Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells

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IL-17 is implicated in the pathogenesis of rheumatoid arthritis (RA) and has previously been shown to be induced by tumor necrosis factor (TNF) in vitro. The aim of this study was to assess the impact of TNF inhibition on IL-17 production in collagen-induced arthritis, a model of RA. TNF blockade using TNFR-Fc fusion protein or anti-TNF monoclonal antibody reduced arthritis severity but, unexpectedly, expanded populations of Th1 and Th17 cells, which were shown by adoptive transfer to be pathogenic. Th1 and Th17 cell populations were also expanded in collagen-immunized TNFR $p55^{-/-}$ but not $p75^{-/-}$ mice. The expression of IL-12/IL-23 p40 was up-regulated in lymph nodes (LN) from $p55^{-/-}$ mice, and the expansion of Th1/Th17 cells was abrogated by blockade of p40. Treatment of macrophages with rTNF also inhibited p40 production in vitro. These findings indicate that at least one of the ways in which TNF regulates Th1/Th17 responses in arthritis is by down-regulating the expression of p40. Finally, although TNF blockade increased numbers of Th1 and Th17 cells in LN, it inhibited their accumulation in the joint, thereby providing an explanation for the paradox that anti-TNF therapy ameliorates arthritis despite increasing numbers of pathogenic T cells.

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Rheumatoid arthritis (RA) is a chronic autoimmune disease in which proinflammatory cytokines, such as TNFα, IL-6, and IL-1, play dominant pathological roles. More recently, IL-17 has been suggested to play an important additional role in the induction and maintenance of RA (1, 2). Thus, IL-17 is present in the synovium of RA patients and contributes to the production of IL-6 and MMP-1 in the joint (2, 3), whereas treatment of human macrophages with IL-17 in vitro stimulates the production of TNFα and IL-1β (4). IL-17 can also synergize with TNFα to induce cytokine and chemokine production by synovial fibroblasts and cartilage destruction in vitro and can promote osteoclastogenesis (1, 5, 6).

IL-17 is a proinflammatory cytokine produced predominantly by T helper cells (Th17 cells) and, although there is controversy over the signals required for the differentiation of murine and human Th17 cells, both murine and human $CD4^+$ Th17 T cells require IL-23 for their proliferation and maintenance (7). IL-23 is a heterodimeric protein composed of a p19 subunit and a p40 subunit, whereas IL-12, an important cytokine for Th1 cell differentiation, is formed when the p40 subunit dimerizes with p35 (8).

The role of TNFα in RA is well documented, with TNFα-blocking biologics causing amelioration of clinical symptoms (e.g., pain, joint swelling, and stiffness), laboratory parameters of inflammation (e.g., CRP and ESR), and radiological progression of disease (9, 10). Although TNFα plays a direct pathological role in RA, its contribution to disease pathogenesis is amplified by its ability to promote the expression

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of other proinflammatory cytokines. For example, TNFα has been shown in vitro to drive the production of IL-17 by equipping DC with the ability to differentiate T cells toward a Th17 phenotype (11). On this basis, it would be predicted that TNFα blockade would result in reduced IL-17 expression, and to test this hypothesis in vivo, we investigated the dependence of IL-17 expression on TNFα in collagen-induced arthritis (CIA). Surprisingly, our data show that TNFα is an important negative regulator, not only of IL-17 but also of IFNγ production by T cells. We propose that this forms a part of a negative feedback loop that attempts to limit the intensity and/or duration of Th17 and Th1 responses.

RESULTS AND DISCUSSION

To investigate the effect of blockade of TNFα on the production of IL-17, DBA/1 mice were immunized with bovine type II collagen in CFA. After onset of arthritis, mice were treated with soluble TNFR-Fc for 10 d and the production of IL-17 and IFNγ by LN cells was determined by ELISA. Significantly increased IL-17 and IFNγ production was observed after stimulation of LN cells from TNFR-Fc–treated mice with collagen or anti-CD3 mAb in vitro, and a trend toward enhanced production of these cytokines was observed even in unstimulated LN cells (Fig. 1). As expected, arthritis severity was significantly reduced in TNFR-Fc–treated mice despite the increased IL-17 and IFNγ production (Fig. 1).

We next set out to establish whether the increased IFNγ and IL-17 production in vitro by LN cells after blockade of TNFα was paralleled by increased numbers of Th1 and Th17 cells in vivo. In addition, we compared the effect of TNFα blockade on numbers of Th1/Th17 cells during the T cell expansion phase (days 0–14 after immunization) versus the phase of T cell contraction (days 1–14 after disease onset). For this experiment, we used anti-TNFα mAb (TN3-19.12), which has been characterized extensively in CIA (12). Anti-TNFα mAb treatment lead to a significant expansion of the proportion of $CD4^+IFN\gamma^+$ cells in inguinal LN, irrespective of whether it was administered from days 0–14 after immunization or days 1–14 after disease onset (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072707/DC1). There was a trend toward increased numbers of $CD4^+IL\text{-}17^+$ cells during the expansion phase and a significant increase in $CD4^+IL\text{-}17^+$ cells during the contraction phase.

It was concluded that early TNFα blockade led to a significant expansion of Th1 cells, whereas late TNFα blockade led to a significant expansion of both Th1 and Th17 cells. The coordinate expansion of both Th17 and Th1 cells in vivo after blockade of TNFα is surprising and challenges the assumption that the differentiation of these two subsets is mutually antagonistic (13, 14). Less than 0.2% of $CD4^+$ cells from control or anti-TNFα–treated mice were double positive for IFNγ and IL-17 (Fig. S1).

Thus far, the data suggest that signaling via the TNF receptor inhibits Th1/Th17 responses. To confirm this finding, and to establish which of the two TNF receptors (p55 or p75) is responsible for delivering the inhibitory signal, Th1/Th17 responses were assessed in $p55^{-/-}$, $p75^{-/-}$, and WT mice immunized 14 d previously with type II collagen in CFA. This time point was chosen because maximal T cell responses are normally detected around this time.

The results show conclusively that inhibition of Th1/Th17 responses occurs via the p55 and not the p75 TNF receptor. Thus, the production of IL-17 and IFNγ was dramatically higher in collagen or anti-CD3 mAb–stimulated LN cell cultures from $p55^{-/-}$ mice compared with either WT or $p75^{-/-}$ mice (Fig. 2 A). Further analysis by flow cytometry confirmed

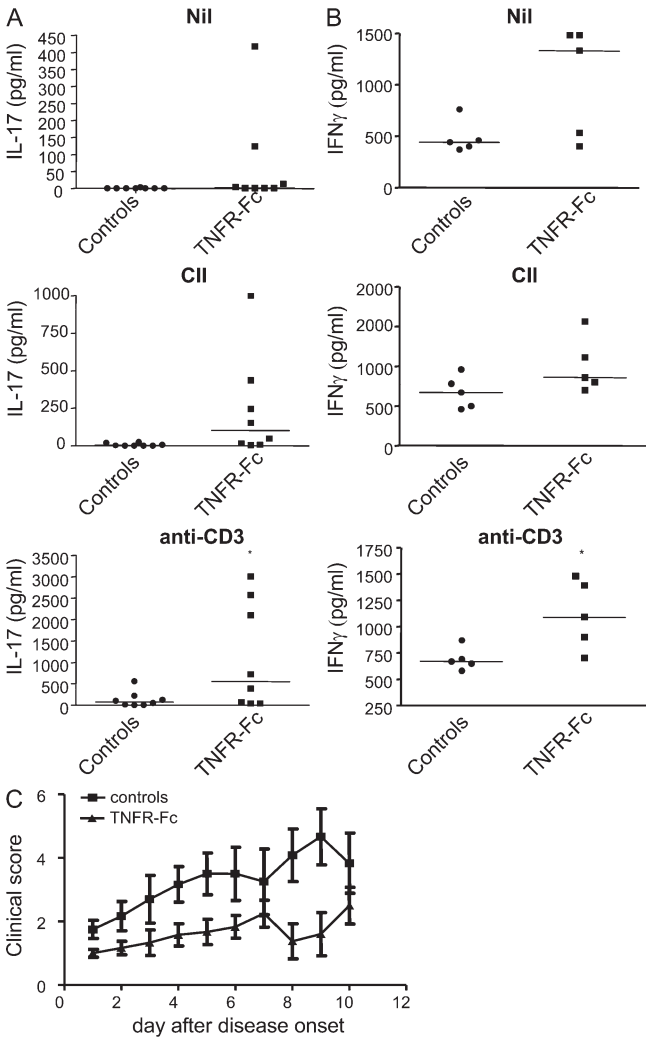

Figure 1. Increased IL-17 and IFNγ production in CIA after blockade of TNFα. DBA/1 mice with CIA were treated with TNFR-Fc or isotype control mAb (100 µg/mouse on alternate days) from the time of disease onset. (A and B) LN cells were taken 10 d after disease onset and levels of IL-17 (A) and IFNγ (B) were determined by ELISA in the supernatants without further stimulation (Nil) or after stimulation with type II collagen (CII) or anti-CD3 mAb (CD3). Data show individual mice ($n = 8$; *, $P < 0.05$). (C) Clinical scores were assessed over the 10-d period in TNFR-Fc–treated and control mice. The data are representative of at least three experiments. Error bars show SEM.

that the proportion of $CD4^+$ T cells producing IL-17 and IFNγ in the LN of $p55^{-/-}$ mice was significantly greater than in those from WT or $p75^{-/-}$ mice (Fig. 2 B). However, increased IFNγ and IL-17 responses were not observed in anti-CD3–stimulated LN cells from nonimmunized $p55^{-/-}$ mice (Fig. 2, A and B), indicating that the T cells were not skewed toward Th1/Th17 responses before immunization. The percentage of $CD4^+$ cells in immunized WT mice coexpressing IFNγ and IL-17 was low (~0.1%) and was not altered in $p55^{-/-}$ or $p75^{-/-}$ mice.

Despite the increase in Th1/Th17 responses observed in immunized $p55^{-/-}$ mice, the proliferative responses of T cells from $p55^{-/-}$, $p75^{-/-}$, and WT mice did not vary significantly in response to collagen or anti-CD3 mAb stimulation (Fig. 2 C), and the percentages of $CD4^+$ T cells in the LN of WT and $p55^{-/-}$ mice were comparable, although slightly reduced in $p75^{-/-}$ mice (Fig. 2 C). The percentage of regulatory $CD4^+Foxp3^+$ T cells remained unchanged in the $p55^{-/-}$ and $p75^{-/-}$ mice when compared with WT mice. IL-4 and IL-5 were undetectable in immunized WT, $p55^{-/-}$, and $p75^{-/-}$ mice, which was attributed to the strongly Th1/Th17-skewing properties of CFA. The fact that expanded populations of Th1 and/or Th17 cells were observed in inguinal LN of $p55^{-/-}$ and anti-TNF–treated mice before the onset of arthritis indicates that there was not simply a redistribution of cells away from the joint to the LN.

We next sought to identify the mechanism by which TNFα reduces Th17 and Th1 cell activity. IL-12 and IL-23 share a common subunit, p40. Dimerization of p40 with p35 forms IL-12, which is involved in the differentiation of Th1 cells, whereas dimerization of p40 with p19 forms IL-23, which has an important role in the generation and/or survival of Th17 cells. Hence, one possible explanation for our findings is that TNFα conditions myeloid cells toward reduced p40 expression, and we set out to address this question using thioglycolate-elicited macrophages stimulated with LPS in vitro. Pretreatment of macrophages before LPS stimulation with 30 or 100 ng/ml of TNFα produced a dose-dependent reduction of p40 upon subsequent stimulation with LPS (Fig. 3). The maximum inhibition of p40 production by LPS-stimulated macrophages was ~50%, and the failure to obtain greater suppression was attributed to the fact that LPS alone would inevitably produce significant quantities of TNFα. TNFα pretreatment also suppressed IL-6 production at 100 ng/ml, but not at 30 ng/ml, but had no effect on IL-1β production at either dose (Fig. 3). This shows that the effect of TNFα on cytokine production was selective and did not cause global suppression of cellular activity.

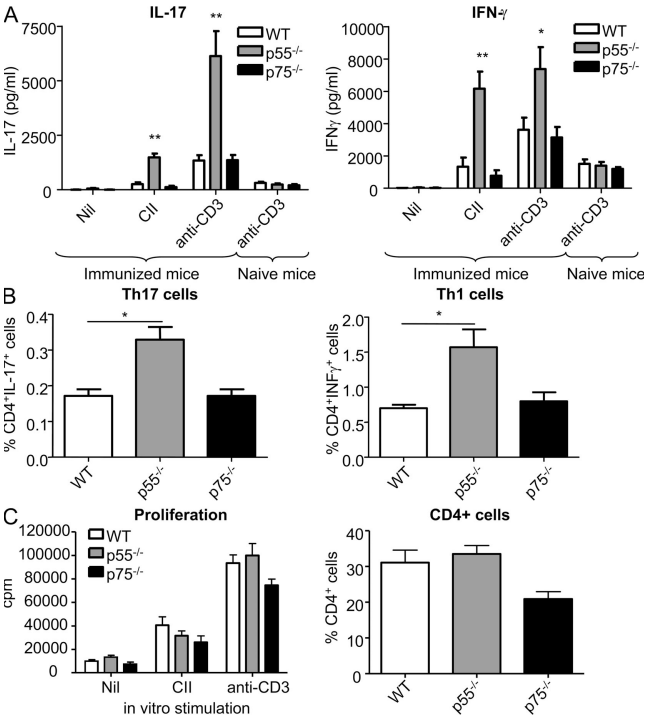

Figure 2. Amplification of Th17 and Th1 cell activity in p55 $TNFR^{-/-}$ mice. LN cells from WT, p55 $TNFR^{-/-}$, and p75 $TNFR^{-/-}$ mice were taken 14 d after immunization with type II collagen in CFA. (A) LN cells were either unstimulated or stimulated with collagen or with anti-CD3 mAb, and the level of proliferation was determined by $[^3H]$thymidine incorporation. The percentage of $CD4^+$ T cells in the LN was determined by flow cytometry on day 14 after immunization. (B) Levels of IL-17 and IFNγ were determined by ELISA. (C) The proportion of $CD4^+$ cells in the LN producing IL-17 and IFNγ were determined by flow cytometry. Histograms show mean ± SEM ($n = 8$). *, $P < 0.05$; **, $P < 0.01$. Data are representative of two experiments.

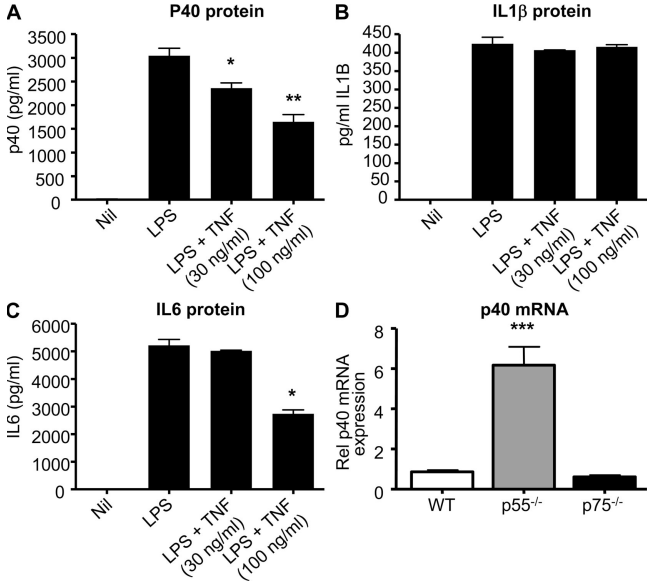

Figure 3. TNFα inhibits expression of IL-12/IL-23 p40. Thioglycolate-elicited macrophages were cultured in the presence or absence of 30 or 100 ng/ml TNFα for 8 h and then stimulated for a further 18 h with 1 ng/ml LPS. (A–C) Levels of p40, IL-1β, and IL-6 protein were determined in the culture supernatants by ELISA. (D) Relative levels of p40 mRNA from WT, p55 $TNFR^{-/-}$, and p75 $TNFR^{-/-}$ LN cells 14 d after immunization were determined by real time PCR. Histograms show mean ± SEM ($n = 4$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Next, we addressed the question of whether p40 expression was elevated in vivo in the absence of signaling via the p55 TNFR. To quantify p40 expression without any form of manipulation in vitro, mRNA was extracted from LN cells immediately postmortem and analyzed by real-time PCR. LN cells from immunized $p55^{-/-}$ mice were found to express sixfold greater levels of p40 mRNA compared with immunized WT and $p75^{-/-}$ mice (Fig. 3). This confirms the ability of TNFα to suppress p40 expression in vivo.

We then questioned whether inhibition of IL-12/IL-23 p40 activity in $p55^{-/-}$ mice would result in a reduction of IFNγ/IL-17 production and reduced numbers of Th1/Th17 cells. Treatment with a blocking anti–mouse p40 mAb in $p55^{-/-}$ mice from the day of immunization to day 14 after immunization partially or completely abrogated both the increase in IL-17 and IFNγ production and the expansion of $CD4^+IL\text{-}17^+$ and $CD4^+IFN\gamma^+$ cells (Fig. 4). These findings strongly suggest that at least one of the mechanisms by which TNFα influences the development and/or survival of Th1 and Th17 cells is by inhibition of IL-12/IL-23 p40 expression, although other mechanisms may also be involved.

These findings raise the important question of why TNFα blockade is effective in reducing disease activity despite increasing numbers of Th1 and Th17 cells. To address this question, we used an adoptive transfer system established previously (15) to confirm that the expanded Th1/Th17 cells were potentially pathogenic. Spleen and LN cells from arthritic mice treated for 10–14 d with anti-TNFα mAb or control Ab were pooled and injected into CB-17 SCID mice (5×10^7 cells/mouse or 10^7 cells/mouse). The SCID recipients were also injected i.p. with 100 μg of type II collagen without adjuvant, which is required for the successful transfer of arthritis (15). The proportion of LN cells to spleen cells was ∼1:10 and was identical in both anti-TNFα–treated and control groups.

Transfer of 5×10^7 cells from either anti-TNFα–treated or control mice led to efficient transfer of arthritis, although onset of arthritis was much earlier when donor cells were derived from anti-TNFα–treated mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072707/DC1). However, when the numbers of donor cells were reduced to 10^7 cells, there was a dramatic difference between the two groups, with the cells from the anti-TNFα–treated donors showing vastly superior transfer of arthritis (Fig. S2). Remarkably, when the recipient SCID mice were treated with anti-TNFα mAb from the time of cell transfer, the development of arthritis was largely abrogated (Fig. S2). This experiment shows conclusively that anti-TNFα therapy expands the population of pathogenic T cells. However, the pathogenicity of these donor cells is largely blocked by anti-TNFα treatment of the recipients.

The enhanced capacity of lymphoid cells from anti-TNFα–treated mice to adoptively transfer arthritis to SCID mice demonstrates that the expansion of Th1/Th17 cells may have pathological consequences when the anti-TNFα "brake" is removed. However, in the presence of TNFα-neutralizing antibodies, the pathogenic potential of these T cells is effectively neutralized.

One of the mechanisms by which anti-TNFα therapy is known to act in human RA is by preventing inflammatory cell infiltration into the joint (16). On this basis, we hypothesized that the mechanism by which TNFα blockade is able to reduce disease activity while expanding populations of Th1 and Th17 cells is by preventing the migration of T cells into the joint. To validate this hypothesis, arthritic DBA/1 mice were treated for 10–14 d with anti-TNFα mAb or control Ab. The mice were then killed and cells harvested from paws and inguinal LN for subsequent analysis by flow cytometry.

Anti-TNFα treatment of established arthritis reduced total numbers of $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}17^+$ cells in joints

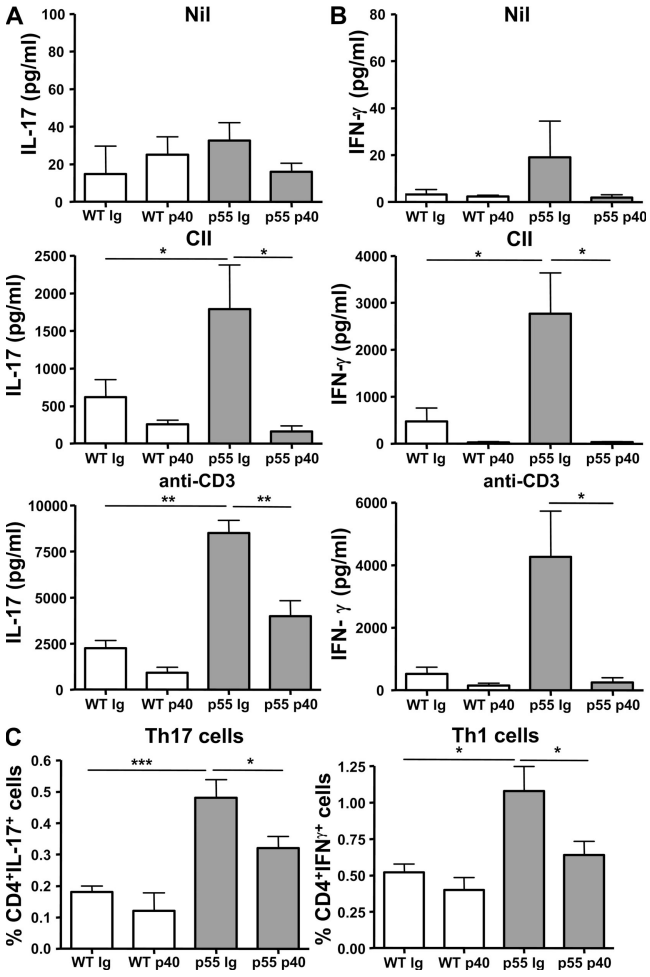

Figure 4. Blockade of IL-12/IL-23 blocks the expansion of Th1/Th17 cells. (A–C) LN cells from immunized WT (white bars) or p55 $TNFR^{-/-}$ (gray bars) mice treated with control Ig (Ig) or rat anti–mouse p40 Ab (p40) were either unstimulated or stimulated with collagen or anti-CD3 mAb. Levels of IL-17 (A) and IFNγ (B) were determined by ELISA, and the proportion of $CD4^+$ cells in the LN producing IL-17 and IFNγ were determined by flow cytometry (C). Histograms show mean ± SEM ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of two experiments.

by 63% and 67%, respectively, despite being present in increased numbers in inguinal LN (Fig. 5). It was concluded that one of the mechanisms of action of anti-TNFα therapy is to inhibit immigration of pathogenic T cells to the joint or prevent their emigration from LN. It was beyond the scope of the present study to investigate the mechanisms by which anti-TNFα prevents T cell accumulation in the joint, but they are likely to include reduced chemokine and adhesion molecule expression in the joint, as has been proposed for human RA (16, 17).

The key finding to emerge from this study is that, in addition to its proinflammatory role, TNFα is also responsible for dampening down Th1 and Th17 responses within the context of autoimmune arthritis. We propose that this represents a negative feedback mechanism that normally serves to limit the duration of T cell–driven inflammatory responses.

There are several published studies that support the findings reported here. For example, as observed in the present study, it was reported that TNFα selectively inhibits p40 expression in human and mouse myeloid cells in an IL-10–independent manner (18, 19). In CIA, it was shown that there was elevated IFNγ production (IL-17 was not measured) and increased numbers of $CD4^+$ T cells in $TNF^{-/-}$ mice compared with WT mice (20). More recently, it was shown that i.p. administration of rTNFα reduced IFNγ production as well as the clinical severity of adjuvant-induced arthritis in rats (21), which is also consistent with our findings. In experimental autoimmune encephalomyelitis (EAE), it was reported that there was enhanced IL-12/IL-23 p40 expression in $p55^{-/-}$ mice and increased Th1 (but not Th17) cells in the CNS compared with WT mice (22).

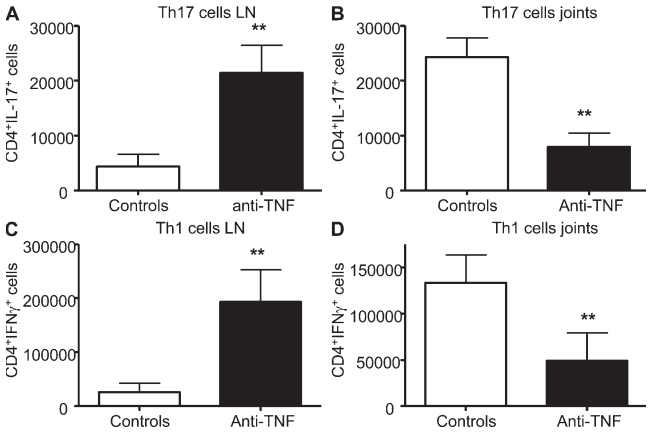

Figure 5. Anti-TNFα therapy prevents the accumulation of Th1/Th17 cells in the joint. Arthritic DBA/1 mice ($n = 6$) were treated once every 3 d for a total of 14 d with anti-TNFα mAb (TN3-19.12; 300 μg/mouse) or control Ab. $CD4^+$ cells from the inguinal LN and joints (obtained by enzymatic digestion of synovial tissue) were analyzed for intracellular cytokine expression by flow cytometry after stimulation with PMA/ionomycin. A, $CD4^+IL\text{-}17^+$ cells in LN; B, $CD4^+IL\text{-}17^+$ cells in joints; C, $CD4^+INF\gamma^+$ cells in LN; D, $CD4^+INF\gamma^+$ cells in joints. **, $P < 0.01$. Data are representative of two experiments. Error bars show SEM.

Anti-TNFα therapy is now undergoing evaluation in an increasing number of diseases and its effect is not universally beneficial. For example, TNFα blockade was shown to increase both the rate and frequency of relapse in patients with existing multiple sclerosis (23). In EAE, $TNF\alpha^{-/-}$ mice developed enhanced inflammation and demyelination, whereas treatment of susceptible mice with TNFα reduced the severity of disease (24). In another study, EAE failed to resolve in $TNF\alpha^{-/-}$ or $TNFR^{-/-}$ mice, suggesting that TNFα plays an important role in resolution of inflammation (25). Similarly, in murine lupus, administration of rTNFα was found to be protective (26), whereas TNFα deficiency was associated with increased production of antinuclear antibodies and accelerated onset of disease (27). Hence, an important question is whether the deleterious effects of TNFα blockade in these diseases are mediated via the expansion of Th1 and/or Th17 cells.

As discussed in a previous paragraph, TNFα has been reported to decrease p40 expression in human macrophages (18). Therefore, the findings presented in this paper may have implications for human RA. As in CIA, the therapeutic efficacy of TNFα blockade in human RA is indisputable (28), but it is possible that the rare occurrence of side effects, such as antinuclear autoantibodies and demyelination (29), could be explained by an amplification of Th17 and/or Th1 responses. However, it is also possible that the increased Th1/Th17 responses in the periphery versus the joint after anti-TNFα therapy may have beneficial consequences by increasing resistance to infection. This may help to explain the relatively low impact of TNFα blockade on susceptibility to infection (30).

In conclusion, the results of this study show that TNFα plays at least two distinct and opposing roles in CIA. First, it contributes to the accumulation of Th1 and Th17 cells in arthritic joints, and second, it plays an inhibitory role by limiting total numbers of these pathogenic T cell subsets in peripheral lymphoid organs.

MATERIALS AND METHODS

Mice. DBA/1, C57BL/6, and CB-17 SCID mice were purchased from Harlan. $p55^{-/-}$ and $p75^{-/-}$ mice were bred in house on a C57BL/6 background. All experimental procedures were approved by the Ethical Review Process Committee.

Immunization. DBA/1 and C57BL/6 mice were immunized with CFA plus bovine or chicken type II collagen, respectively (31). Arthritis severity was assessed as follows: 0, normal; 1, slight swelling and/or erythema; 2, pronounced edematous swelling; and 3, ankylosis. Each limb was graded, giving a maximum score of 12.

Anticytokine therapy. Blockade of TNFα was achieved using murine p75 TNFR-Fc (donated by GlaxoSmithKline) or hamster anti–mouse TNFα mAb (TN3-19.12; provided by R.D. Schreiber, Washington University School of Medicine, St. Louis, MO). Blockade of IL-12/IL-23 was achieved using rat anti–mouse p40 mAb (c17.8; donated by G. Trinchieri, then at Wistar Institute, Philadelphia, PA).

LN cell culture. LN cells were cultured at a density of 2×10^6 cells/ml in RPMI-1640 with L-glutamine plus 10% FCS, penicillin/streptomycin, sodium pyruvate, and β_2-mercaptoethanol and stimulated with 50 μg/ml

of type II collagen or 0.1 μg/ml of anti–CD3 mAb (145-2C11). Supernatants were collected for cytokine analysis after 48 h. Cells were then incubated for a further 16 h in the presence of 1 μCi/well of [^{3}H]thymidine to quantify proliferation.

Thioglycolate-elicited macrophages. Mice were injected i.p. with 1 ml of 3% thioglycolate. After 3 d, mice were killed and peritoneal macrophages collected by PBS lavage. After overnight adherence, cells were incubated for 8 h in the presence or absence of 30 ng/ml rTNFα (PeproTech), followed by 18 h in the presence of 1 ng/ml LPS (Sigma-Aldrich).

Isolation of cells from joints. The skin was removed from arthritic hind paws, and synovial cells were liberated by digestion with 1.6 U/ml Liberase C (Roche) and 0.2 mg/ml DNase I (Roche) for 40–60 min at 37°C.

Cytokine measurement. Cytokines were measured using commercially available kits as follows: IFNγ (BD Biosciences); IL-17A (R&D Systems); and IL-12/IL-23 p40, IL-1β, and IL-6 (eBioscience).

Flow cytometry. For intracellular cytokine staining, cells were stimulated for up to 10 h with PMA and ionomycin. Brefeldin A was added for the last 4 h. For surface staining, cells were incubated with anti-CD4 or anti-CD8 (BD Biosciences) for 30 min at 4°C, washed, and then fixed in Cytofix (BD Biosciences). Cells were permeabilized using PBS containing 1% FCS, 0.01% sodium azide, and 0.05% saponin and stained with anti–mouse IFNγ (BD Biosciences), anti–mouse IL-4 (BD Biosciences), and anti–mouse IL-17 (Cambridge BioScience) and analyzed on FACS Canto II using FACSDIVA software (BD Biosciences).

Real-time quantitative RT-PCR. RNA was isolated using the RNeasy protect mini kit (QIAGEN) and cDNA transcribed using the reverse transcription system (Promega). p40 gene expression was determined by real-time PCR using predesigned TaqMan primers and probe (Applied Biosystems) by the comparative method of relative quantitation. HPRT mRNA was used as an endogenous control to check for RNA and cDNA differences within samples. Differences in the mean threshold cycle (C_t) for the target gene p40 and the C_t for HPRT RNA, indicated by ΔC_t, were calculated to normalize differences in the mRNA extractions and the efficiency of the reverse transcription. The relative mRNA amount for each target gene was calculated as $\Delta\Delta C_t$ and expressed as fold change compared with a control sample.

Statistical analysis. The unpaired *t* test or one way ANOVA with Dunnett's multiple comparison test was used to test statistical significance. A p-value of <0.05 was considered significant.

Online supplemental material. Fig. S1 shows the expansion of Th1 and Th17 cells after treatment with anti-TNFα mAb. Fig. S2 uses an adoptive transfer model to demonstrate that the expanded population of Th1/Th17 cells is pathogenic. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20072707/DC1.

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