specific T cells would not have had previous exposure to CII in a proinflammatory context. Activation of CII-specific T cells in the presence of OVA driven inflammation could perhaps be likened to the CII immunization regimen preceding CII-pulsed DC induction of erosive arthritis previously reported (16).

Consistent with receiving CFA/OVA immunizations, all animals demonstrated OVA-specific recall and Ab responses (Fig. 3C, 3E). Elevated proliferative responses on OVA restimulation of pLN were observed in groups receiving OVA in the challenge (HAO, DCs OVA and CII, DCs OVA, and mixed DCs) compared with DCs alone and DCs CII (Fig. 3C), demonstrating further expansion or recruitment of OVA specific cells to the LNs in these groups.

Thus, cDC are activated and present Ag at the earliest stages of the breach of tolerance in experimental arthritis and their adoptive transfer can trigger autoreactivity leading to joint pathology.

Transient depletion of CD11c⁺ cells reduced articular pathology and prevented breach of CII tolerance

To definitively demonstrate a central role for cDC in our model of arthritis, we used CD11cDTR transgenic mice, which have been used to reveal the importance of these cells in a variety of experimental systems (22, 30–33). It is reported that depletion is not

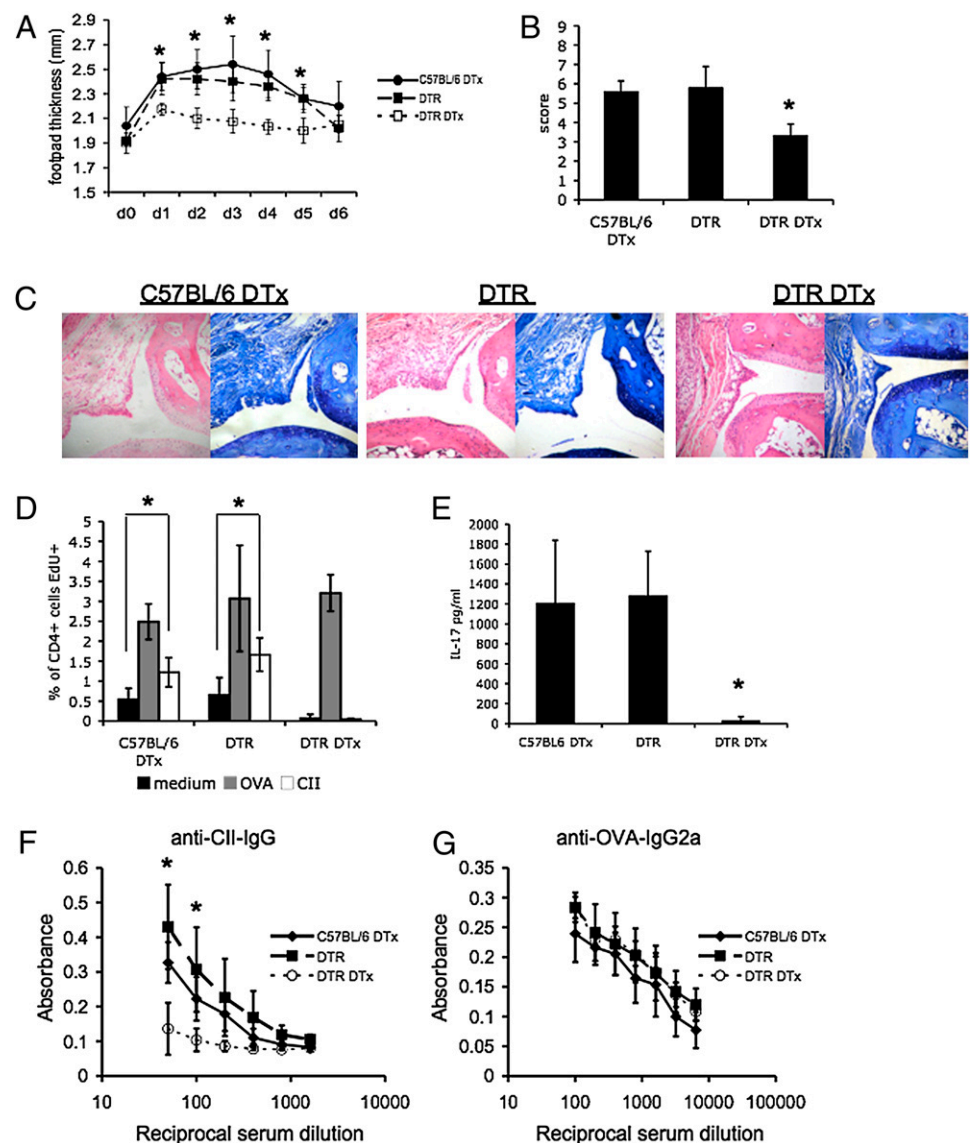
entirely specific to cDCs, impacting on low expressers of CD11c (34). Local DTx treatment in CD11cDTR transgenic mice at prior to challenge depleted the CD11c^{hi} population but did not influence the presence of pDCs or B cells present in the draining pLNs (30, 35; data not shown). HAO challenged, cDC-depleted mice exhibited significantly reduced footpad swelling (Fig. 4A) and overall histological score (Fig. 4B) compared with either nondepleted or DTx treated C57BL/6 control groups, with reduced signs of synovial thickening, inflammatory infiltrate and mild erosion (Fig. 4C). pLNs were also harvested and recall responses to OVA and CII assessed (Fig. 4D). Each group manifested similarly robust responses to OVA. This reflected the prior transfer of Th1 OVA specific cells and OVA/CFA immunization before DTx treatment and the depletion of cDCs. Importantly, HAO-challenged mice depleted of CD11c^{hi} cells did not exhibit significantly different OVA-specific responses compared with either control group, indicating that HAO-driven OVA-specific expansion had still occurred in vivo. In vitro recall responses by CD4⁺ T cells to OVA were consistently higher in mice that had received HAO versus PBS challenge (Fig. 2D), indicating that additional expansion/recruitment of OVA-specific T cells occurs on subsequent Ag encounter. In contrast, no proliferation on in vitro restimulation with CII could be detected, whereas DTx treated

C57BL/6 arthritic mice and nondepleted CD11cDTR arthritic mice exhibited CD4⁺ T cell proliferation to CII (Fig. 4D).

Accumulating evidence supports the importance of IL-17 in the pathogenesis of autoimmune disease, particularly in joint destruction in inflammatory arthropathies (36). Elevated IL-17 was evident in both control arthritic groups but not in DTx-depleted CD11cDTR mice 7 d after challenge (Fig. 4E). Lack of IL-17 may specifically relate to the absence of autoreactive T cell responses but equally, this cytokine may be required to facilitate breach of self-tolerance. To further confirm breach of self-tolerance was cDC dependent we measured autoreactive Ab production. DTx-treated C57BL/6 and nondepleted CD11cDTR arthritic mice had equivalent levels of anti-CII-IgG (Fig. 4F), whereas DTx treatment prior to HAO challenge significantly reduced titers of anti-CII-specific IgG (Fig. 4F). Significantly, titers of anti-OVA-specific IgG2a were unaffected by DTx treatment (Fig. 4G) implying that depletion of cDCs had not significantly impacted on the in vivo anti-OVA response.

Depletion of cDCs at the time of challenge in our model prevented development of CII-specific immune responses and ameliorated subsequent joint pathology. These effects were apparent, whereas OVA-specific T and B cell responses were still recalled in vivo in the absence of cDCs. These data support the hypothesis

FIGURE 4. Reduced joint pathology and failure to develop anticollagen responses in the absence of cDCs. C57BL/6 or CD11cDTR transgenic mice received Th1 cells and were immunized with OVA/CFA as before. C57BL/6 mice were DTx (C57BL/6 DTx) treated and CD11cDTR transgenics received PBS or DTx (CD11cDTR and CD11cDTR DTx, respectively). All mice were footpad challenged 24 h later with HAO. Footpad swelling was monitored for the duration of the experiment (A). A total histology score (B) was ascribed to each group based on day 7 histology (examples of H&E toluidine blue-stained sections presented in C). Original magnification $\times 10$. pLNs were harvested 7 d after footpad challenge and restimulated in vitro with medium, OVA, or CII (D). Serum samples were also assayed for the presence of IL-17 (E), anti-CII-IgG (F), and anti-OVA-IgG2a (G). The average of $n = 5$ mice for a single experiment is shown \pm SD in the mean. * $p < 0.05$; ** $p < 0.01$.



that cDCs directly contribute to the induction of CII-specific responses, independently of indirect effects of cDCs on the development of recall OVA responses.

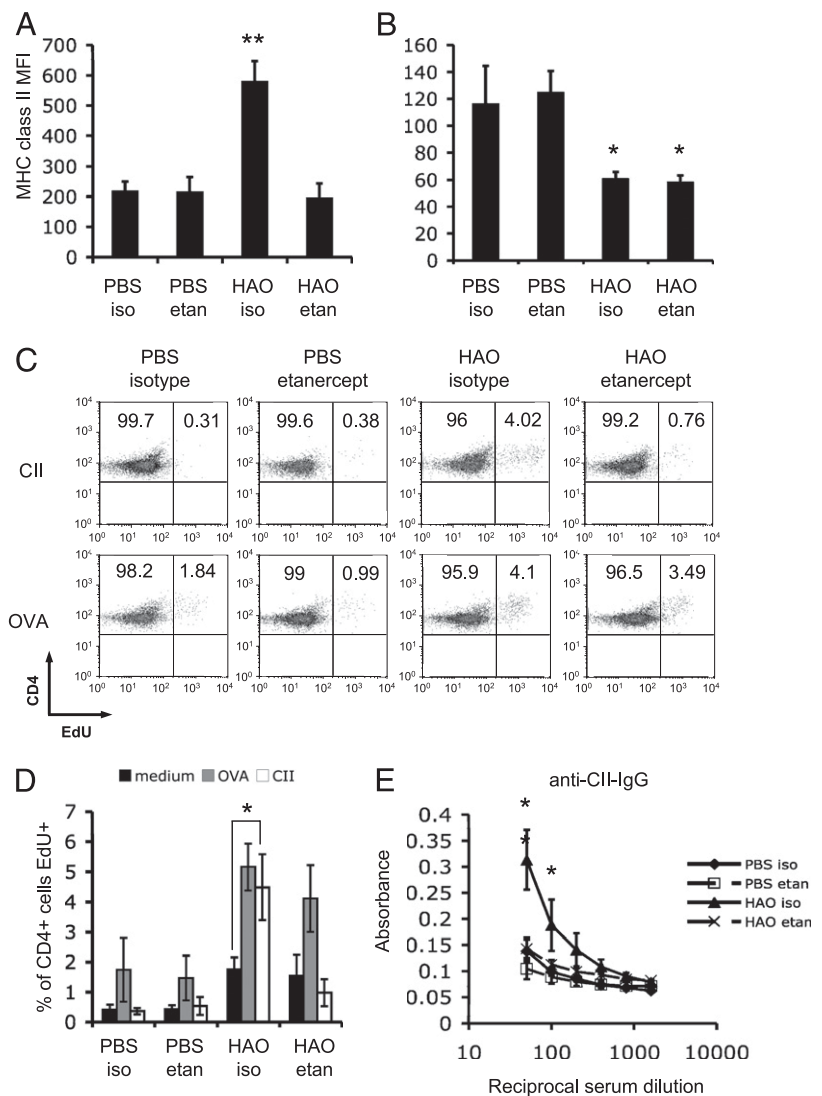
TNF blockade inhibits elevated expression levels of MHC class II by cDCs and prevents breach of self-tolerance

Early and aggressive blockade of TNF- α in RA can result in clinical remission (37, 38) but there is a paucity of data pertaining to possible mechanisms of action. TNF- α blockade can prevent the maturation of circulating myeloid DCs in ankylosing spondylitis (39); patients exhibited decreased T cell reactivity and it was concluded that this related directly to reduced activation by DCs. Indeed, reduced disease severity in RA after anti-TNF- α therapy correlates with decreased DC maturation (12). As we have attributed maturation of cDCs in our model to the stimulation of autoreactive responses we sought to assess whether reduced pathology and long-term clinical remission seen after TNF- α blockade in patients could reflect reduced cDC maturation and subsequently, a failure to prime autoreactive T cell responses in our model.

Mice were treated with the dimeric recombinant human TNF receptor p80 Fc fusion protein (sTNFR-Fc) or control Fc matched human Ig (hIgG). Treatment with sTNFR-Fc prevented cDC upregulation of MHC class II expression after HAO challenge (Fig.

5A). No significant differences in MHC class II expression were detected between cDCs from nonarthritic mice treated with either control hIgG or sTNFR-Fc (Fig. 5A). The pDCs in the pLN of arthritic mice expressed lower levels of MHC class II compared with nonarthritic animals, with no detectable influence of sTNFR-Fc treatment (Fig. 5B). Having demonstrated that cDCs are a major contributor to Ag presentation, can mediate and are critical for, the development of autoreactive responses in this model, we tested whether sTNFR-Fc mediated inhibition of elevated cDC MHC class II could also prevent development of collagen-specific T cell and Ab responses. LN cells were restimulated in vitro with OVA or CII. Only HAO-challenged, hIgG-treated mice showed CD4⁺ proliferative responses to CII (Fig. 5C, 5D), whereas no responses were detected in the HAO challenged sTNFR-Fc treated or PBS challenged groups. The OVA recall response was significantly increased in HAO challenged arthritic groups, with no discernable effect of treatment with sTNFR-Fc (Fig. 5C, 5D). The sTNFR-Fc inhibited production of anticollagen-IgG with only background levels, comparable with PBS footpad challenged mice, being detected (Fig. 5E). Treatment with sTNFR-Fc did not alter titers of anti-OVA-IgG, with all groups demonstrating comparable levels of these Abs (data not shown). In vitro treatment of bone marrow DCs with sTNFR-Fc prior to addition of EaGFP did not impact on Ag presentation (Supplemental Fig. 3A,

FIGURE 5. Anti-TNF- α therapy prevents cDC increased MHC class II expression in draining LN, avoiding T and B cell breach of tolerance to CII. Flow cytometric analysis of MHC class II expression by cDCs (A) and pDCs (B) in the pLN of both nonarthritic and arthritic mice treated with either control hIgG iso or the sTNFR-Fc fusion protein, Etan. pLN and serum were harvested 7 d after footpad challenge. pLNs were restimulated in vitro with medium, OVA, or CII. The percentage of CD4⁺ T cells responding was determined by flow cytometric analysis of EdU incorporation. FACS plots representative of responses to CII and OVA are shown (5 mice per group) (C, D). Anti-CII-specific serum IgG was detected by ELISA (E). The mean \pm the SD for $n = 5$ animals representative of two experiments is shown. * $p < 0.05$ ** $p < 0.01$. Etan, etanercept; iso, isotype;



3B), as detected by YAc staining. As such the *in vivo* effects observed may relate TNF-dependent tissue maturation and migration rather than reduced Ag presentation.

Thus, reduction of MHC class II expression by cDCs found in the draining LN of arthritic mice treated with sTNFR-Fc was followed by a failure to mount both CII-specific T cell and Ab responses. As such, limiting the maturation of cDCs by TNF- α blockade and preventing/limiting autoreactive T cell responses may represent one of the mechanisms whereby early intervention with anti-TNF- α biologics can achieve long-term clinical remission.

Discussion

Understanding the factors triggering the activation of previously unresponsive, self-reactive T cells will be crucial in delineating the pathological response seen in RA and development of new therapeutic regimes. Central to this will be the identification of the APC population initiating the breach of self-tolerance underlying not just RA, but all autoimmune diseases. Using a recently developed model of experimental arthritis (17), we were able to delineate the critical events surrounding the development of autoreactive T and B cell responses and the ensuing joint pathology. These studies have demonstrated cDC activation and Ag presentation associated with breach of self-tolerance, an ability of these cells to transfer autoimmunity, together with a central role for cDCs in the TNF- α -dependent development of self-reactivity and RA pathogenesis.

After challenge with HAO in our model of arthritis, similarly increased numbers of cDCs and pDCs were observed in the draining LN. However, expression of MHC class II was elevated in the cDC compartment compared with pDCs. Detection of this activated cDC population could have resulted from either stimulation of LN resident cells, influx of tissue activated cDCs or, most likely, a combination of both. We therefore used E α GFP and the mAb YAc (23, 40) to identify *in vivo* the major APC populations associated with the development of autoimmunity and pathology in RA. Coadministration of E α GFP and HAO identified cDCs as the primary population presenting E α peptide, particularly as this population increased significantly in size in arthritic mice. As cDCs were the main cell presenting exogenous Ag, we hypothesized that they were the most likely candidates to be presenting tissue-derived Ag in our model.

Previous studies demonstrated that adoptive transfer of arthritogenic T cells into naive recipients is sufficient to induce disease, suggesting constitutive presentation of articular Ags (29). Joint related Ag may drain directly via lymphatics but may also be presented in the draining LN via the homeostatic migration of DCs. This raises the possibility that the requirement for cDCs in priming a CII response in our model may partly reflect a role in Ag transport to the draining LN. One hypothesis would be that the stimulation of the existing OVA-specific memory response alters cDC characteristics sufficiently to allow reversal of their usual tolerogenic interaction with self-specific T cells and priming of autoreactivity. Indeed, a model of RA in which self-Ag presented by activated DCs stimulates autoreactive T cells that have escaped thymic selection has been proposed (25, 26). In support of this hypothesis, adoptive transfer of Ag-bearing DCs can incite autoimmunity underlying diabetes, experimental allergic encephalitis, and arthritis (16, 27, 28). In this study, we were able to induce CII-specific responses in the OVA TCR transgenic transfer model by challenge with Ag-bearing DCs. Activation of OVA-specific T cells by DCs was a prerequisite for, and could support activation of, collagen-specific autoreactive T cells when endogenous autoantigen was available, whereas addition of exogenous CII could not. This likely reflects key differences in the prior context in which CII reactive T cells have seen their specific Ag. In the

forementioned study by Leung et al. (16), CII-pulsed DCs reactivated CII-specific T cells in mice previously immunized with CII. In our OVA TCR transgenic transfer model, CII-specific T cells would not have had previous exposure to CII in a proinflammatory context. Activation of CII-specific T cells in the presence of OVA driven inflammation could perhaps be likened to the CII immunization regimen preceding CII-pulsed DC induction of erosive arthritis previously reported (16).

Thus, cDC are activated and present Ag at the earliest stages of the breach of tolerance in experimental arthritis and their adoptive transfer can trigger autoreactivity leading to joint pathology. To definitively demonstrate a central role for cDC in our model of arthritis, we used CD11cDTR transgenic mice that have been used to reveal the importance of these cells in a variety of other systems (22, 30–33). Depletion of these cells at the time of challenge in our model prevented development of CII-specific immune responses and ameliorated subsequent joint pathology. These effects were apparent, whereas this treatment had little effect on OVA-specific responses, indicating that OVA-specific expansion had still occurred *in vivo*. Our finding does suggest that memory responses can be efficiently mounted in the absence of cDCs. Indeed, the presence of other APCs appeared sufficient to ensure activation of the memory OVA response, potentially being driven by an increased frequency of high-affinity B cells generated from the OVA/CFA immunization (41). It therefore seems unlikely that a reduced OVA response could account for the lack of autoreactivity in CD11c^{hi}-depleted mice.

TNF- α blockade prevented the increase of MHC class II expression by draining LN cDCs and development of autoreactive responses. TNF- α may be required for migration of mature cDCs to the draining LN, preventing the second influx of Ag-presenting DCs important in priming T cell response (23). Equally, maturation of LN resident cDCs may also be TNF- α dependent. The most likely explanation will no doubt prove a combination of both, with failure of either scenario preventing activation of pathogenic CII-specific T cells. The importance of TNF- α to DC maturation in models of both type 1 diabetes (42) and alloresponses (43) supports this hypothesis. Indeed, in these two studies, tolerance induction was observed after inhibition of TNF- α activity, a tantalizing prospect given the success of current anti-TNF- α biologics in RA.

This study demonstrates for the first time that cDCs play a central role in driving arthritogenic autoimmunity and no other APC is sufficient for breach of self-tolerance arising via endogenous pathways initiated by an irrelevant nonarticular Ag. Accumulating phenotypic studies have identified and characterized DC populations in RA and highlighted their potential for contribution to disease in RA. This study directly demonstrates that cDCs mature and present Ag in the context of murine inflammatory arthritis and that development of autoreactivity in this model is crucially dependent on their presence. These studies clarify the processes by which immunological regulation can be overcome by responses to infectious agents that have been implicated in the pathogenesis of RA (44–47).

Disclosures

The authors have no financial conflicts of interest.

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