Lack of TNFR p55 Results in Heightened Expression of IFN-γ and IL-17 during the Development of Reactive Arthritis

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Reactive arthritis (ReA) is a type of arthritis originating from certain gastrointestinal or genitourinary infections. In previous studies, we reported the development of progressive *Yersinia enterocolitica*-induced ReA in mice lacking TNFR p55; however, the mechanisms underlying this effect are still uncertain. In this study, we investigated the impact of TNFR p55 deficiency in modulating Ag-specific Th1 and Th17 responses during this arthritogenic process. We found more severe ReA in *TNFRp55*$^{-/-}$ mice compared with their wild-type (WT) counterparts. This effect was accompanied by increased levels of *Yersinia* LPS in the joints of knockout mice. Analysis of the local cytokine profile revealed greater amounts of IFN-γ and IL-17 in arthritic joints of *TNFRp55*$^{-/-}$ mice compared with WT mice at day 21 postinfection. Moreover, altered IL-17 and IFN-γ production was observed in mesenteric and inguinal lymph nodes of *Yersinia*-infected *TNFRp55*$^{-/-}$ mice, as well as in spleen cells obtained from infected mice and restimulated ex vivo with bacterial Ags. Increased levels of cytokine secretion were associated with a greater frequency of CD4$^+$ IL-17$^+$, CD4$^+$IFN-γ$^+$, and IL-17$^+$IFN-γ$^+$ cells in *TNFRp55*$^{-/-}$ mice compared with WT mice. Remarkably, Ab-mediated blockade of IL-17 and/or IFN-γ resulted in reduced joint histological scores in *TNFRp55*$^{-/-}$ mice. A mechanistic analysis revealed the involvement of p40, a common subunit of heterodimeric IL-12 and IL-23, in the generation of augmented IFN-γ and IL-17 production under TNFR p55 deficiency. Taken together, these data indicate that, in the absence of TNFR p55 signaling, Th1 and Th17 effector cells may act in concert to sustain the inflammatory response in bacterial-induced arthritogenic processes. ***The Journal of Immunology*****, 2010, 185: 4485–4495.**

Reactive arthritis (ReA), a group of arthritides also known as spondyloarthropathies, represents a broad spectrum of inflammatory diseases that arise upon infection with certain types of gastrointestinal or genitourinary pathogens (1). Infections with Gram-negative bacteria, including *Yersinia enterocolitica*, are associated with a risk for ReA in ~1−15% of patients (2). Because the pathogenesis of ReA is incompletely understood, appropriate treatments are not available (1).

TNF is a proinflammatory cytokine that signals via two cell surface receptors, TNFR p75 (TNFR2; CD120b) and TNFR p55 (TNFR1; CD120a); the latter is implicated in most TNF effects (3, 4). This pleiotropic cytokine has been involved in the pathogenesis of a variety of rheumatic and inflammatory diseases. However, TNF has also been considered a critical cytokine capable of activating macrophages during the protective host response against bacterial infections, such as *Y. enterocolitica* (5). Interestingly, low TNF production has been detected in sera of a high percentage of patients with ReA (6, 7). Given the dual role of TNF as a pathogenic or host-protective factor in autoimmune or microbial settings, the pathological relevance of this cytokine in ReA remains unclear. In this regard, we previously found that TNFR p55-driven immune-protective mechanisms prevent the evolution to ReA upon oral infection with *Y. enterocolitica* O:3, the most frequently associated human arthritogenic serotype (8). Interestingly, TNFR p55-deficient (*TNFRp55*$^{-/-}$) mice develop severe chronic arthritis compared with their wild-type (WT) counterparts (8); however, the mechanisms underlying these pathogenic effects remain uncertain.

Th17 cells have been recognized as the third effector T cell subset in the portfolio of Th effector cells (9). Th17 cells play a pathogenic role in several models of inflammatory and autoimmune disorders, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, uveitis, and multiple sclerosis (10, 11). Although mouse Th17 cells require TGF-β_1 and IL-6 for their differentiation, human Th17 cells can be generated in the presence of IL-1 and IL-6 (12). Remarkably, expansion of human and mouse Th17 cells requires IL-23, a heterodimeric cytokine consisting of a unique p19 subunit linked to a common p40 chain (12). Of note, p40 can also be associated with the p35 chain to generate IL-12, a critical cytokine responsible of promoting Th1 cell differentiation (13). Interestingly, we found that *Yersinia*-infected *p40*$^{-/-}$ mice develop acute ReA (14), in contrast to *TNFRp55*$^{-/-}$ mice, which show more severe and chronic arthritis (8). However, IL-17 was also shown to act inde-

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Abbreviations used in this paper: μA, microabsorbance; C, cartilage; HKY, heat-killed *Yersinia*; ILN, inguinal lymph node; JS, joint space; MEKC, micellar electrokinetic chromatography; MLN, mesenteric lymph node; P, pannus; ReA, reactive arthritis; S, synovium; WT, wild-type.

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pendently of TNF during the course of streptococcal cell wall-induced arthritis (15). Furthermore, a recent study demonstrated increased levels of IL-17, IL-6, TGF-β_1, and IFN-γ in synovial fluids and sera of patients with ReA (16), and emerging data indicate an essential role of the IL-23/IL-17 axis in the pathogenesis of spondyloarthritis (17). Although *Y. enterocolitica* induces strong Th1 responses (18), the possibility that IFN-γ and/or IL-17 might play a role in *Yersinia*-induced ReA remains largely unexplored. In addition, it is still uncertain whether Th1- and Th17-differentiation pathways are regulated by TNF signaling during the course of bacterial-induced inflammatory disease. In the current study, we aimed to dissect the mechanisms underlying the increased severity of the arthritogenic process in *TNFRp55*$^{-/-}$ mice following infection with *Y. enterocolitica*. Given the essential role of IL-17 in the host mucosal immune response (19), as well as the link frequently observed between gut inflammation and arthritis (20), we also wished to examine the in vivo cytokine profile observed in mucosal lymph nodes compared with articular lymph nodes during the reactive arthritogenic process. Our data showed an augmented local inflammatory response dominated by IL-17, IFN-γ, IL-12p70, and IL-23 (p19/p40) in *TNFRp55*$^{-/-}$ mice compared with WT mice. Ab-mediated blockade of IL-17 and/or IFN-γ resulted in significantly reduced histopathological changes in the joints of these mice, suggesting that IL-17 and IFN-γ participate in the severe arthritogenic process occurring in *TNFRp55*$^{-/-}$ mice. Collectively, these data support an in vivo regulatory role for TNFR p55 signaling in fine-tuning Th17 and Th1 programs during bacterial-induced ReA through modulation of the common p40 subunit of IL-23 and IL-12.

Materials and Methods

Mice

TNFRp55$^{-/-}$ mice (C57BL/6) were kindly provided by the Max von Pettenkofer Institute (Munich, Germany). C57BL/6 WT mice were purchased from the Animal Facilities of the National University of La Plata, La Plata, Argentina. Breeding colonies were established at the Animal Facilities of the National University of San Luis. Mice were kept under specific pathogen-free conditions in positive-pressure cabinets (EHRET, Emmendingen, Germany) and provided with sterile food and water ad libitum. Male mice (6-wk old) were used for the experiments. At least three independent experiments were performed. Protocols were approved by the Institutional Review Board of the Faculty of Medical Sciences, National University of Cuyo. Animal work was approved by the Animal Care and Use Committee of National University of San Luis.

Bacterial culture and infection

Strain MHC 700 *Y. enterocolitica* O:3 (kindly provided by Dr. G. Kapperud, Department of Bacteriology, Oslo, Norway) was used for infection. Bacteria were cultured as described earlier (8). Mice were starved for 8 h and then were infected orogastrically, with 1–5 $\times$ 10^8 yersiniae in 200 μl saline, using a gastric tube. The number of inoculated bacteria was controlled by plating serial dilutions of the inoculated suspension on Mueller–Hinton agar and counting the CFU following incubation at 26°C for 48 h.

Assessment of arthritis

Mice were examined daily for visual appearance of arthritis in peripheral joints, and the arthritis score was calculated until day 21 postinfection, as described earlier (21). Clinical severity was classified for each paw: 0, normal joint; 1, slight inflammation and redness; 2, severe erythematic and swelling affecting the entire paw; and 3, deformed joint paw with ankylosis, joint rigidity, and loss of function. Total score was based on the assessment of all paws, with a maximum score of 12 per animal, as described (21).

Histopathological evaluation

Histopathological examination of the joints was carried out at day 21. After routine fixation, decalcification, and paraffin embedding, 5-μm-thick sections were cut and stained with H&E. Histopathological scoring was performed, as described by Kyo et al. (22). Ankles from different groups of mice were assigned histopathological scores of 0–5, where 0, normal; 1, minimal infiltration; 2, mild infiltration; 3, moderate infiltration; 4, marked infiltration; and 5, severe infiltration. The same ankles were assigned a score for cartilage/bone resorption, according to the following criteria: 0, normal; 1, minimal (small areas of resorption); 2, mild (numerous areas of resorption); 3, moderate (obvious resorption of trabecular and cortical bone without full-thickness defects in the cortex; loss of some trabeculae); 4, marked (full-thickness defects in the cortical bone and marked trabecular loss without distortion of the remaining cortical surface profile); and 5, severe (full-thickness defects in the cortical bone and marked trabecular bone loss, with distortion of the remaining cortical surface profile). The total score was defined as the sum of the scores for inflammation and for cartilage/bone destruction. Each slide was scored by two independent observers, and the average score was recorded.

Micellar electrokinetic chromatography in joint homogenates

The presence of bacterial Ags was examined in homogenates of joints obtained from *Yersinia*-infected *TNFRp55*$^{-/-}$ mice and WT mice, as described (22). The presence of LPS in these samples was determined by micellar electrokinetic chromatography (MEKC). LPS from *Y. enterocolitica* O:3 was extracted by the hot phenol-water method, as described (23), and used as positive control. For MEKC, we used a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, CA) equipped with a diode array detector and a data-handling system comprising an IBM personal computer and P/ACE System MDQ Software. The fused-silica capillaries (57 cm total length, 50 cm effective length, 75 μm internal diameter, 375 μm OD) were obtained from MicroSolv Technology (Eatontown, NJ). Detection was performed at 197 nm, the capillary temperature was maintained at 18°C, and the voltage was set at 25 kV. Samples were pressure-injected at the cathodic side at 0.5 ψ for 10 s. To achieve high reproducibility of migration times, the capillary was washed between analyses with sodium hydroxide for 2 min and then equilibrated with running buffer for 4 min. The background electrolyte used contained 20 mM phosphate buffer (pH 8) and 10 mM SDS. The electroendosmotic flow marker was prepared by diluting 1 ml acetone with the background electrolyte (1:1), and sonication was applied for 5 min prior to injection.

In vitro cultures

At days 7, 14, and 21 postinfection, spleens were isolated, and erythrocytes were lysed in 0.16 M NH_4Cl (pH 7.2). Cells (2×10^6 cell/well) were cultured in 24-well plates for 72 h at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 (HyClone, Logan, UT), supplemented with 10% FBS, 1 mM pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μmol/ml 2-ME in the presence or absence of 10^7 bacteria/ml heat-killed *Yersinia* (HKY), as an Ag-specific stimulus (24), or ConA (5 μg/ml), as a mitogenic stimulus. Culture supernatants were obtained and stored at −20°C until cytokine determination.

Cytokine determination by ELISA

Mouse IL-17, IFN-γ, IL-6, IL-1β, TGF-β_1, IL-12/IL-23 p40, IL-12p70, IL-23 (p19/p40), and IL-10 were determined in the joint, mesenteric lymph node (MLN), and inguinal lymph node (ILN) homogenates, prepared as described previously (8), as well as in spleen cell culture supernatants using capture ELISA kits (eBioscience, San Diego, CA), according to the manufacturer's instructions. The limit of detection for the above-mentioned cytokines was 4 pg/ml for IL-17, 0.7 pg/ml for IFN-γ, 4 pg/ml for IL-6, 8 pg/ml for IL-1β, 60 pg/ml for TGF-β_1, 2 pg/ml for IL-12/IL-23 p40, 30 pg/ml for IL-23 (p19/p40), and 30 pg/ml for IL-10. IL-12p70 was determined using the BD OptEIA set.

Flow cytometry

Splenocytes were isolated from spleens of *TNFRp55*$^{-/-}$ mice and WT mice 21 d postinfection. Cells (1×10^6 cells/ml) were cultured for 72 h at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 (HyClone), supplemented with 10% FBS and stimulated with HKY (6×10^7 bacterial/ml). Cells were restimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) for 6 h in the presence of GolgiPlug (BD 555029; BD Biosciences, San Jose, CA) for the last 5 h. After washing, cells were stained with PE-Cy5–conjugated anti-CD4 (RM4-5; BD Biosciences), fixed in 1% paraformaldehyde, and permeabilized using BD FACS Permeabilizing solution (BD 340973; BD Biosciences), followed by staining with PE-conjugated anti–IL-17 (eBio17B7) or FITC-conjugated anti–IFN-γ (XMGG1.2; both from eBioscience). Samples were analyzed on a FACSAria flow cytometer (BD Biosciences). WinMDI 2.9 software was used for data analysis. A gate was established to define $CD4^+$ T cells.

Anti-cytokine–blocking experiments

IFN-γ was blocked with a hamster anti-mouse IFN-γ mAb (MAB4851), and IL-17 was neutralized using a rat anti-mouse IL-17 mAb (MAB421; both from R&D Systems, Minneapolis, MN). As control, the same doses of

isotype-control Ab were injected. Briefly, *TNFRp55*$^{-/-}$ or WT mice were treated in vivo with an anti–IFN-γ mAb, an anti–IL-17 mAb, or a combination of both. Mice received anti-cytokine mAb (2.5 mg/kg) i.p. on days 16 and 18 postinfection. On day 21, ankle joints were obtained for histological analysis.

Statistical analysis

Differences between the groups were tested for significance using the Mann–Whitney *U* test or one-way ANOVA with the Dunnett multiple-comparison test, as appropriate. A p value <0.05 was considered statistically significant. The results are expressed as the mean ± SD.

Results

Impact of TNFR p55 deficiency on the development of chronic ReA following Yersinia *infection*

Through activation of TNFR p55 signaling, TNF was demonstrated to be a critical cytokine in the protective host response to *Yersinia* (5, 24). Moreover, in recent studies, we found that TNFR p55 signaling also plays a protective role against ReA induced by *Y. enterocolitica* O:3 (8). To further dissect the mechanisms involved in this protective effect, we first examined disease progression in *TNFRp55*$^{-/-}$ mice compared with WT mice. *TNFRp55*$^{-/-}$ mice that did not succumb to *Yersinia* infection (survival: 60%) developed a severe and progressive arthritis, with considerably increased clinical score compared with WT mice, at day 14 (Fig. 1*A*). Moreover, increased inflammatory swelling, disorganization of the synovial membrane, proliferation of synovial lining cells with pannus formation, and bone/cartilage degradation were clearly evident when histopathological changes were assessed in the joints of *TNFRp55*$^{-/-}$ mice on day 21 after *Yersinia* infection (Fig. 1*B*). Joint inflammation was observed until day 55, suggesting the development of chronic arthritis in TNFR p55-deficient hosts (data not shown). Interestingly, although WT mice showed a peak of arthritis on day 14, clinical severity was markedly attenuated in comparison with *TNFRp55*$^{-/-}$ mice (Fig. 1*A*), and histopathological changes were considerably reduced on day 21 postinfection (Fig. 1*B*). Taken together, these results indicate a protective and tempering function of normal TNFR p55 signaling in the development of *Yersinia*-induced chronic ReA.

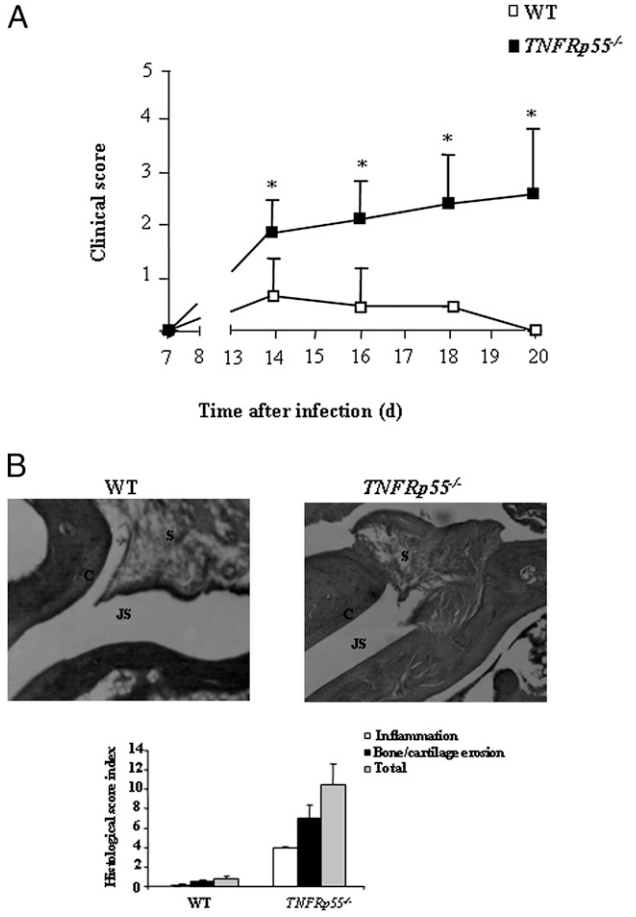

FIGURE 1. *TNFRp55*$^{-/-}$ mice develop severe and progressive *Yersinia*-induced ReA. WT ($n = 6$) and *TNFRp55*$^{-/-}$ ($n = 10$) age-matched male mice were infected orally with *Y. enterocolitica* O:3 and monitored daily for arthritis onset and clinical severity. *A*, The clinical score was calculated in WT ($n = 6$) and surviving *TNFRp55*$^{-/-}$ mice ($n = 6$), as described in *Materials and Methods*, and represents the total paw swelling score for each mouse. *B*, Histopathological examination of representative joints of WT and *TNFRp55*$^{-/-}$ mice on day 21 postinfection (original magnification ×100). Histological index was calculated at day 21 postinfection. Results (mean score ± SD) are representative of two or three independent experiments. $*p < 0.05$. C, cartilage; JS, joint space; P, pannus; S, synovium.

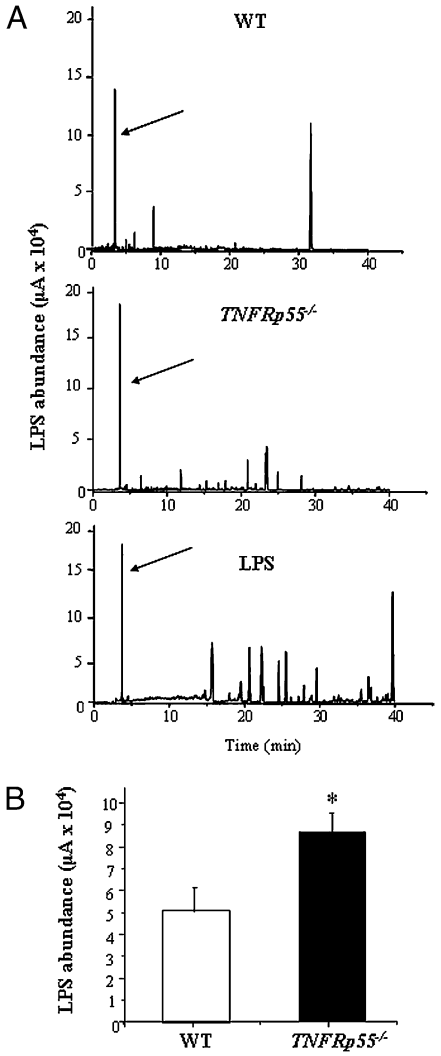

FIGURE 2. TNFR p55 deficiency influences the abundance of *Yersinia* LPS in the joints. *A*, MEKC was performed in joint homogenates of WT and *TNFRp55*$^{-/-}$ mice at day 14 postinfection, and chromatograms were compared with purified *Yersinia* LPS as positive control. A sharp peak in LPS (arrow) was detected in homogenates of joints, but it was higher in *TNFRp55*$^{-/-}$ mice compared with WT mice. Data are representative of three independent experiments. *B*, Mean ± SD of WT and *TNFRp55*$^{-/-}$ LPS abundance measured by MEKC in three independent experiments. µA, microabsorbance. $*p < 0.001$.

Influence of TNFR p55 deficiency in the control of bacterial LPS in the joints of infected mice

Given the presence of outer membrane-specific Abs in sera from *TNFRp55*$^{-/-}$ infected mice (8), we further examined whether an increased bacterial Ag load in the joints of arthritic mice might contribute to the increased severity of ReA in these mice. For this purpose, we examined, by MEKC, the presence of LPS, the main outer membrane component of the bacteria, in joint homogenates of WT and *TNFRp55*$^{-/-}$ mice at days 14 and 21 postinfection. We observed a sharp peak corresponding to purified *Yersinia* LPS in joint homogenates of *TNFRp55*$^{-/-}$ and WT mice (Fig. 2), suggesting that *Yersinia* LPS may trigger articular inflammation in the early phase of arthritis in both groups of mice (day 14 postinfection). However, LPS levels were significantly higher in *TNFRp55*$^{-/-}$ mice compared with WT mice ($p < 0.001$) (Fig. 2*B*), suggesting increased bacterial Ags in mice devoid of TNFR p55 signaling. However, LPS was no longer detected at day 21 in knockout or WT mice (data not shown). Thus, despite the presence of a pronounced arthritogenic disease triggered by TNFR p55 deficiency, bacterial Ags seem to be cleared from the joints of *TNFRp55*$^{-/-}$ and WT mice during the ongoing infectious process. These results suggest that immune-mediated mechanisms, activated early during bacterial invasion, may operate in concert to trigger chronic ReA in TNFR p55-deficient mice.

Differential expression of proinflammatory cytokines in the joints of Yersinia*-infected TNFRp55*$^{-/-}$ *and WT mice*

Compelling evidence indicates a critical role for IL-17 in sustaining the inflammatory response in several models of arthritis (25–27). This cytokine was shown to be fundamental in turning acute synovitis into a chronic destructive arthritis (28, 29). In contrast, IFN-γ is strongly induced by *Yersinia* infection (18, 24) and is essential for initiating joint inflammation in several arthritis models (30). Hence, we examined the relative abundance of IL-17 and IFN-γ in joint homogenates of *TNFRp55*$^{-/-}$ mice versus WT mice. Although we observed a comparable induction of articular IL-17 in WT and *TNFRp55*$^{-/-}$ mice at days 7 and 14 postinfection, the amounts of IL-17 were substantially higher in *TNFRp55*$^{-/-}$ mice when analyzed 21 d after bacterial infection ($p < 0.05$) (Fig. 3*A*). Thus, disruption of the TNFR p55 pathway results in uncontrolled articular inflammation that persists at day 21 and is accompanied by high amounts of IL-17 in the arthritic joints. In addition, IFN-γ was increased 7 d postinfection in WT and knockout mice; however, this cytokine was upregulated in inflamed joints of *TNFRp55*$^{-/-}$ mice but not in WT mice at days 14 and 21 postinfection ($p < 0.05$) (Fig. 3*B*). The increased arthritis severity, together with the higher IFN-γ levels and the lack of difference in IL-17 at day 14 postinfection of *TNFRp55*$^{-/-}$ mice, suggests a dominant contribution of IFN-γ to joint inflammation during this time period.

Given the role of IL-6 and TGF-β_1 in promoting Th17 cell differentiation, we next examined the levels of these cytokines in the joints of *Yersinia*-infected WT and *TNFRp55*$^{-/-}$ mice. We found higher amounts of IL-6 in *TNFRp55*$^{-/-}$ mice compared with WT mice, an effect that started as early as day 7 ($p < 0.05$), peaked at day 14 ($p < 0.05$), and was sustained until day 21 ($p < 0.05$) after bacterial infection (Fig. 4*A*). Furthermore, the concentration of TGF-β_1 was significantly higher in the joints of *TNFRp55*$^{-/-}$ mice at day 21 postinfection ($p < 0.05$) (Fig. 4*B*). Interestingly, lack of TNFR p55 led to upregulation of TGF-β_1, even in the absence of bacterial Ags (Fig. 4*B*). Given the cooperative effects of IL-17

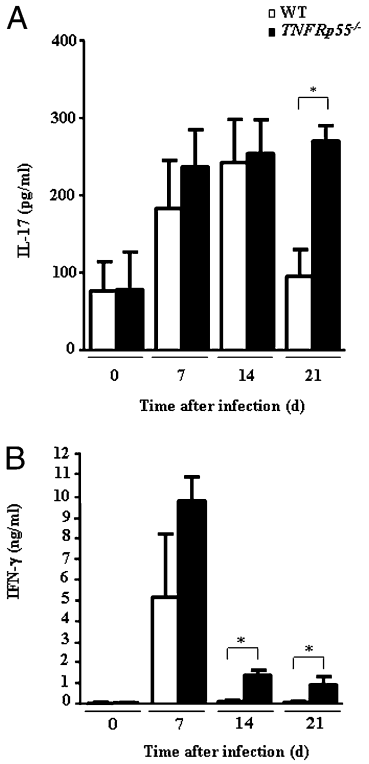

FIGURE 3. Lack of TNFR p55 augments IL-17 and IFN-γ production in the joints of mice with *Yersinia*-induced ReA. IL-17 (*A*) and IFN-γ (*B*) in joint homogenates of WT and *TNFRp55*$^{-/-}$ mice were prepared at different time points postinfection and assayed by ELISA. Results are shown as the mean ± SD of three independent experiments, with four or five mice per group. *$p < 0.05$.

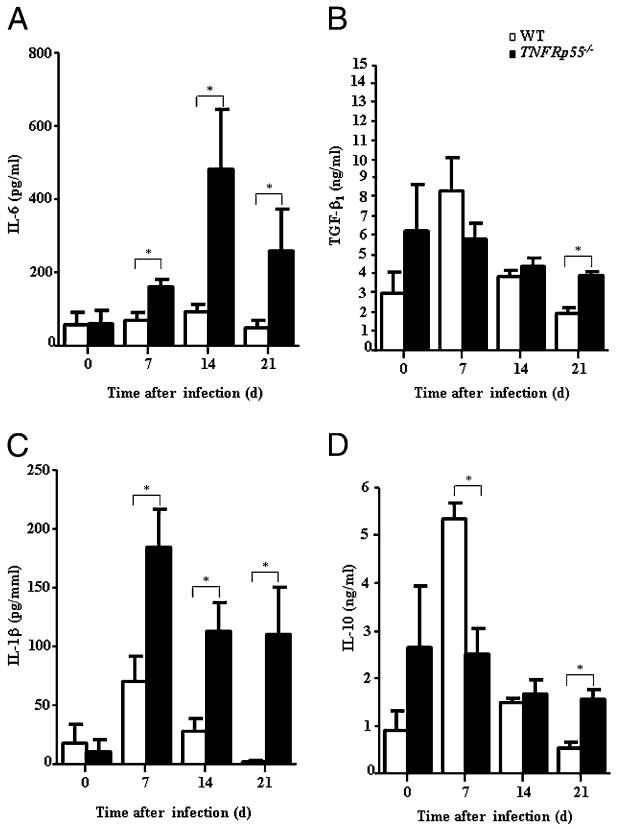

FIGURE 4. Deficiency of TNFR p55 affects pro- and anti-inflammatory cytokines in the joints of mice with *Yersinia*-induced ReA. Levels of IL-6 (*A*), TGF-β_1 (*B*), IL-1β (*C*), and IL-10 (*D*) in joint homogenates of WT and *TNFRp55*$^{-/-}$ mice obtained at different time points postinfection. Results are the mean ± SD of three independent experiments, with four to five mice per group. *$p \leq 0.05$.

and IL-1β in sustaining joint inflammation (27), we next wished to examine the concentrations of IL-1β in the joints of both groups of mice. We found increased amounts of IL-1β in arthritic joints of *TNFRp55*$^{-/-}$ mice versus WT mice at days 7, 14, and 21 following *Yersinia* infection ($p < 0.05$) (Fig. 4*C*). The increased amounts of IL-6 (Fig. 4*A*) and IL-1β (Fig. 4*C*) in *TNFRp55*$^{-/-}$ mice compared with WT mice at all times analyzed suggested that these cytokines may also be targets of regulation by TNFR p55 signaling. However, in contrast to IFN-γ and IL-17, the kinetics of IL-6 and IL-1β were not in accordance with clinical expression of arthritis, suggesting a secondary role of these cytokine in arthritis progression in *TNFRp55*$^{-/-}$ mice. Interestingly, only when IL-6 and TGF-β_1 increased in *TNFRp55*$^{-/-}$ mice (day 21 postinfection) (Fig. 4*B*), IL-17 increased in the joints of *TNFRp55*$^{-/-}$ mice (Fig. 3*A*). Given the immunoregulatory role of IL-10 at the peak and resolution of inflammatory diseases (31), we further examined the regulation of this cytokine in the joints of arthritic mice. Although the levels of IL-10 increased significantly in WT mice at day 7 ($p < 0.05$), this cytokine was substantially decreased in the joints of WT mice at 21 d postinfection, when joint inflammation was already resolved ($p < 0.05$) (Fig. 4*D*). Collectively, these data indicate that different pro- and anti-inflammatory cytokines are differentially regulated in the joints of *Yersinia*-induced ReA in *TNFRp55*$^{-/-}$ mice and may operate in concert to modulate the progression and severity of the inflammatory disease.

Impact of TNFR p55 deficiency on systemic production of IL-17 and IFN-γ during Yersinia*-induced ReA*

To determine whether Th17- and Th1-type cytokine profiles predominate at the systemic level in *TNFRp55*$^{-/-}$ mice infected with *Yersinia*, spleen cells from WT and *TNFRp55*$^{-/-}$ mice obtained 7, 14, and 21 d postinfection were stimulated ex vivo with HKY, and supernatants were examined for Ag-specific IFN-γ and IL-17 production (Fig. 5). Although discrete amounts of IFN-γ and IL-17 were detected in spleen cell cultures of *Yersinia*-infected WT mice, substantially higher concentrations of both cytokines were observed in *TNFRp55*$^{-/-}$ mice after HKY stimulation (Fig. 5*C*, 5*D*). However, no considerable changes were observed when spleen cells were obtained from both groups of mice and stimulated ex vivo with ConA (Fig. 5*E*, 5*F*). Interestingly, IL-17 reached concentrations significantly higher only in supernatants of *TNFRp55*$^{-/-}$ spleen cells isolated 14 d postinfection ($p < 0.05$; Fig. 5*C*). In contrast, the amounts of IFN-γ were abundant in supernatants of *TNFRp55*$^{-/-}$ spleen cells at all times tested ($p < 0.05$, $p < 0.01$, and $p < 0.001$ days 7, 14, and 21 postinfection versus WT spleen cells; Fig. 5*D*). These results indicate augmented Ag-specific Th1 and Th17 responses in spleen cells of *TNFRp55*$^{-/-}$ versus WT mice challenged with *Yersinia.* Thus, in the absence of TNFR p55 signaling, Th17- and Th1-type cytokines may play a role in driving the inflammatory response observed in *Yersinia*-induced ReA, suggesting an immunoregulatory role of the TNFR p55 pathway in attenuating Ag-specific Th1 and Th17 responses in vivo. Remarkably, the time-course study of IL-17 expression correlated well with the clinical outcome of progressive arthritis developed in *TNFRp55*$^{-/-}$ mice, suggesting a critical role for IL-17 in *Yersinia*-induced chronic ReA.

Impact of TNFR p55 signaling in the cytokine profile of mucosal and regional lymph nodes of Yersinia*-infected arthritic mice*

Because mice developed joint inflammation following oral delivery of *Y. enterocolitica*, we further studied the cytokine profile in mucosal lymph nodes compared with joint regional lymph nodes.

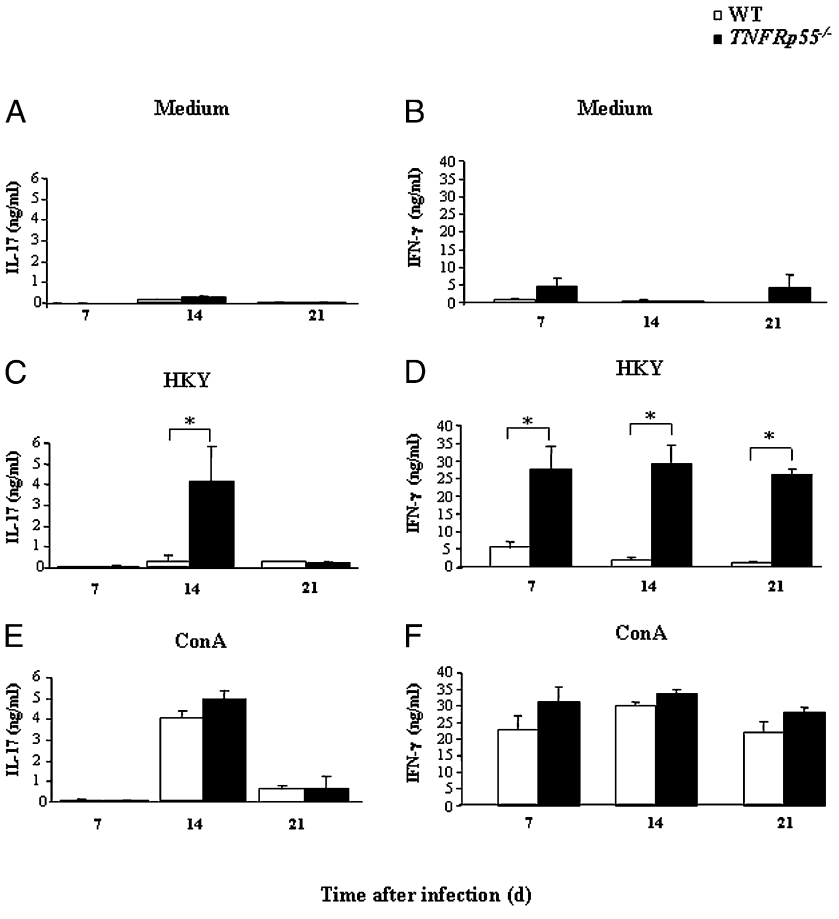

FIGURE 5. Lack of TNFR p55 augments Ag-specific IL-17 and IFN-γ production by spleen cells of mice with *Yersinia*-induced ReA. Spleen cells from infected WT and *TNFRp55*$^{-/-}$ mice were obtained at days 7, 14, and 21 postinfection and cultured in the absence (*A*, *B*) or presence of HKY (Ag-specific stimulation) (*C*, *D*) or ConA (polyclonal activation) (*E*, *F*) for 3 d. Supernatants were collected and assayed by ELISA for IL-17 (*A*, *C*, *E*) and IFN-γ (*B*, *D*, *F*). Values represent the mean ± SD of two representative experiments with four mice per group at each time point postinfection. $*p \leq 0.05$.

For this purpose, we examined the amounts of IL-17, IFN-γ, IL-6, and TGF-β_1 in tissue homogenates of MLNs and ILNs. Remarkably, IL-17 was significantly higher in MLNs of *TNFRp55*$^{-/-}$ mice versus WT mice at day 7; this effect was in line with that found in ILNs ($p < 0.001$ and $p < 0.01$ compared with WT mice in MLNs and ILNs, respectively; Fig. 6). Although similar levels of IL-17 were detected in MLNs and ILNs in WT and *TNFRp55*$^{-/-}$ mice at day 14 postinfection, reduced levels of this cytokine were observed in MLNs of *TNFRp55*$^{-/-}$ mice compared with WT mice at day 21 postinfection ($p < 0.05$ versus WT mice), probably reflecting migration of effector Th17 cells to target tissues. In contrast, the differences in the levels of IL-17 in ILNs paralleled those found in the joints of *TNFRp55*$^{-/-}$ and WT mice ($p < 0.01$; Fig. 6*B* versus Fig. 3*A*). However, IL-17 expression in ILNs showed a peak at day 14 postinfection and a tendency toward diminished expression at day 21 postinfection, in contrast to the expression profile in the joints. In addition, IFN-γ was more abundant in MLNs from *TNFRp55*$^{-/-}$ mice versus WT mice early postinfection (day 7; $p < 0.01$; Fig. 6*A*). In contrast, an increase in IFN-γ could only be detected late postinfection (day 21) in ILNs of mice devoid of TNFR p55 ($p < 0.01$). In keeping with the essential role of IL-6 and TGF-β_1 in promoting Th17 differentiation, these cytokines showed a similar profile to that of IL-17. Hence, substantially higher amounts of IL-6 and TGF-β_1 were detected in MLNs from *TNFRp55*$^{-/-}$ mice compared with WT mice at day 7 postinfection ($p < 0.01$ versus MLNs from WT mice; Fig. 6*A*). Likewise, we also detected a strong correlation among IL-17 and the Th17-promoting cytokines IL-6 and TGF-β_1 in ILNs, which showed a higher concentration in ILNs of *TNFRp55*$^{-/-}$ mice at day 7 ($p < 0.05$) or at day 21 ($p < 0.01$ and $p < 0.05$ for IL-6 and TGF-β_1, respectively) postinfection (Fig. 6*B*). Taken together, these data indicate a significant correlation among cytokines produced in mucosal-associated lymph nodes and those prevailing in the joint regional lymph nodes of *TNFRp55*$^{-/-}$ mice early after bacterial infection, supporting a link between the intestinal mucosa and the local immune response operating in the arthritic joints.

Increased numbers of IFN-γ– and IL-17–producing cells in TNFRp55$^{-/-}$ *mice*

To determine the cell type responsible of producing IL-17 and IFN-γ during *Yersinia* infection, flow-cytometry analysis was performed in splenic cells obtained from *TNFRp55*$^{-/-}$ and WT mice on day 21 postinfection (Fig. 7*A*). A significantly higher frequency of CD4$^+$IL-17$^+$ ($p < 0.05$), CD4$^+$IFN-γ$^+$ ($p < 0.05$), and IL-17$^+$ IFN-γ$^+$ ($p < 0.01$) cells were detected in *TNFRp55*$^{-/-}$ mice compared with WT mice (Fig. 7*B*). These results support the concept that Th1 and Th17 cells may contribute to sustain joint inflammation during the arthritogenic process triggered by bacterial Ags. Moreover, the increased number of IL-17$^+$IFN-γ$^+$CD4$^+$ T cells in *TNFRp55*$^{-/-}$ mice suggested that these cytokines are produced by double-positive T cells producing IL-17 and IFN-γ.

Reduced joint inflammation and bone/cartilage erosion by Ab-mediated neutralization of IFN-γ and IL-17

To investigate the functional relevance of IFN-γ and IL-17 in progressive *Yersinia*-induced ReA, blocking experiments were conducted in *TNFRp55*$^{-/-}$ and WT mice by neutralizing IFN-γ, IL-17, or both cytokines in vivo. Remarkably, blockade of IFN-γ, IL-17,

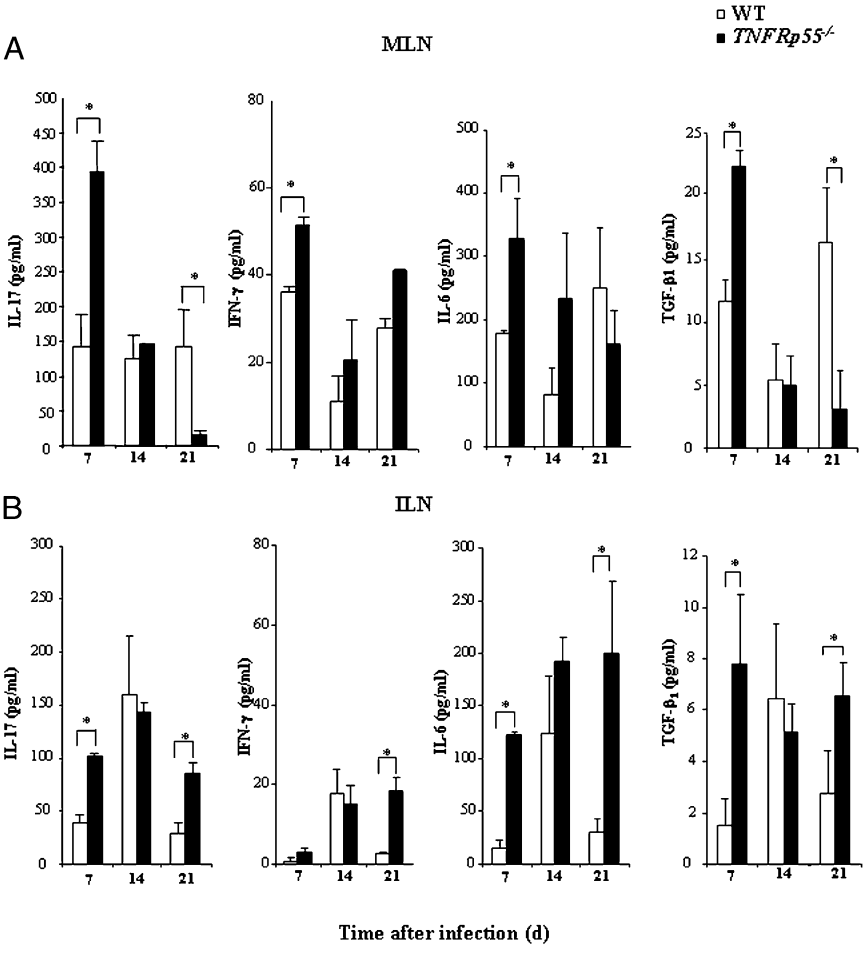

FIGURE 6. Lack of TNFR p55 influences cytokine production in mucosal lymph nodes and ILNs of mice with *Yersinia*-induced ReA. Levels of IL-17, IFN-γ, IL-6, and TGF-β1 in homogenates of MLNs (*A*) and ILNs (*B*) from WT and *TNFRp55*$^{-/-}$ mice at days 7, 14, and 21 postinfection. Values represent the mean ± SD of four or five mice per group evaluated each day postinfection. Results are representative of three experiments. $*p \leq 0.05$.

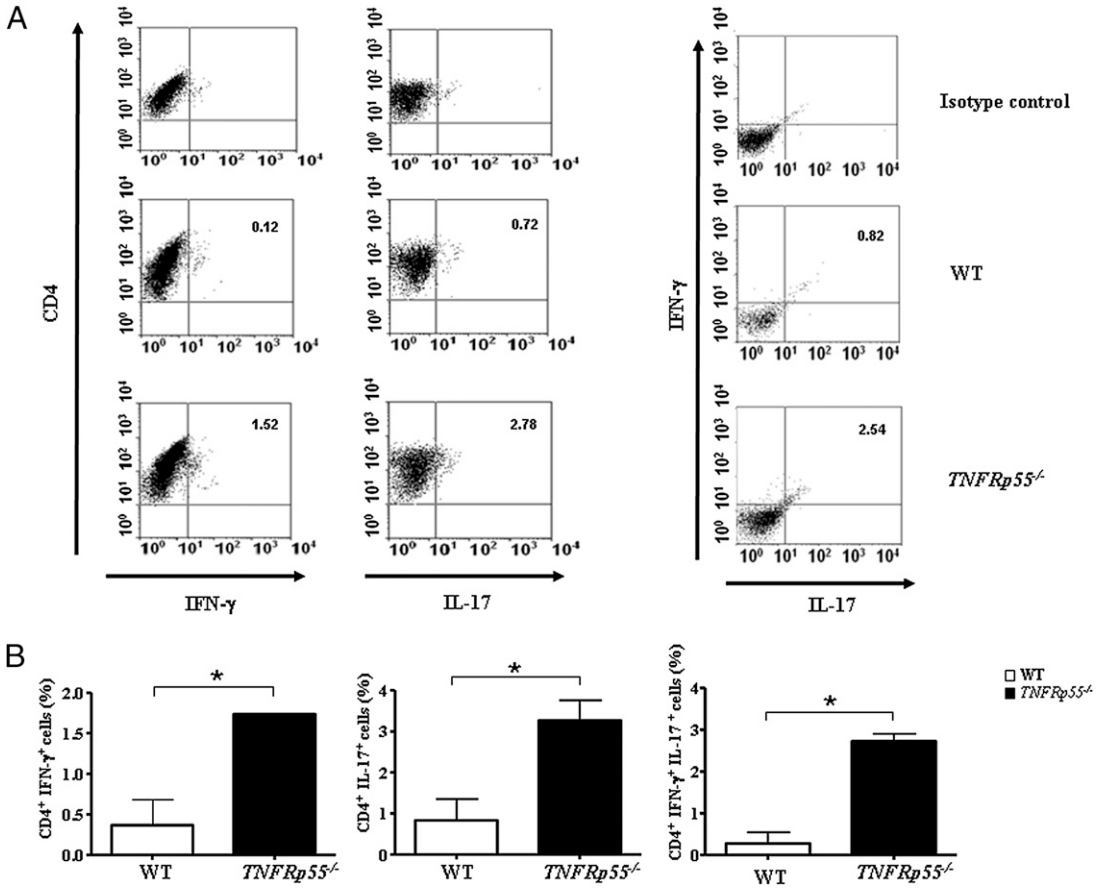

FIGURE 7. Increased IFN-γ– and IL-17–producing cells in *TNFRp55*$^{-/-}$ mice. *A*, Splenic cells from *TNFRp55*$^{-/-}$ and WT mice were obtained 21 d after *Yersinia* infection. After ex vivo restimulation, cells were stained for cell surface CD4, intracellular IFN-γ, and intracellular IL-17 and analyzed by flow cytometry. Dot plots are representative of three mice per group. *B*, Data show mean ± SD of the percentage of $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}17^+$, or $CD4^+IFN\text{-}\gamma^+IL\text{-}17^+$ cells (three independent experiments with $n = 3$ mice per group in each experiment). $*p < 0.05$.

or both cytokines successfully suppressed joint inflammation in ankle joints isolated on day 21 postinfection and markedly reduced cartilage and bone erosion (Fig. 8). These data support the essential roles of IL-17 and IFN-γ in ReA progression in *TNFRp55*$^{-/-}$ mice.

TNFR p55 signaling regulates p40 expression in MLNs and ILNs

To identify possible mechanisms by which TNFR p55 deficiency leads to increased Th17- and Th1-type cytokines, we next sought to examine a potential convergent pathway that could be a common denominator to these divergent Th effector subsets. Hence, we examined the expression levels of the p40 common chain that is shared by the Th1-promoting cytokine IL-12 and the Th17-promoting cytokine IL-23 (9, 13, 31). Expression of p40 was shown to be critical in the pathogenesis of organ-specific autoimmune disorders, particularly multiple sclerosis (10) and chronic inflammatory arthritis (31, 32). Analysis of p40 expression in MLNs and ILNs at day 21 postinfection revealed critical differences in *TNFRp55*$^{-/-}$ mice versus WT mice (Fig. 9). In fact, considerably higher levels of p40 were detected in MLNs and ILNs of *TNFRp55*$^{-/-}$ mice compared with WT mice ($p < 0.05$; Fig. 9). To dissect further the role of IL-23 and IL-12 in these effects, IL-12(p70) and IL-23 (p19/p40) were also determined. We found increased levels of IL-12(p70) and IL-23 (p19/p40) in ILNs of *TNFRp55*$^{-/-}$ mice compared with WT mice ($p < 0.05$; Fig. 9*B*). The correlation of these results with IFN-γ and IL-17 production strongly suggests that the TNFR p55-signaling pathway can suppress p40 protein expression in mucosal and joint regional lymph nodes to counteract the development of Th1 and Th17 responses. Thus, a potential cross-talk among distinct proinflammatory pathways could be proposed that controls the amplification and resolution of pathogenic responses during *Yersinia*-induced ReA.

TNFR p55 signaling regulates IL-10 production in MLNs and ILNs

IL-10, a potent anti-inflammatory cytokine, can antagonize Th1 and Th17 responses through inhibition of IFN-γ and IL-17 (33, 34). In addition, IL-10 plays a critical role in mucosal homeostasis, because IL-10 deficiency leads to spontaneous colitis due to aberrant activation of dendritic cells (35). Thus, we compared the amounts of IL-10 in MLNs and ILNs of *TNFRp55*$^{-/-}$ mice versus WT mice. Notably, lack of TNFR p55 led to substantial differences in IL-10 kinetics and expression pattern (Fig. 10). IL-10 peaked earlier in MLNs of *TNFRp55*$^{-/-}$ mice, as shown by its expression levels on day 7 after *Yersinia* infection ($p < 0.01$ compared with MLNs of WT mice; Fig. 10*A*). In contrast, augmented IL-10 production was detected in MLNs of WT mice for only 21 d postinfection ($p < 0.05$ versus MLNs of *TNFRp55*$^{-/-}$ mice; Fig. 10*A*). In contrast, the IL-10 peak was observed in ILNs from WT mice on day 14 postinfection, coincident with the onset of the recovery of the synovial inflammatory response ($p < 0.05$ versus ILNs of *TNFRp55*$^{-/-}$ mice; Fig. 10*B*). In contrast, higher amounts of IL-10 were detected on days 7 and 21 postinfection in ILNs from *TNFRp55*$^{-/-}$ mice compared with WT mice ($p < 0.05$ versus WT mice; Fig. 10*B*). Altogether, these results indicate that an altered production of IL-10 in MLNs and ILNs may contribute to the chronic progression of *Yersinia*-induced ReA in the absence of TNFR p55 signaling. Given the distinct roles of IL-10

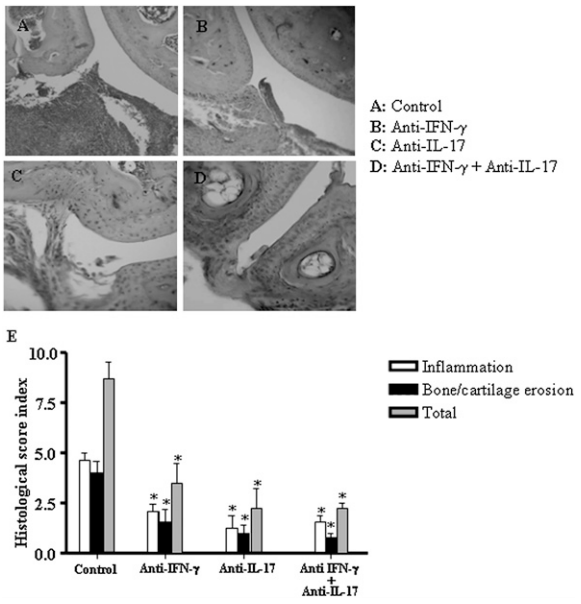

FIGURE 8. Blockade of IFN-γ and/or IL-17 reduces inflammation and cartilage/bone erosion in ankle joints of *TNFRp55*$^{-/-}$ mice. On days 16 and 18 postinfection, *TNFRp55*$^{-/-}$ mice were treated with isotype-control Ab (IgG1 or IgG2a) (*A*), anti–IFN-γ (*B*), anti–IL-17 (*C*), or anti–IFN-γ plus anti–IL-17 (*D*) mAbs. *E*, Inflammatory cell infiltrates, bone/cartilage erosion, and total histological scores were assessed at day 21 postinfection. Results are the mean score ± SD of at least three mice per group. $*p < 0.05$ versus control group; one-way ANOVA with the Dunnett multiple-comparison test.

during immunoregulatory processes (35), this pleiotropic cytokine may negatively or positively regulate the inflammatory response based on its expression levels and the synergistic or additive actions of other cytokines.

Discussion

TNF is well-known as a master proinflammatory cytokine capable of amplifying or modulating cytokine cascades in organ-specific autoimmune diseases, including a broad range of rheumatic disorders. TNF Abs and antagonists favor amelioration of clinical symptoms and laboratory parameters by restoring homeostasis of innate and adaptive immune cells (36). However, TNF is also a critical cytokine in the protective host response against bacterial infection, including *Y. enterocolitica* (5, 24), raising the question as to whether this cytokine plays a pathogenic or protective role in bacterial-induced ReA, a pathological entity that combines components of infection and autoimmunity. We recently demonstrated that TNFR p55 signaling confers protection against ReA induced by *Y. enterocolitica* O:3 (8). In addition, unfavorable evolution has been reported in patients with spondyloarthropathies under treatment with TNF blockers (37). In the present work, we confirmed the protective role of TNFR p55 signaling in *Yersinia*-induced ReA, demonstrating that, in contrast to WT mice, *TNFRp55*$^{-/-}$ mice developed joint inflammation with progression to a chronic arthritogenic process. Because ReA is an aseptic synovitis (1, 2) and, no bacteria are commonly detected in the joints of arthritic mice (8), we investigated the presence of bacterial Ags in the joints using MEKC, a high-resolution separation technique widely used for trace analysis in biological samples (38). We detected *Yersinia* LPS at day 14 postinfection in the joints of WT and *TNFRp55*$^{-/-}$ mice, with a higher concentration of this bacterial component in mice lacking TNFR p55. This result is compatible with the impaired bacterial clearance previously found in these mice (8). As a consequence, bacterial Ags can trigger joint inflammation, as in other pathological conditions, such as Lyme arthritis (39). These results are in keeping with our previous observations on the arthritogenic properties of *Yersinia* LPS (23) and the relevance of *Yersinia* outer membrane Ags in triggering ReA in *TNFRp55*$^{-/-}$ mice (8). Importantly, LPS could not be detected at day 21 postinfection, suggesting that early stimulation with *Yersinia* Ags may be responsible for triggering the immunopathogenic mechanisms implicated in the chronicity of ReA (28).

The identification of the pathogenic Th17 subset capable of inciting and perpetuating tissue injury via the release of IL-17 (9, 40) suggested the possibility that IL-17 may be involved in chronic *Yersinia*-induced ReA observed in *TNFRp55*$^{-/-}$ mice. In addition, *Y. enterocolitica* induces Th1 response in C57BL/6 mice

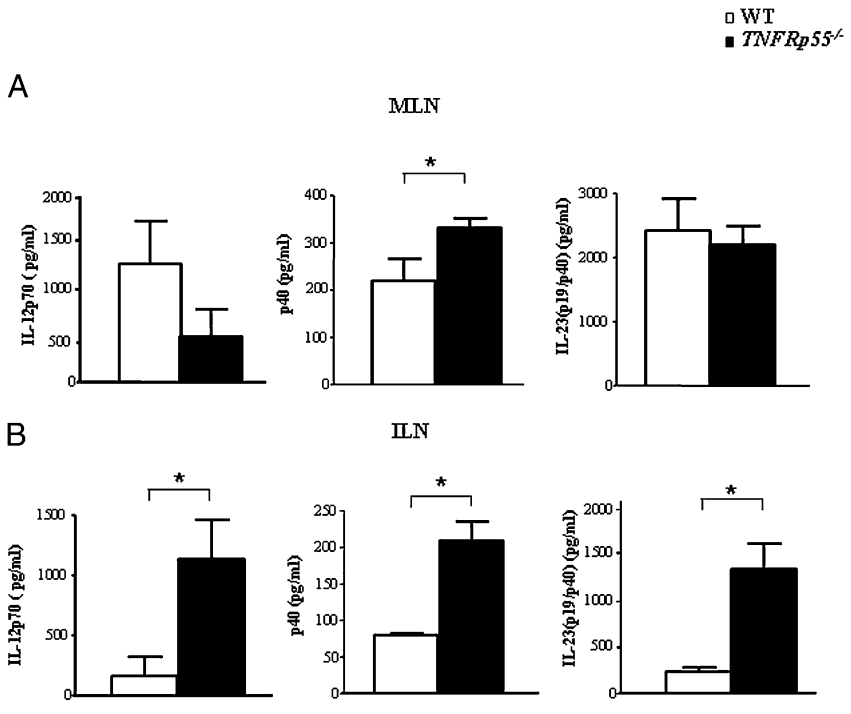

FIGURE 9. Lack of TNFR p55 increases the IL-12/IL-23 p40 common subunit, IL-12p70 and IL-23(p19/p40) during *Yersinia*-induced ReA. Levels of p40, IL-12p70, and IL-23 (p19/p40) were assessed in homogenates of MLNs (*A*) and ILNs (*B*) from WT and *TNFRp55*$^{-/-}$ mice at day 21 postinfection. Values represent the mean ± SD of four or five mice per group evaluated each day postinfection. Results are representative of three experiments. $*p < 0.05$.

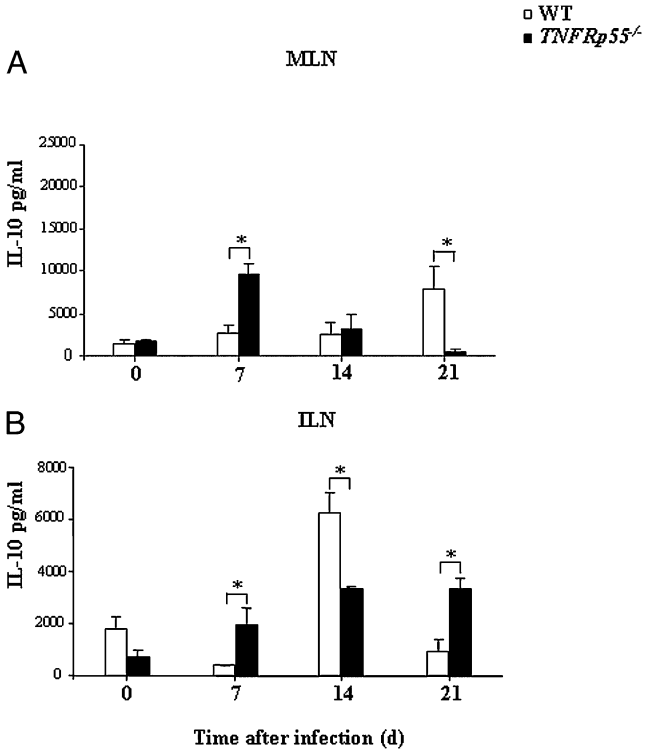

FIGURE 10. Lack of TNFR p55 regulates IL-10 production during *Yersinia*-induced ReA. IL-10 concentration was assessed in cellular homogenates of MLNs (*A*) and ILNs (*B*) from WT and *TNFRp55*$^{-/-}$ mice at days 7, 14, and 21 postinfection. Values represent the mean ± SD of four or five mice per group evaluated each day postinfection. Results are representative of three experiments. $*p < 0.05$.

(18), and IFN-γ regulates IL-17–producing T cells (41). To investigate the pathogenic role of Th17 and Th1 responses as potential mechanisms underlying the exuberant inflammatory responses unleashed by TNFR p55 deficiency, we performed a comparative kinetic analysis of the levels of IL-17 and IFN-γ in the joints of WT and *TNFRp55*$^{-/-}$ mice. Remarkably, *Yersinia* infection triggered an increase in IL-17 and IFN-γ; however, IL-17 closely paralleled the clinical score of arthritis at day 21 postinfection (a readout indicating chronic arthritis). These results are in keeping with those obtained by Abdollahi-Roodsaz et al. (28), who demonstrated IL-17–dependent T cell-driven processes during the chronic phase of arthritis in other experimental models. On the contrary, IFN-γ was higher at day 14 postinfection in mice lacking TNFR p55 signaling, suggesting that Th1-type cytokines might contribute to the more acute joint inflammation observed in *TNFRp55*$^{-/-}$ mice. Whether these cytokines may arise as a result of the absence of protective TNF signaling and whether they may synergize to compensate for the lack of TNF functions remain to be ascertained. Notably, the coexistence of Th17- and Th1-type cytokines in the absence of TNFR p55 signaling contrasts with the widely recognized counterregulation of IL-17–producing cells by IFN-γ (9); however, it was recently reported that these pathogenic effector cells may indeed coexist, particularly in the absence of other regulatory signals, such as those triggered by TNF (42). In fact, the tendency toward reduced levels of IFN-γ, but higher levels of IL-17, on day 21 in the joints of *TNFRp55*$^{-/-}$ mice may indicate a compensatory mechanism of counterregulation of proinflammatory cytokines (43). The higher Ag-specific IL-17 and IFN-γ production detected in *TNFRp55*$^{-/-}$ mice was also confirmed in cultures of splenocytes. Interestingly, the substantially higher amounts of IL-17 and IFN-γ in *TNFRp55*$^{-/-}$ mice indicate a role of the TNF–TNFR p55 pathway as a negative regulator of Th1- and Th17-type cytokines. These results are in line with those recently reported in a model of collagen-induced arthritis (42). Of note, our data support the concept that, under certain circumstances, proinflammatory cytokines may also display double-edged effects and have regulatory attributes (44). Interestingly, we also found a substantial increase in IL-6 and IL-1β expression in *TNFRp55*$^{-/-}$ mice. However, the kinetics of these cytokine were not in line with arthritis progression, suggesting a secondary role for IL-6 and IL-1β in *Yersinia*-induced ReA in *TNFRp55*$^{-/-}$ mice.

The differentiation of naive CD4 T cells into specialized effector subsets is controlled, in part, by cytokines present at sites of Ag presentation (45). Although mouse Th17 cells require IL-6 and TGF-β_1 for their initial in vitro differentiation and IL-23 for their expansion (46), the major cytokines that prime commitment of this lineage in response to in vivo infection are poorly understood (45). The concomitant increase in IL-17, as well as in IL-6 and TGF-β_1, in the joints of *TNFRp55*$^{-/-}$ mice confirmed the requirement of the latter for sustained IL-17 secretion (46).

Because ReA is induced following oral infection, we also conducted a comparative analysis of the cytokine profile in MLNs and ILNs, as representative of mucosal and joint draining lymph nodes, respectively. Interestingly, we found a strong correlation among IL-17, IL-6, and TGF-β_1 in MLNs and ILNs at early stages of *Yersinia* infection (day 7) and during the arthritis onset (day 14), supporting a link between gut and joint immune responses.

In accordance with Notley et al. (42), the coexistence of IL-17 and IFN-γ challenges the assumption that differentiation of these two subsets is mutually exclusive. In fact, more CD4$^+$IL-17$^+$ cells and CD4$^+$IFN-γ$^+$ cells was concurrently found in *TNFRp55*$^{-/-}$ mice, indicating that Th1 and Th17 cells might contribute to sustain joint inflammation in these mice. Moreover, the higher number of IL-17$^+$IFN-γ$^+$CD4$^+$ T cells in *TNFRp55*$^{-/-}$ mice indicates that these cytokines are produced by double-positive (Th1/Th17) T cells, suggesting a plasticity in T cell cytokine secretion and/or a possible developmental association between the Th1 and Th17 phenotypes; this finding is supported by in vivo studies in other mouse models (47). Importantly, Ab-mediated blocking experiments revealed a considerable protection from joint damage when IL-17, IFN-γ, or both cytokines were neutralized in vivo. These protective effects support a role for IL-17 and IFN-γ in ReA progression in the absence of TNFR p55 signaling, as demonstrated for other inflammatory diseases (48, 49).

Interestingly, recent studies reported defects in IFN-γ regulation in patients with other types of spondyloarthropathies, including ankylosing spondylitis (50). Thus, in a broader sense, our findings contribute to the understanding of the pathogenesis of ReA, and they have profound therapeutic implications, because specific blockade of the TNF pathway in rheumatic disorders might lead to exacerbation of pathogenic Th1 and Th17 responses (44). In contrast, recent studies suggested that a cross-talk among distinct regulatory T cell populations in the control of autoimmune inflammation (51). Accordingly, it was demonstrated that IL-6 and TGF-β_1 upregulate IL-10 (52), and TGF-β_1 amplifies IL-10–producing regulatory-type 1 cells (53). Given the alterations observed in these cytokines in our model, further experiments are warranted to elucidate the influence of TNFR p55 deficiency in regulatory T cell expansion in chronic ReA.

In the search for potential mechanisms responsible for the augmented Th17 and Th1 responses in *TNFRp55*$^{-/-}$ mice, we assessed the expression of p40, a common subunit of IL-12 and IL-23 heterodimeric cytokines, with respective Th1- and Th17-promoting activity (13). Given the upregulation of Th1 and Th17 responses in *TNFRp55*$^{-/-}$ mice, we hypothesized that p40 may act as a common mediator linking TNF signaling with Th1/Th17 responses.

Importantly, we detected a substantial increase in p40 at day 21 in MLNs and ILNs of *TNFRp55*$^{-/-}$ mice, and this effect correlated with increased amounts of IL-12p70 and IL-23(p19/p40) in ILNs of these mice. These data substantiate a role for the TNFR p55 pathway in silencing p40 production in vivo and provide a rational explanation for the hyper-Th1 and hyper-Th17 responses observed in *TNFRp55*$^{-/-}$ arthritic mice. These findings are consistent with recent studies implying a functional cross-talk between TNF and IL-12p40 in in vitro studies (54), collagen-induced arthritis (42), experimental autoimmune encephalomyelitis (55), and streptococcal cell wall-induced arthritis (32). In this regard, we observed a self-limiting *Yersinia*-induced ReA in *p40*$^{-/-}$ mice (14). Taken together, these data and the severe and progressive arthritis that developed in *TNFRp55*$^{-/-}$ mice indicate a critical role for this common cytokine subunit in modulating Th1 and Th17 responses during the development of ReA.

In conclusion, our findings demonstrate that, in the absence of TNF signaling, redundant pathways, particularly Th17 and Th1 effector cells, may act in concert to sustain inflammation in bacterial-induced ReA, suggesting caution in the use of TNF blockers in cases of acute or chronic ReA.

Disclosures

The authors have no financial conflicts of interest.

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