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Research Article **Immunology**

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Transcription factor T-bet regulates inflammatory arthritis through its function in dendritic cells

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Introduction

Inflammatory arthritis has traditionally been regarded as the consequence of systemic autoimmune responses arising primarily from the adaptive immune system (1, 2). However, recent evidence indicates that components of the innate immune system also play a critical role in pathogenesis. Experimental arthritis can be induced in the absence of mature T and B cells (3), and multiple cell types from the innate immune system such as macrophages, DCs, NK cells, and mast cells infiltrate synovial tissue of RA patients (4). In animal models, inflammatory arthritis has been shown to be dependent on IgG Fcγ receptor type III (FcrRIII), complement receptors, and mast cells (5–7). Furthermore, the stunning success recently achieved by blocking the TNF pathway with the biologics infliximab and etanercept, a therapy originally developed to block the function of cytokines secreted by macrophages, attests to the importance of innate immunity in RA pathogenesis (8). Nevertheless, a significant number of patients do not respond to TNF blockade, highlighting the need for additional therapeutics.

The transcription factor T-bet (T-box expressed in T cells) is required for the generation of IFN-γ–producing Th1 cells (9–15), and provision of T-bet to either mouse or human Th2 cells redirects them to the Th1 lineage (9, 10, 16). Mice lacking T-bet are protected from the development of many different Th1-driven autoimmune diseases, including inflammatory bowel disease, systemic lupus erythematosus and EAE, but develop spontaneous asthma, consistent with a Th1/Th2 shift in vivo toward the Th2 subset (17–20).

Recent data from our laboratory indicate that T-bet is also required for optimal production of IFN-γ by DCs under certain conditions but not for development or maturation of DCs (21). T-bet also controls the terminal maturation of NK cells (22). These observations suggest that T-bet regulates type 1 immunity by influencing genetic programs in both adaptive and innate immunity. Here we explore the role of T-bet in the collagen antibody–induced arthritis (CAIA) model of inflammatory arthritis. Our experiments reveal that T-bet expression in DCs is both necessary and sufficient for disease development.

Results

T-bet is expressed in inflammatory infiltrates in human rheumatoid synovium. Immunohistochemical analysis of synovial tissue from 7 patients with established RA who fulfilled American College of Rheumatology (ACR) criteria for the diagnosis of RA revealed a predominance of CD3$^+$ cells and a large number of cells immunoreactive for T-bet (Figure 1, B and C), consistent with previous observations that RA tissue infiltrates consist predominantly of T cells. However, B cells (Figure 1D) and macrophages (Figure 1E) were also present as well as a small number of DCs (Figure 1F). T-bet has functions in adaptive immunity outside of the T helper cell. It controls IgG2a class switching in B cells (18), and T-bet together with another T-box family member, *Eomes*, regulates the production of IFN-γ from CD8 and NK cells (20, 22–24). Thus, some of the T-bet immunoreactivity we observed may reflect T-bet expression in these latter cell types. While the synovial samples are

Nonstandard abbreviations used: CAIA, collagen antibody–induced arthritis; DKO, double KO; IL-1Ra, IL-1 receptor antagonist; KLH, keyhole limpet hemocyanin; MIP-1α, macrophage inflammatory protein-1α; TARC, thymus- and activation-related chemokine; T-bet, T-box expressed in T cells.

Conflict of interest: L.H. Glimcher has equity in and is on the corporate board of the Bristol-Myers Squibb Co. and has equity in and is a paid consultant for MannKind Corp., a biopharmaceutical company that, with Harvard University, owns the rights to the T-bet technology.

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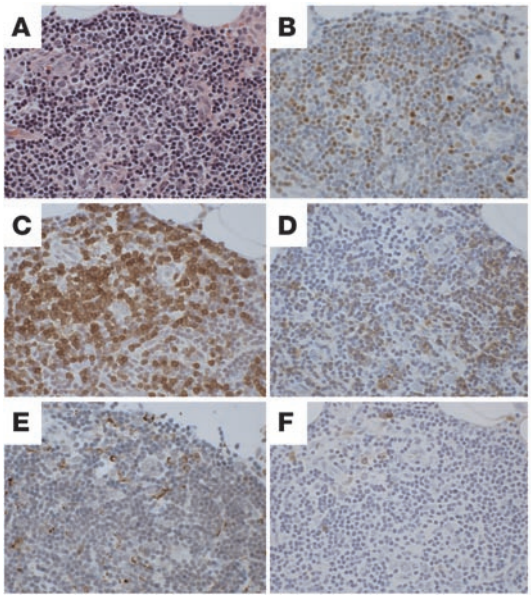

Figure 1
T-bet is expressed in human rheumatoid synovium. Immunohistochemical analysis of synovial tissue from 7 patients with established RA who fulfilled American College of Rheumatology criteria for the diagnosis of RA. (**A**) H&E staining. (**B**–**F**) Representative immunostaining of inflammatory synovium for (**B**) T-bet, (**C**) CD3 (T cells), (**D**) CD20 (B cells), (**E**) CD68 (macrophages), and (**F**) S100 (DCs).

mostly from patients with advanced RA requiring surgical intervention and thus may not reflect the cell composition of early disease, nevertheless, the prominent expression of T-bet in affected tissues in human RA prompted evaluation of T-bet's role in a mouse model of arthritis.

Both innate and adaptive immunity contribute to inflammatory arthritis in the CAIA model. A variant of collagen-induced arthritis can be readily evoked by administering a combination of mAbs to type II collagen together with LPS (CAIA) (6, 25, 26). This model provides some advantages compared with classical collagen-induced arthritis, including (a) rapid induction with joint inflammation occurring within several days and peaking within a week, (b) strain independence, and (c) high reproducibility. Although the underlying mechanisms that lead to arthritis in CAIA are still under intensive investigation, the innate immune system, at least in the early phase, plays a significant role, as evidenced by the development of severe arthritis in SCID mice in the 7 days after arthritis induction (27).

To further explore the contribution of innate immunity to the early phase of CAIA, we assessed the severity of arthritis in RAG2$^{-/-}$ mice, which completely lack mature T and B cells and are therefore less "leaky" than the SCID strain. We titrated the amount of LPS and anti-CII mAb cocktail to identify the lowest dose (1 mg and 2 mg/mouse for BALB/c and C57BL/6 backgrounds, respectively) that induced full-fledged arthritis. Figure 2A shows that WT BALB/c mice developed mild joint inflammation (mean score of 0.75) with subsequent increasing severity of inflammation, peaking (mean score of 10.25) on days 5 to 7 and continuing unabated until day 14, when mice were sacrificed. Notably, RAG2$^{-/-}$ mice developed early joint inflammation and paw swelling (Figure 2D) comparable to WT mice. However, the arthritis that persisted in the later phase (day 14) was attenuated (mean score of 5.75). CAIA is thus a bipartite disease characterized by an early innate component and a later adaptive immune phase.

T-bet$^{-/-}$ mice are markedly protected from the development of inflammatory arthritis. To test the function of T-bet in CAIA, BALB/cT-bet$^{-/-}$ or WT mice were injected with the anti-CII mAb cocktail and LPS as above. Onset of inflammation in T-bet$^{-/-}$ mice was delayed by approximately 24 hours compared with WT controls. Disease severity was also markedly reduced throughout the dis-

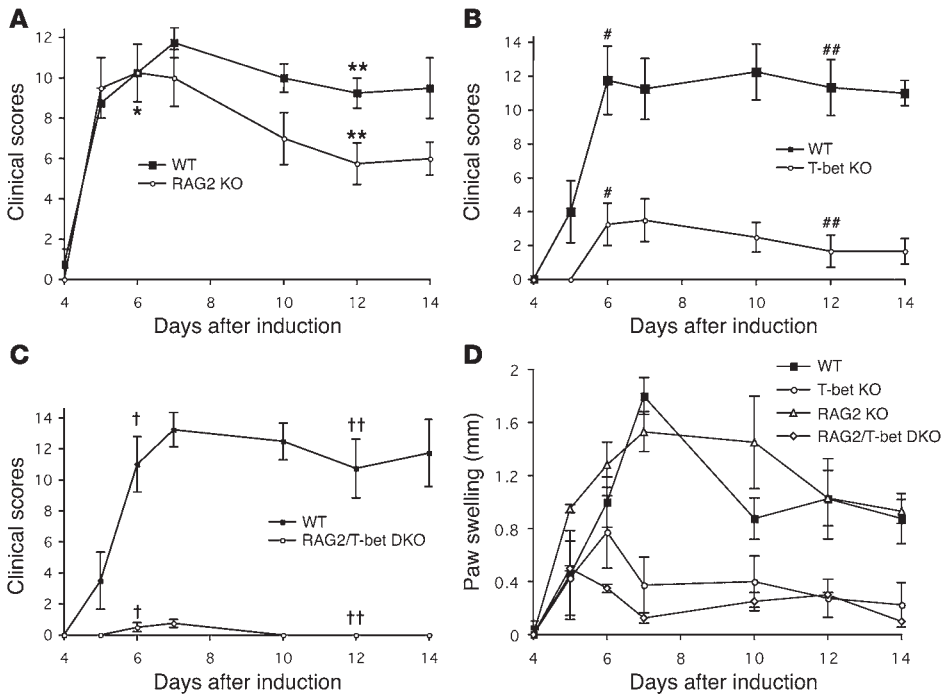

Figure 2
CAIA can be induced in both WT and RAG2$^{-/-}$ mice and is diminished in the absence of T-bet. Seven-week-old BALB/c mice (WT) and (**A**) RAG2$^{-/-}$ mice on BALB/c background (RAG2 KO), (**B**) T-bet$^{-/-}$ mice (T-bet KO), and (**C**) RAG2$^{-/-}$T-bet$^{-/-}$ DKO mice (RAG2/T-bet DKO) were subjected to arthritis induction. Clinical signs of inflammation (**A**–**C**) and paw swelling (**D**) were scored and plotted at different time points as indicated. The values are expressed as mean ± SEM. Each group consists of 4 mice. The results shown are representative of 3 to 5 separate experiments. Unpaired Student's *t* tests were performed on days 6 and 12 after arthritis induction. $^{*}P > 0.3$; $^{**}P < 0.05$; $^{\#}P < 0.02$; $^{\#\#}P < 0.002$; $^{\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.001$.

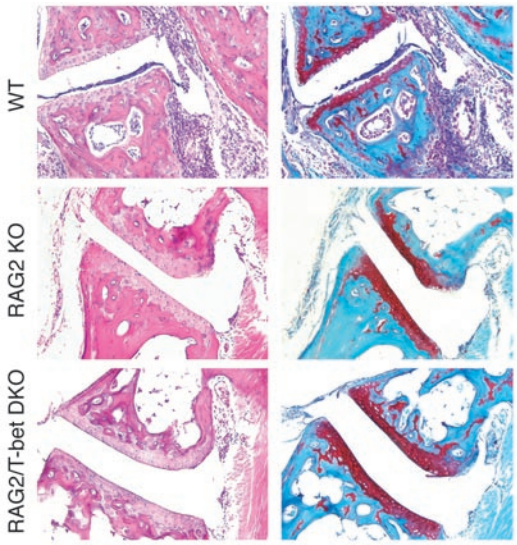

Figure 3
Histologic analysis of CAIA. Tissue sections obtained from paw joints of WT (upper), RAG2$^{-/-}$ (middle), and RAG2$^{-/-}$T-bet$^{-/-}$ DKO (lower) mice on day 14 after arthritis induction were stained with H&E (left panels) and safranin O red (right panels). Inflammatory cell accumulation in synovium and loss of safranin O red staining were abundant in WT but not in RAG2$^{-/-}$ or RAG2$^{-/-}$T-bet$^{-/-}$ DKO mice. The results shown are representative images from 3 to 6 independent experiments with 4 mice per group in each experiment.

ease course (Figure 2B). Since the inflammation scores were significantly decreased both at the initial and late phases (Figure 2B), we concluded that T-bet expression and function in both the innate and adaptive immune system cells control the development of inflammatory arthritis.

A function for T-bet in both innate and adaptive immunity is revealed by the profound resistance of RAG2$^{-/-}$T-bet$^{-/-}$ double-deficient mice to CAIA. To evaluate the role of T-bet in the innate immune system only, we generated mice lacking both RAG2 and T-bet. RAG2$^{-/-}$T-bet$^{-/-}$ double-KO (DKO) mice were essentially completely resistant to the development of arthritis throughout the 14-day evaluation course (Figure 2C). A transient mild inflammation appeared from days 6 to 7 (mean score 0.5–0.75 versus 11–13.25 for WT). Since DKO mice were more resistant to disease than T-bet$^{-/-}$ mice, we concluded that (a) T-bet in the innate immune system influences arthritis development and (b) T-bet–dependent and –independent pathways in the adaptive immune system may also contribute to disease.

We also quantitated paw swelling with calipers (Figure 2D). The results were consistent with the inflammation scores of arthritis (total number of joints involved) at early time points as they were markedly decreased in T-bet$^{-/-}$ but not in RAG2$^{-/-}$ mice. For T-bet$^{-/-}$ and T-bet$^{-/-}$RAG2$^{-/-}$ mice, this decrease was also observed at later time points. Interestingly, although at later time points, the arthritis of RAG2$^{-/-}$ mice as measured by the inflammation score declined as compared with WT, the paw swelling did not appreciably differ from WT.

We evaluated inflammation by H&E staining in paw joints (Figure 3) and articular cartilage damage by safranin O red staining for proteoglycans (Figure 3). In paw joints of WT mice, synovial lining hyperplasia with inflammatory cellular infiltrates, loss of safranin O red staining, and bony erosions was present (Figure 3). In contrast, RAG2$^{-/-}$T-bet$^{-/-}$ DKO (Figure 3) mice had no inflammatory infiltrates and had no signs of cartilage damage. Interestingly, RAG2$^{-/-}$ mice also had reduced inflammatory infiltrates and cartilage damage, consistent with decreased clinical score indicating attenuated arthritis at day 14, the time at which joints were harvested. Thus, although the innate immune response is essential, the adaptive immune system plays a role in inflammation and cartilage damage at later time points.

Further, the absence of T-bet in the innate immune system, coupled to the absence of adaptive immunity, leads to abrogation of inflammatory arthritis in this model.

Transfer of WT DCs into T-bet$^{-/-}$ mice restores susceptibility to inflammatory arthritis. Macrophages, DCs, and mast cells have all been considered relevant components of the innate immune system in the initiation of inflammatory arthritis (2, 7, 28). Preliminary attempts to quantitate the inflammatory infiltrates by FACS analysis in knee synovial tissue harvested at day 6 after antibody injection revealed approximately equal total cell number and percentage of specific cell types (T cells, B cells, DCs, macrophages, and mast cells) between WT and T-bet$^{-/-}$ mice (not shown). We wished to identify the arthritis-inducing T-bet–expressing innate immune cell. T-bet is not expressed in macrophages (21). Although

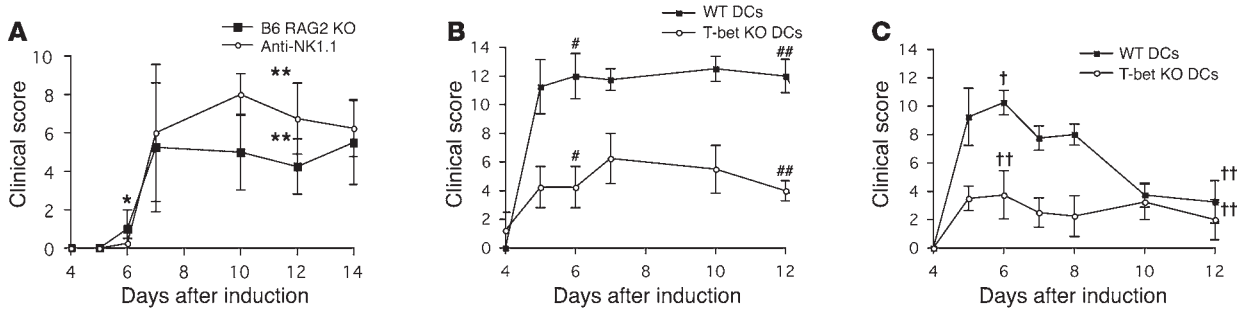

Figure 4
Adoptive transfer of WT but not T-bet KO DCs restores inflammatory arthritis. Clinical signs of inflammation were scored and plotted at different time points as indicated. Individual cell components of the innate immune system were evaluated for their role in the development of arthritis. (**A**) RAG2$^{-/-}$ on C57BL/6 background (B6 RAG2 KO) and the same strain of mice depleted of NK cells in vivo by repeated injection of anti-NK1.1 (anti-NK1.1). (**B**) T-bet KO mice and (**C**) RAG2$^{-/-}$T-bet$^{-/-}$ DKO mice received 0.3 × 10^6 purified WT or T-bet KO splenic DCs. Unpaired Student's *t* tests were performed on days 6 and 12 after arthritis induction. $^*P > 0.5$; $^{**}P > 0.3$; $^{\#}P < 0.02$; $^{\#\#}P < 0.001$; $^{\dagger}P < 0.003$; $^{\dagger\dagger}P > 0.2$.

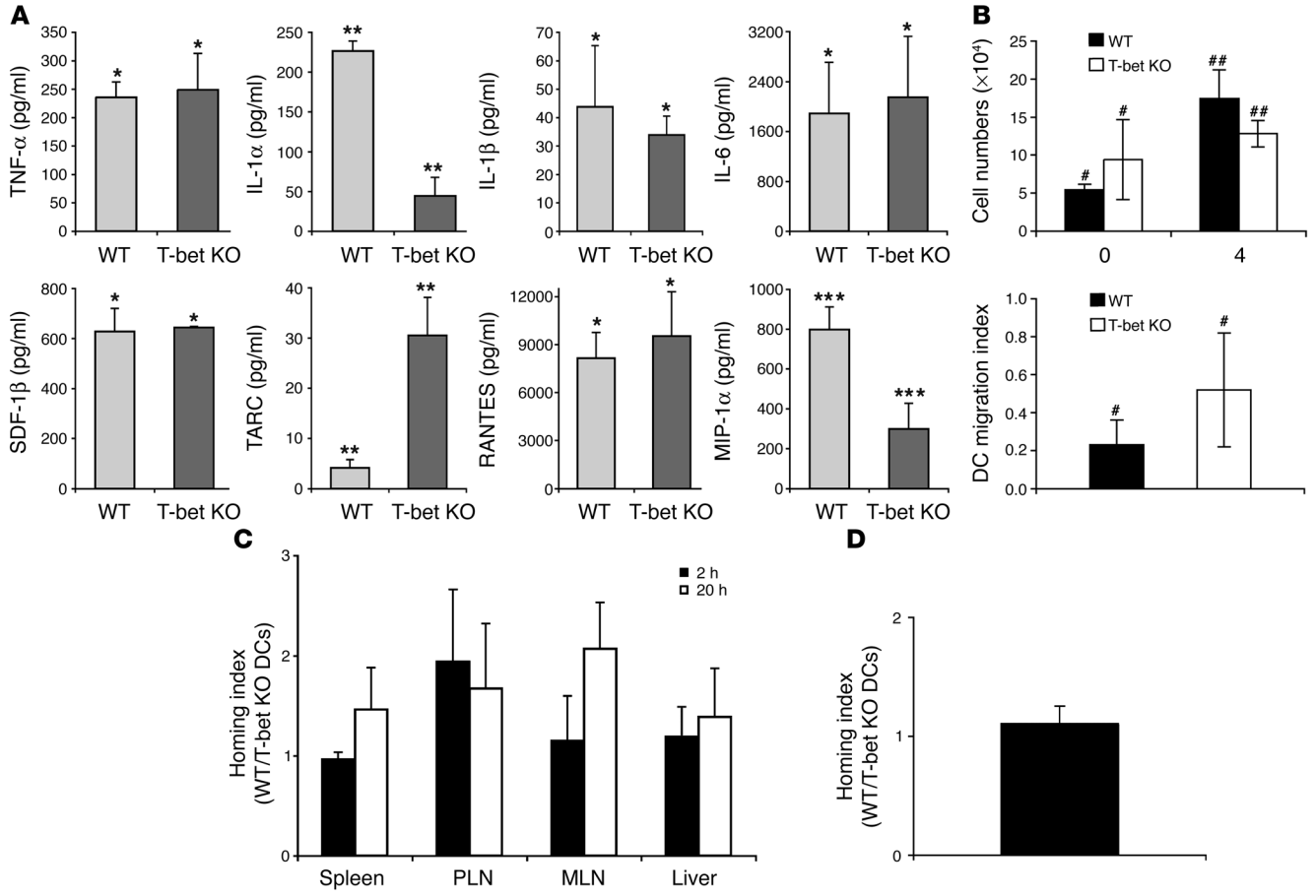

Figure 5

T-bet controls the production of inflammatory cytokines and chemokines in DCs but does not regulate DC migration and life span in vivo. (**A**) Cytokine and chemokine levels in DC culture supernatants were measured with SearchLight high dynamic range imaging and analysis system. Culture supernatants were collected from T-bet$^{-/-}$ or WT DCs after in vitro culture with LPS for 20 hours. Results are from 3 independent experiments with DCs pooled from 8 mice per group in each experiment. SDF-1β, stromal cell–derived factor-1β. (**B**) CD11c^{+} DC counts in knee joint synovium of WT and T-bet$^{-/-}$ mice at days 0 and 4 after arthritis induction (top panel). On day 4 after arthritis induction, ratios of CD11c^{+} DCs in popliteal LNs and knee joint synovium were calculated as migration index in WT and T-bet$^{-/-}$ mice (bottom panel). $^{*}P > 0.05$; $^{**}P < 0.005$; $^{***}P < 0.03$; $^{\#}P > 0.05$; $^{\#\#}P > 0.05$. (**C**) Homing ratio (WT/T-bet KO) measurement of i.v.-injected DCs in WT and T-bet KO mice. PLN, peripheral LNs; MLN, mesenteric LNs. Results are from 9 independent experiments. (**D**) Homing ratio (WT/T-bet KO) of footpad-injected DCs in popliteal LNs at 20 hours after injection. Results are from 6 independent experiments.

T-bet is important in NK cells (22), CAIA-induced arthritis was not diminished in RAG2$^{-/-}$ common gamma chain–deficient mice (not shown) or mice depleted of NK cells in vivo by anti-NK1.1 mAbs (Figure 4A). Preliminary experiments with a serum transfer arthritis model mediated by mast cells (7) revealed modest protection from arthritis in T-bet$^{-/-}$ mice, indicating a possible but restricted arthritogenic function for T-bet in mast cells.

DCs serve as a link between innate and adaptive immunity in the setting of inflammation. To evaluate the role of T-bet^{+} in DCs, we adoptively transferred 0.3×10^{6} purified splenic WT or T-bet$^{-/-}$ DCs into T-bet$^{-/-}$ hosts on day 1. Transfer of T-bet$^{-/-}$ DCs resulted in only minimal levels of arthritis that resembled that observed in T-bet$^{-/-}$ mice injected with the CII mAbs cocktail/LPS directly. Remarkably, adoptive transfer of WT DCs into T-bet$^{-/-}$ hosts restored inflammatory arthritis (Figure 4B). We also transferred WT DCs into DKO mice to interrogate the role of the adaptive immune system in disease induction and maintenance. Transfer of WT DCs restored inflammatory arthritis at early time points as above. However, disease activity was not maintained beyond day 7 (Figure 4C). We concluded that the expression of T-bet in DCs is required for the development of inflammatory arthritis but that cells in the adaptive immune system are required for disease maintenance.

T-bet regulates production of select proinflammatory cytokines and chemokines in DCs. DCs are an important source of inflammatory cytokines and chemokines, pivotal both to elicit local inflammation and sensitize the adaptive immune system. We measured cytokine and chemokine production from T-bet$^{-/-}$ DCs stimulated with LPS in vitro. T-bet$^{-/-}$ DCs produced significantly less IL-1α and chemokine macrophage inflammatory protein-1α (MIP-1α) but produced higher amounts of thymus- and activation-related chemokine (TARC), a ligand for the Th2-specific chemokine receptor CCR4 (Figure 5A). No significant differences in the production of other inflammatory cytokines, IL-1β, IL-6, TNF-α, IFN-γ, and RANTES, were noted (Figure 5A and not shown), consistent with our preliminary data showing lack of T-bet involvement in an IL-1

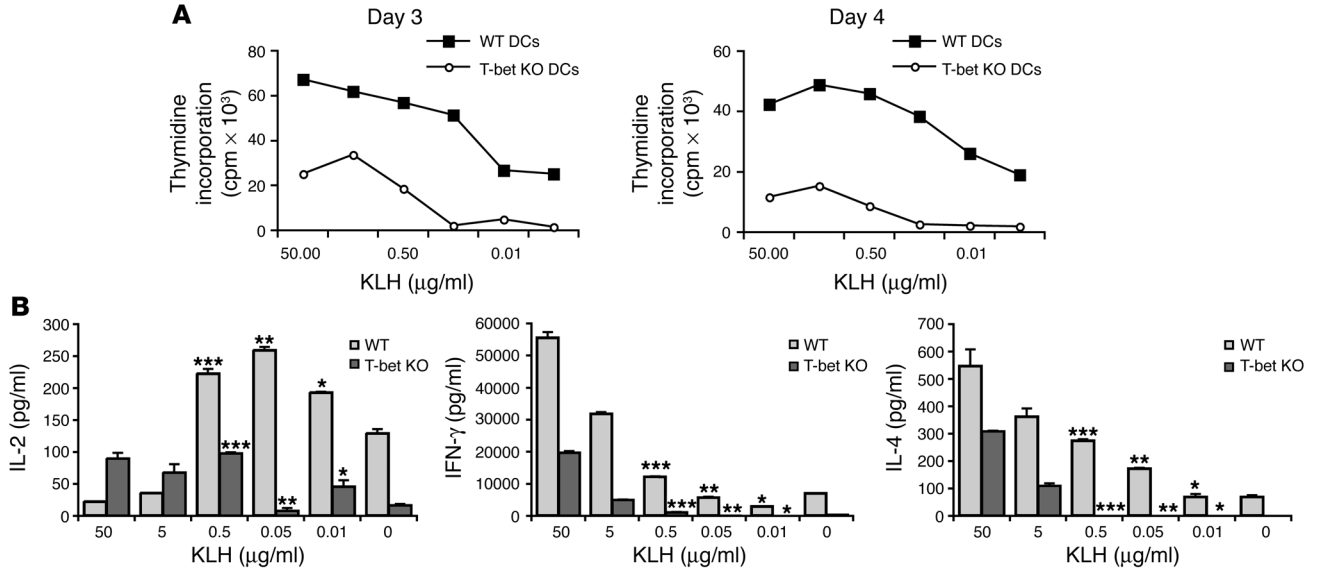

Figure 6
T-bet$^{-/-}$ DCs are defective in activating T cells in vivo. (**A**) LN cell proliferation to KLH on day 3 of popliteal LN cells harvested from WT mice primed with KLH-pulsed T-bet$^{-/-}$ or WT DCs 3 and 4 days earlier. The results shown are representative of 3 independent experiments. (**B**) Cytokine production from LN cells primed with KLH-loaded WT and T-bet KO DCs and cultured in the presence of various concentrations (from 0.01 to 50 μg/ml) of KLH at the time points indicated. *P < 0.05; **P < 0.01; ***P < 0.05.

receptor antagonist–driven (IL-1Ra–driven) model that is dependent on IL-6 and TNF-α (29). It is unlikely that any 1 factor will account for the function of the DCs; indeed, we have established that MIP-1α KO mice are not protected from CAIA (not shown).

T-bet does not regulate the distribution, migration, or lifespan of transferred DCs in vivo. In addition to inflammatory mediator production, DCs migrate to local tissues and home to draining LNs, where they mature. While the effector function of T-bet$^{-/-}$ DCs was compromised, we found no evidence for impaired DC migration. There were equivalent numbers of T-bet$^{-/-}$ and WT CD11c^{+} DCs in inflamed joints (Figure 5B) and draining popliteal LNs (Figure 5B) at the onset of inflammation. We examined the integrity of DC migration both from blood to draining LNs and from tissue to draining LNs using competitive in vivo migration assays and found no impairment in the absence of T-bet (Figure 5, C and D). Consistent with these results, we found normal expression of CCR1, CCR6, CCR7, and CXCR4 receptors in T-bet$^{-/-}$ DCs (not shown). We concluded that there is no impairment in DC migration in vivo either at the steady state or in the setting of inflammatory arthritis in the absence of T-bet. These experiments also rule out differences in apoptosis and life span since they utilized competitive migration and showed that the transferred WT and T-bet KO DCs behaved similarly.

Impaired LN cell activation by T-bet$^{-/-}$ DCs. Activation of immature DCs in peripheral tissues induces their maturation and migration to LNs, where they prime naive T cells. To test the capacity of T-bet$^{-/-}$ DCs to prime naive Th cells in vivo, we performed adoptive transfer to WT recipients of antigen-loaded (keyhole limpet hemocyanin–loaded [KLH-loaded]) maturing WT or T-bet$^{-/-}$ DCs injected in the footpad to mimic the route of cell migration during arthritis development. After in vivo priming for 3 or 4 days, draining popliteal LNs were removed and restimulated in vitro with varying doses of KLH for 3 days and proliferation and cytokine production assayed. Proliferation of cells from popliteal LNs of WT mice that had received KLH-pulsed T-bet$^{-/-}$ DCs was markedly reduced compared with LN cells from mice primed with KLH-pulsed WT DCs (Figure 6A). Further, this reduced proliferation was accompanied by decreased levels of multiple cytokines (IL-2, IL-4, IFN-γ) (Figure 6B).

Discussion

Here we report an essential role for the transcription factor T-bet in the development of an inflammatory arthritis controlled by both the innate and adaptive immune systems. Given the prominent role of T-bet in directing Th1 differentiation, a contribution of T-bet to adaptive immune system–mediated autoimmunity was expected. Remarkably, however, we found that the primary site for T-bet activity in controlling inflammation resided in the DC population. Thus, adoptive transfer of WT DCs but not T-bet$^{-/-}$ DCs into the protective environment of a T-bet–deficient host restored inflammatory arthritis. Our studies show that both proinflammatory mediators secreted by DCs and the ability of DCs to prime naive T cells was compromised in the absence of T-bet.

Despite accumulating evidence that DCs play a critical role in the pathogenesis of RA, the exact mechanism has not been well elucidated (30). The presentation of arthrogenic antigen to T cells and the promotion of B cell activation and immunoglobulin class switching in rheumatoid synovial tissue by DCs may be the main driver of memory T cell– and B cell–mediated responses in RA. These events can then influence macrophage and synoviocyte responses (31). T-bet may influence the above events at multiple checkpoints due to the failure of T-bet$^{-/-}$ DCs (a) to produce certain inflammatory cytokines, which leads to an altered cytokine milieu both locally in the RA joint and systemically, and (b) to present antigen to naive T cells. Our data demonstrate that DCs and a specific transcription factor, T-bet, in DCs can control the

initiation and course of inflammatory arthritis. Thus, we have provided not only the cell type involved but also the gene in the DCs that regulates its function in inflammatory arthritis. Further, we have identified 3 candidate factors, MIP-1α, IL-1α, and TARC, that are downstream of T-bet. The current, most advanced therapy for inflammatory arthritis blocks the biologic function of various mediators, but our experiments identify a gene that regulates a cluster of those mediators.

Mouse models of any human disease have to be examined with a suitable degree of skepticism. Nevertheless, they have proved to be exceptionally valuable tools whose investigation has led to many insights about disease pathogenesis. Further, they are essential since human experimentation is, of course, not possible. Individual models may reflect unique aspects and features of the human disease, and this will be true in mouse RA models. No single animal model can reflect all aspects of human disease. Thus, the K/BXN serum transfer model (7) suggests a role for mast cells — and there are mast cells in human rheumatoid synovium — but it is not possible to establish their importance directly in the human disease. This model has provided the field with a new focus on the mast cell, and novel compounds that target mast cell function are being developed and will be tested in human clinical trials. We have examined the function of T-bet in the K/BXN serum transfer model. In preliminary experiments, T-bet–deficient mice developed robust arthritis that was comparable to the WT controls when the amount of serum administered was according to published reports (7). However, at lower doses of serum, modest protection from arthritis was observed in T-bet–deficient mice, suggesting that T-bet may play a role in mast cell function in inflammatory arthritis (J. Wang and L.H. Glimcher, unpublished data). Additional careful titrations of pathogenic sera need to be performed.

Although the relevance of any mouse model of inflammatory arthritis to human RA is uncertain, there are a number of features of the CAIA model that resemble the human disease. Human RA appears to develop in 2 stages in which an initial response induced by foreign antigens (most likely infectious organisms) activates innate immunity accompanied by proinflammatory cytokine and chemokine secretion and subsequently develops into a self-sustaining T cell–driven autoimmune process (2). An increased incidence of infections preceding early RA has been reported recently in a prospective population-based study (32). Our findings indicate that CAIA is also a bipartite disease characterized by an early component mediated through the innate immune system and a later phase influenced by the adaptive immune system. The presence of T-bet expressing $CD3^+$ cells in rheumatoid synovium is consistent with a role for T-bet in late-stage RA.

We also examined the function of T-bet in the IL-1 signaling pathway in the spontaneous arthritis that develops in the absence of the IL-1Ra on the BALB/c background (29, 33). We crossed the T-bet–deficient strain onto the IL-1Ra–deficient strain and monitored arthritis in the DKO. Mice that lacked IL-1Ra developed polyarticular arthritis as expected, but interestingly, the absence of T-bet did not reduce or alter the disease course (J. Wang and L.H. Glimcher, unpublished data). Thus, mice that lacked both IL-1Ra and T-bet developed polyarticular arthritis similar to the IL-1Ra single–deficient strain. These data indicate that, at least in this model, T-bet does not regulate IL-1 pathways in controlling the development of arthritis.

Macrophages, DCs, and mast cells have all been considered relevant components of the innate immune system in the initiation of inflammatory arthritis (2, 7, 28). Antigen-pulsed DCs induce experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis (34), and play a pivotal role in the development of inflammatory arthritis (35). DCs pulsed with type II collagen peptides were sufficient for arthritis induction through effects on both innate and adaptive immunity. The importance of DCs in animal models of inflammatory arthritis has also been demonstrated by the effectiveness of genetically modified DCs expressing IL-4 in preventing arthritis development (36, 37). Our experiments using the CAIA model provide evidence for the involvement of the DC in inflammatory arthritis.

The 2 major functions of DCs in inflammation — to serve as effector cells by virtue of the production of proinflammatory mediators and to act as antigen-presenting cells to prime naive T cells (38–40) — both require T-bet. In contrast, DC maturation (21) and migration are T-bet independent. We would suggest that the reduced production of IL-1α and MIP-1α likely accounts for or contributes to the diminished early phase inflammation in $T\text{-}bet^{-/-}$ and $T\text{-}bet^{-/-}$ $RAG2^{-/-}$ DKO mice. MIP-1α, a major inducible proinflammatory chemokine and a Th1 cell chemoattractant (39), is elevated in RA synovial fluids consistent with the expression of its target chemokine receptor CCR5 in synovium (41). Although the total number of T cells in inflamed joints was not reduced in the absence of T-bet (not shown), the simultaneous decrease in MIP-1α but increase in the Th2-recruiting chemokine TARC raise the interesting possibility of selective recruitment of Th2 but not Th1 cells to the site of inflammation. Further, DC-derived IL-1, whose secretion is also reduced in the absence of T-bet, is a known enhancer of T cell clustering and activation (38, 42), and local IL-1α administration promotes Th1 differentiation (43). Whether MIP-1α and IL-1α are direct or indirect targets of T-bet remains to be determined.

Genetic background and environmental triggers, such as bacterial or viral infections, likely cooperate to precipitate the onset of disease. Continuing exposure to environmental triggers may explain the ongoing involvement of both the innate and adaptive immune systems. In light of the central position occupied by DCs as the cells responsible for priming naive T cells, they serve as a link between innate and adaptive immunity in the setting of inflammation. Most current therapies target only 1 component of the immune system. The success of TNF-α blockade compared with the failure of anti–T cell blockade in past clinical trials underscores the importance of targeting both the innate and adaptive immune systems. T-bet provides a novel target to control inflammation at multiple checkpoints in both the innate and adaptive immune systems.

Methods

Immunohistochemical staining of synovial tissue from RA patients. Case material was obtained from Brigham and Women's Hospital in accordance with institutional policies. Immunostaining for T-bet was performed on formalin-fixed paraffin-embedded tissue sections following microwave antigen retrieval in 1 μM EDTA, pH 8.0, with a previously described antihuman T-bet monoclonal antibody (4B10) using a standard indirect avidin-biotin horseradish peroxidase method and diaminobenzidine color development, as previously described (44). T-bet staining was compared with that of mouse IgG isotype control antibody diluted to identical protein concentration for all cases studied to confirm staining specificity. Staining for the T cell marker CD3, B cell marker CD20, histocyte marker CD68/Kp-1, and S100, which marks interdigitating DCs, was performed as described (44).

Mice. The generation of T-bet$^{-/-}$ mice has been described (10). T-bet$^{-/-}$ mice were backcrossed 8 generations onto the BALB/c (H-2^{d}) or C57BL/6 (B6) (H-2^{b}) background. RAG2$^{-/-}$ mice (BALB/c or B6 background) were obtained from the Jackson Laboratory. T-bet$^{-/-}$ x RAG2$^{-/-}$ mice were generated by crossing T-bet$^{-/-}$ and RAG2$^{-/-}$ mice on a BALB/c background. In all experiments, we rigorously controlled for genetic background. All mice were housed in a pathogen-free facility at the Harvard School of Public Health, and all animal studies were performed according to institutional and NIH guidelines for animal use and care. Animal experimentation was approved by the Standing Committee on Animals at the Harvard Medical School.

CAIA in mice. The arthritogenic mAb cocktail containing equal amounts of 4 mAbs (3 IgG2a, 1 IgG2b) to type II collagen (anti-CII) and LPS (*Escherichia coli* 0111:B4) (Chemicon International) was used to induce arthritis as described by Terato et al. (25) with modifications. Briefly, 7- to 8-week-old female mice were injected i.v. with 1 mg (BALB/c background) or 2 mg (B6 background) of the anti-CII mAb cocktail. Three days later, mice were injected i.p. with 25 μg (BALB/c background) or 50 μg (B6 background) of LPS. Inflammation of the paws and knee joints was graded as described (25): 0, normal; 1, swelling of 1 digit or the knee joint; 2, swelling of 2 or more digits or 1 digit plus the knee joint; 3, swelling of the entire paw; and 4, swelling of the entire paw plus the knee joint. The cumulative score of all 4 extremities of each mouse was used as clinical score to represent overall disease severity. Paw thickness was measured with a pocket thickness gauge (Mitutoyo America Corp.). Paw swelling represents the increment in paw thickness before and after the onset of arthritis.

Histological assessment of mouse arthritis. Mice were euthanized by CO_2 inhalation. The hind paws and knees were collected and fixed with 4% neutral buffered paraformaldehyde (Electron Microscopy Sciences) for 4 days, decalcified in 5% formic acid (Sigma-Aldrich) for 2 weeks, and embedded in paraffin. Sections (5 μm) were stained with H&E and with safranin O red (Sigma-Aldrich).

Splenic DC preparation and stimulation. Splenic DCs were isolated by collagenase treatment and enriched by anti-CD11c magnetic beads (Miltenyi Biotec) (21) and FACS with Abs to I-A^{d} and CD11c. Postsorting CD11c^{high}/MHC II^{+} cell purity was greater than 97%. Purified DCs (1×10^{6}/ml) were cultured in 6-well plates and stimulated with LPS (Sigma-Aldrich) at 100 ng/ml.

DC isolation from mouse joints and LNs. At various times after arthritis induction, mice were sacrificed, knee joints dissected with scissors to expose synovia, and joints washed 3 times with PBS. Popliteal draining LNs were passed through a 70- mm cell strainer and cells digested with collagenase D (1 mg/ml; Roche Applied Science) for 60 minutes at 37°C. The eluted synovial cells and single LN cells were stained with PE-labeled anti-CD11c antibody and the number of CD11c^{+} cells determined by FACS.

Competitive in vivo DC migration and homing assays. Homing assays were performed as described (45). Briefly, DCs from WT and T-bet$^{-/-}$ mice were fluorescently labeled with TRITC or CFSE (Invitrogen Corp.). These cells were mixed in a ratio of 1:1 and coinjected into the tail vein of recipient WT mice. After 2 hours or 20 hours of the adoptive transfer, recipient mice were sacrificed. Spleens, peripheral LNs, mesenteric LNs, and Peyer patches were harvested and passed through wire mesh. Livers were digested for 30 minutes at 37°C with 0.5% collagenase type 2 (Worthington Biochemical Corp.) before passing through wire mesh. Single-cell suspensions were stained with anti-CD11c mAbs (BD) and analyzed by flow cytometry. The homing index in the organs of interest was calculated as the ratio between the number of CD11c^{+}TRITC^{+} and CD11c^{+}CFSE^{+} cells divided by the ratio of CD11c^{+}TRITC^{+} and CD11c^{+}CFSE^{+} cells in the input population.

Measurement of cytokines and chemokines. Purified DCs were cultured at 1×10^{6}/ml in DMEM buffer containing 10% fetal calf serum plus 100 μg/ml LPS for 20 hours. Cytokines and chemokines were measured in culture supernatants utilizing SearchLight high dynamic range (HDR) imaging and analysis system (Pierce Biotechnology Inc.).

Arthritis induction by adoptive DC transfer. Splenic DCs were purified as described above and injected via tail vein at 0.3×10^{6} per mouse. Two hours after DC transfer, 1 mg of anti-CII mAb cocktail was injected i.v. followed 48 hours later with 25 μg of LPS injected i.p.

In vivo NK cell depletion. To deplete NK cells in vivo, mice were injected i.p. daily with 0.5 ml of PBS containing 250 μg of anti-NK 1.1 as described. After 5 days, depletion of NK cells was confirmed by FACS.

LN cell proliferation from mice primed in vivo with adoptively transferred DCs. Purified DCs were resuspended in HL-1 medium (BioWhittaker Inc.) and cultured with KLH (100 μg/ml) in vitro for 2 hours to load antigen, then with LPS (100 ng/ml) for 4 hours to mature the DCs. KLH-pulsed DCs (3.5×10^{5}) from WT or T-bet$^{-/-}$ mice were injected into footpads of WT recipients. After 3 or 4 days, popliteal LN cells were collected and cultured in HL-1 medium in 96-well plates (5×10^{5}/well) with varying concentrations of KLH for 3 days. Twelve hours prior to the end of the culture period, cells were pulsed with [^{3}H]-thymidine (1 μCi/well; NEN Life Science Products), harvested onto glass fiber filters using the Filtermate Harvester (Packard Bioscience Co.), and counted in a microplate scintillation and luminescence counter, TopCount, NTX (Packard Bioscience Co.). Cytokine secretion in the culture supernatants was assessed by ELISA (10).

Statistics. Two-tailed Student's *t* test was used for all comparisons, with a *P* value of < 0.05 set as statistically significant.

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