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Inducing Experimental Arthritis and Breaking Self-Tolerance to Joint-Specific Antigens with Trackable, Ovalbumin-Specific T Cells

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The importance of T cell Ag specificity and Th1 vs Th2 phenotype in synovial inflammation remains controversial. Using OVA-specific TCR transgenic T cells from DO11.10 mice, we demonstrate that mice receiving Th1, but not Th2, cells display a transient arthritis following immunization that is characterized by synovial hyperplasia, cellular infiltration, and cartilage erosion. OVA-specific T cells also accumulated in inflamed joints, suggesting that they could exert their inflammatory effect locally in the joint or in the draining lymph node. Importantly, this pathology was accompanied by a breakdown in self-tolerance, as evidenced by the induction of collagen-specific T and B cell responses. This model directly demonstrates a pivotal role for Th1 cells of an irrelevant specificity in the development of inflammatory arthritis. Furthermore, the ability to track these cells in vivo will make feasible studies revealing the dynamic role of T cells in arthritis.

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Materials and Methods

Animals

Six- to 8-wk-old male BALB/c mice purchased from Harlan Olac (Bicester, U.K.) were used as adoptive transfer recipients. DO11.10 BALB/c TCR Tg mice (9) contain CD4+/H1001.

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The Journal of Immunology, Infection and Inflammation, Glasgow, University of Glasgow, and we performed all procedures according to U.K. Home Office regulations.
Flow cytometry

We pooled peripheral LNs (axillary, inguinal, cervical) and mesenteric LNs from DO11.10 mice and forced through Nitex mesh (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. We stained aliquots of these cells with PE-labeled anti-CD4 mAb CD4 (L3T4) (RM4-5) (BD Pharmingen, San Diego, CA) and biotinylated KJ1.26 mAb, followed by FITC-labeled streptavidin (BD Pharmingen) for the detection of DO11.10 T cells. We analyzed stained cells (20,000 per sample) with flow cytometry using CellQuest software (BD Biosciences, Oxford, U.K.).

T cell purification and T cell cultures

We purified naive CD4⁺ T cells from pooled LN cells from DO11.10 mice by negative selection. We removed B cells, monocytes, and CD8⁺ T cells using anti-CD19, anti-CD11c, and anti-CD8 mAbs (all Serotec, Oxford, U.K.), respectively, which were then bound by anti-IgG MACS beads (Miltenyi Biotec, Auburn, CA), according to manufacturer’s instructions. We obtained APCs irradiating DO11.10 spleen cells (2000 rad). We induced T cell differentiation culturing 2 × 10⁵/ml CD4⁺ T cells with 2 × 10⁶/ml APC and 0.3 μM OVA 323–339 (Genosys, Cambridge, U.K.). In addition, Th1 cell cultures included 5 ng/ml IL-12 (PeproTech, Rocky Hill, NJ) and 10 μg/ml anti-IL-4 mAb (R&D Systems, Minneapolis, MN), whereas Th2-polarizing cultures incorporated 5 ng/ml IL-4 (R&D Systems) and 1/500 anti-IL-12 (R&D Systems), as described previously (10). After 3 days of culture, we washed and harvested the cells for transfer.

Adoptive transfer

A total of 2 × 10⁶ naive, Th1, or Th2 DO11.10 T cells were injected i.v. into BALB/c recipients, as described previously (10). Control mice received PBS only.

Ag administration

One day following adoptive transfer, recipients were immunized s.c. with 100 μg of OVA in CFA (Sigma-Aldrich, Poole, U.K.). We prepared heat-aggregated OVA (HAO), as described previously (11).

Assessment of arthritis

Ten days after challenge with OVA in CFA, all animals (n = 10 per group) were injected s.c. close to both ankle joints with 100 μg of HAO in 50 μl of saline. Some control mice received a periarticular injection of PBS instead of HAO (sham controls). We monitored mice subsequently for signs of arthritis, as described previously (12). Disease was scored based on erythema, swelling, or loss of function present in each paw on a scale of 0–3, giving a maximum score of 6 per mouse. We measured paw thickness with a dial caliper (Kroeplin, Munich, Germany). For histological assessment, mice were sacrificed, hind limbs were removed and fixed in 10% neutral-buffered formalin, and 6-μm sections were stained with H&E or toluidine blue (both from Sigma-Aldrich).

FIGURE 1. Arthritis in recipient mice. Hind paw thickness (a), mean clinical score (b), and incidence (c) of inflammatory arthritis in BALB/c mice receiving PBS (○), unpolarized T cells (naive (●)), Th1 cells (■), or Th2 cells (▲). Th1 recipient mice developed arthritis of greater severity than PBS, naive, and Th2 recipient mice. The disease was prolonged in Th1-transferred animals. Data represent mean ± SEM (n = 10). Similar results were obtained in two additional experiments. *, p < 0.05 (Th1 vs Th2). Histological analysis: staining H&E (d–g); toluidine blue (h–k). Ankle joints of Th1 recipient mice demonstrated extensive synovial hyperplasia, inflammatory cell infiltration (f), and cartilage and bone erosion (j). Th2 recipients developed a less severe inflammation compared with the Th1 recipients (g and k). PBS recipient mice and naive mice revealed no apparent inflammatory cell involvement (d and e) and a normal cartilage integrity (h and i). Original magnification, ×40.
Restimulation of draining LN cells in vitro
At the conclusion of in vivo experiments (day 7), draining LN cells were
restimulated with either medium alone, 1 mg/ml OVA, or 50 µg/ml col-
lagen II (CII) for proliferation and cytokine production, as described pre-
viously (4, 10–13).

ELISA
Cytokines, anti-OVA, and anti-collagen Ab levels were detected by
ELISA, as described previously (4, 10–13).

Immunohistochemistry
Draining LNs were snap frozen in liquid nitrogen in OCT embedding me-
dium (Miles Diagnostic Division, Elkart, IN) and stored at −70 °C. Six- to
to 10-µm joint sections were cut from hind limbs decalcified in a 5.5% EDTA
solution in a phosphate buffer, pH 7.4, for 2 wk.

Single staining for the DO11.10 TCR
DO11.10 T cells were detected in tissue sections with the clonotypic mAb
KJ1.26, as described previously (13).

Statistics
Statistical analysis was done using Minitab software (Minitab, State Col-
lege, PA), and results were compared using Student’s unpaired t test. We
applied Bonferroni’s correction for multiple comparisons, as appropriate.
A p value of <0.05 was regarded as significant.

Results
Phenotype of polarized cells
The cytokine profiles of aliquots of the in vitro polarized T cells
were determined by ELISA. Th1 cells produced high levels of
IFN-γ (9213 ± 1040 pg/ml; p < 0.001, Th1 vs Th2) and little IL-5
(66 ± 8 pg/ml), whereas Th2-polarized cells produced significant
amounts of IL-5 (500 ± 100 pg/ml; p < 0.001, Th2 vs Th1) and
low levels of IFN-γ (145 ± 7.8 pg/ml). Therefore, after polariza-
tion under the appropriate conditions in vitro, DO11.10 T cells
adopted the expected Th1 and Th2 phenotypes, as described pre-
viously (10).

Arthritis in recipient mice
We immunized control and cell-recipient mice, 24 h after the trans-
fer, with OVA/CFA, and subsequently challenged them by peri-
articular injection in right and left hind ankle joints with HAO 10
days later. The subsequent development of inflammatory arthritis
was monitored by paw swelling (Fig. 1a), severity (mean clinical
score; Fig. 1b), and incidence (Fig. 1c). Transfer of Th1 cells into
recipient mice caused the induction of arthritis, first evident at day
1 postimmunization with HAO. In contrast, control mice, mice
receiving unpolarized or Th2 cells, developed only low levels of
inflammation. In addition, the disease was prolonged in Th1-trans-
ferred animals. Finally, Th1 mice displayed a significantly greater
clinical score and paw swelling compared with Th2 mice. Mice
that received a periarticular injection of PBS rather than HAO
(sham controls) failed to develop inflammation (data not shown).
We performed sequential histological analyses (Fig. 1, d–k). Con-
trol mice and mice receiving unpolarized cells exhibited no appar-
ent local inflammatory reaction (Fig. 1, d and e) and normal carti-
ilage integrity (Fig. 1, f and i). Th1-transferred mice exhibited
extensive synovial hyperplasia and inflammatory cell infiltration
(Fig. 1f) in the involved joint. We observed partial or complete
erosion of the cartilage matrix in both central and peripheral
regions of joint surfaces, as assessed by loss of toluidine blue
staining (Fig. 1j). Appearances resembled those of CFA-induced
collagen-induced arthritis (14) and RA synovial tissue (15). Th2-
transferred mice exhibited a less severe inflammation than the

FIGURE 2. Ag-specific T cells in vivo. a, Percentage of CD4+ KJ1.26+ T cells in the draining LNs of mice receiving PBS (○), unpolarized T cells (●), Th1 (■), or Th2 cells (▲) assessed by flow cytometry. b, Number of TCR Tg T cells in popliteal LNs. Mice receiving PBS, unpolarized T cells (○), Th1 (■), or Th2 cells (●). Each time point represents the mean ± SEM for at least three mice per group. Similar results were obtained in two additional experiments. c–f, Immunohistochemistry. c–f, Draining LNs at day 7 from mice receiving PBS (c), unpolarized T cells (d), Th1 (e), or Th2 cells (f). KJ1.26+ T cells (brown) had expanded above control level in all the transferred groups. Transferred T cells observed in the inflamed joint of Th1 recipient
mice from the beginning of the pathology (g, day 1; h, day 7). Original magnification, ×40.
Th1-transferred group, with a modest reduction of toluidine blue staining only in peripheral cartilage regions that were in direct contact with inflamed synovial tissue (Fig. 1, g and k).

Clonal expansion of Tg T cells in vivo
We injected BALB/c mice with $2 \times 10^6$ CD4+ KJ1.26+ unpolarized (naive), Th1, Th2 T cells or PBS on day $-11$. On day $-10$, mice were immunized s.c. with 100 μg of OVA/CFA. On day 0, mice were challenged with 100 μg of HAO close to both ankle joints. Draining LNs were removed on days $-7$, 0, 3, and 7 for flow cytometric analysis. Th1, Th2, and unpolarized Tg T cells were detected immediately ex vivo, indicating that TCR Tg T cells can survive and respond to Ag in vivo (Fig. 2a). In agreement with our previous studies (10), we observed peak clonal expansion on day 3 after immunization, which declined thereafter. Th1 and Th2 cells expanded to similar levels, whereas unpolarized cells proliferated to a lesser extent. The number of TCR Tg T cells in the popliteal LN at different time points is shown in Fig. 2b.

Localization of Tg T cells by immunohistochemistry
Immunohistochemical analysis of draining LNs confirmed that the population of KJ1.26+ T cells had expanded above control levels in all transferred groups (Fig. 2, c–f). This expansion was highest at day $-7$ and declined thereafter. Using immunohistochemistry, we directly examined the localization of KJ1.26+ T cells in the inflamed paw. We have been unable to find Tg T cells in the joints of mice transferred with unpolarized T cells. Moreover, we only ever observed Tg T cells in the joint of one Th2-transferred mice at day 1 (data not shown). Interestingly, we observed transferred T cells in the inflamed joint of Th1-transferred mice from the beginning (Fig. 2g, day 1) until the end (Fig. 2h, day 7) of the pathology. These results suggest that the transferred Th1 cells could exert their inflammatory effect in the joint.

Cytokine profile and cellular proliferation of OVA-specific cells ex vivo
LNs were removed from control and transferred mice 7 days post-challenge with HAO and cultured in vitro with or without 1 mg/ml OVA or with 50 μg/ml CII, and their cytokine production profile and proliferation were determined. OVA323–339 peptide-specific T cells from animals receiving Th1-polarized cells and OVA/CFA continued to produce IFN-γ (Fig. 3a), but little IL-5 (Fig. 3b) after stimulation with OVA or CII, whereas peptide-specific T cells from animals transferred with Th2-polarized cells and immunized with OVA/CFA produced IL-5 (Fig. 3b), but little IFN-γ (Fig. 3a). Cells from control mice and T cells from animals receiving unpolarized cells produced little cytokine of any description when re-stimulated in vitro with CII (Fig. 3, a and b). Little or no cytokine production was apparent in the absence of stimulation (Fig. 3, a and b). TNF-α and IL-6 were not detectable in the culture supernatant of all of the groups of mice. OVA323–339 peptide-specific T cells from animals receiving Th1- or Th2-polarized cells and OVA/CFA proliferated to similar levels after in vitro OVA stimulation, whereas cells from control mice and T cells from animals receiving unpolarized cells proliferated to a lesser extent (Fig. 3c). Little proliferation was apparent in the absence of stimulation in all the groups, except Th2 recipient group (Fig. 3c). Interestingly, only draining LN cells from OVA-specific Th1 and Th2 recipient mice proliferated when cultured in vitro with CII (Fig. 3c).

Anti-OVA and anti-collagen Ab production in vivo
We took serum from animals transferred with unpolarized, Th1, Th2 cells or PBS at day 7. As expected, all of the animals were able to produce high levels of anti-OVA Abs. IgG1 (Fig. 4a) and IgG2a (Fig. 4b) Abs were higher in the Th1 recipient group compared with the Th2 group. Lower level of anti-OVA IgG2a Abs was detectable in serum from mice receiving unpolarized Tg T cells or PBS.

Finally, we sought evidence for anti-collagen Ab production at day 7. IgG1 Abs (Fig. 4c) were higher in the Th2 recipient group compared with the Th1 recipient group. Collagen-specific IgG2a Abs (Fig. 4d), which are typically produced during a Th1 response, were higher in the Th1 recipient group compared with the Th2 group. We detected little anti-collagen IgG1 Abs and no IgG2a Abs in serum from mice receiving unpolarized Tg T cells or PBS.

Discussion
A number of animal models of RA have been developed to characterize the cell types and mechanisms involved in the pathogenesis of RA, with the ultimate objective of therapeutic intervention in the disease process (16). The system described in this study, based upon the adoptive transfer of TCR Tg T cells, has allowed us to track, in detail, the ability of Th1 and Th2 cells of known Ag specificity to produce cytokines and to collaborate with other cell types in the disease process. In the case of the Th1 transplants, we observed a T cell response that was able to produce high levels of the Th1 cytokines IFN-γ and TNF-α. These cytokines are known to be involved in the pathogenesis of RA and could explain much of the observed joint pathology. In contrast, the Th2 transplants were unable to produce high levels of these cytokines. These results are in agreement with previous studies that have shown a correlation between the production of Th1 cytokines and the severity of the joint pathology in the draining LN of these animals (10). The higher levels of IL-5 produced by the Th2 cells may be responsible for the lower level of joint pathology observed in these animals. The production of IL-5 by Th2 cells has been shown to be important in both the production of antibodies to OVA and the induction of arthritis in this model (10). The production of antibodies to OVA is higher in Th1 recipients than in Th2 recipients, and the production of antibodies to collagen is higher in Th2 recipients than in Th1 recipients. The lower levels of joint pathology observed in Th2 recipients may be due to the lower levels of IL-5 produced by these cells. The production of IL-5 by Th2 cells has been shown to be important in the induction of arthritis in this model (10). The production of antibodies to OVA is higher in Th1 recipients than in Th2 recipients, and the production of antibodies to collagen is higher in Th2 recipients than in Th1 recipients. The lower levels of joint pathology observed in Th2 recipients may be due to the lower levels of IL-5 produced by these cells.
specificity to induce arthritis. Not only could this have implications for a variety of therapeutic approaches, but it also provides an ideal system for studying the mechanisms and cellular interactions underlying RA induction in vivo.

We believe that tissue-infiltrating T lymphocytes play a pivotal role in the inflammatory response in RA (17, 18). The abundance of T cells, in RA synovium, expressing differentiation and activation markers supports this concept (18). This evidence suggests that Ag-specific T lymphocytes act as initiators of the inflammatory process and that T cell-derived cytokines play a major role in maintaining inflammation and in mediating matrix destruction of the synovium (17–19). Moreover, T cells can also directly trigger osteoclastogenesis through the TNF family molecule osteoprotegerin ligand (OPGL), which regulates bone loss, LN organogenesis, lymphocyte development, and interaction between T cells and DCs in the immune system (20, 21). Thus, in a model of rat adjuvant arthritis, systemic activation of T cells in vivo leads to an OPGL-mediated increase in osteoclastogenesis and bone loss, and blocking OPGL through treatment with its decoy receptor osteoprotegerin prevents bone and cartilage destruction (22). However, the Ag specificity of the T cells that initiate and maintain this pathology remains unclear.

Murine and human T lymphocytes may be identified as Th1 or Th2 cells depending on their cytokine production profile. The balance between these subsets strongly influences many inflammatory responses, although this may also be influenced by regulatory subsets (23). The Th1/Th2 imbalance in RA joints is associated with high numbers of activated macrophages, leading to an aggressive form of arthritis with rapidly occurring joint destruction (24). Moreover, the demonstration that RA synovial T cells produce cytokines with anti-inflammatory properties confirms the hypothesis that the course of RA synovitis may be the expression of a balance between pro-(Th1) and anti-(Th2) inflammatory cytokines (2).

We have developed an adoptive transfer system in the mouse that has allowed us to further characterize and localize the Th cells involved in the induction of arthritis. In our current study, we stably polarized naïve TCR Tg T cells toward a Th1 or Th2 phenotype in vitro before adoptive transfer. Our transferred T cells secreted the expected pattern of cytokines (IFN-γ for Th1 and IL-5 for Th2) before adoptive transfer and maintained their phenotypes, over the course of the experiment, following restimulation after recovery from immunized animals, as we have described previously (10).

We have demonstrated directly in vivo that transfer of Th1, and not Th2, cells into recipient mice caused the induction of a transient arthritis, first evident at day 1 postchallenge with HAO. Th1-transferred mice exhibited extensive synovial hyperplasia, inflammatory cell infiltration, and cartilage and bone erosion in the involved joint.

FACS analysis revealed Ag-specific T cell expansion in the draining LN, which peaked 3 days after initial Ag exposure, with naive, Th1, and Th2 cells showing similar kinetics of clonal expansion. Immunohistochemistry confirmed that Tg T cell accumulation peaked in the draining LN 3 days after initial immunization with OVA/CFA and declined thereafter, returning to a very low level even after challenge with HAO. Despite this, we could observe accumulation of the transferred OVA-specific T cells in the inflamed joint from the beginning of the pathology, suggesting that the transferred T cells, which expanded in the LN, could exert their inflammatory effect directly or indirectly in the joints where the Ag is present, or possibly in the draining LNs where Ag presentation occurs. Interestingly, Setoguchi et al. (25) recently demonstrated that adoptive transfer of IL-10-transduced DO11.10 splenocytesameliorated OVA-induced arthritis, with a similar accumulation of the transferred Tg T cells in the inflamed joint.

As expected at the end of the experimental procedure, serum from all of the immunized animals contained high levels of anti-OVA IgG1 and IgG2a Abs. Surprisingly and importantly, only Th1 and Th2 recipient groups were able to produce anti-CII Abs, with the Th1 recipients producing higher levels of anti-CII IgG2a Abs at the end of the transient arthritis. Moreover, only draining LN cells from Th1 and Th2 recipient mice proliferated and produced cytokines in response to CII. Thus, we have been able to initiate arthritis and break self-tolerance to a joint-specific Ag with T cells of a specificity entirely unrelated to the joint. This finding has considerable implications for the way we think about the
pathogenesis and therapy of RA and autoimmunity. We hypothesize that by providing an overwhelming number of activated Th1 cells of an irrelevant specificity and a local inflammatory stimulus in the joint, we have been able to overwhelm the usual regulatory circuits and break self-tolerance. We propose that the inflammation initiated by OVA-specific T cells causes normally quiescent, tolerogenic DC carrying self Ag (collagen) from the joint to the local LN to present this in an immunogenic fashion to self-reactive T cells, thereby breaking tolerance and initiating autoimmunity.

A wide spectrum of autoantibodies can be found in RA and related arthritides, not only the rheumatoid factor typical of RA patients, but also a variety of other reactivities. However, the true role of autoantibodies in the pathogenesis of these diseases has been a matter of substantial controversy (26). Several authors consider arthritis due to the loss of tolerance against a cartilage-specific autoantigen. Models have been developed that have proved the arthritogenic potential of cartilage autoantigens such as collagen type II and proteoglycan (27, 28). Moreover, arthritogenic potential has been demonstrated for novel cartilage components such as collagen types IX and XI, cartilage-derived oligomeric protein, and hyaline cartilage gp39 (29, 30). Although both Th1 and Th2 recipient groups were able to produce anti-CII Abs, we saw a fulminant pathology only in the Th1 group. Abs may be sufficient to cause RA in other models (31), and it will now be important to determine why this is not the case in the system we describe.

In summary, we have shown, for the first time, in a TCR Tg trackable Ag-specific system, that following immunization, Th1, but not Th2, cells are able to induce a transient arthritis in BALB/c mice, which is characterized by synovial hyperplasia, cellular infiltration, and cartilage erosion, paralleled by a significant production of anti-CII Abs. This condition mimics some of the clinical and pathological features of human RA. The transferred T cells may exert their inflammatory effect locally in the joint or perhaps more interestingly in the draining LN. This model directly demonstrates a pivotal role of Th1 cells in the development of inflammatory arthritis. Moreover, this will allow us to track the nature and location (e.g., joints vs LN) of the cellular and molecular interactions underlying RA. These results clarify some features of pathogenesis of RA and may have implications for the design of new therapeutic strategies.

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