TNFα but not IL-17 is critical in the pathogenesis of rheumatoid arthritis spontaneously occurring in a unique FcγRIIB-deficient mouse model

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Abstract

Objective TNFα and IL-17 have been shown to be the major inflammatory cytokines involved in the pathogenesis of rheumatoid arthritis (RA). Here, we examined the effect of these cytokines on spontaneously occurring RA in our newly established arthritis-prone FcγRIIB-deficient C57BL/6 (B6) mice, designated KO1, by introducing genetic deficiency of TNFα and IL-17 into KO1 mice.

Methods KO1.TNFα-/- and KO1.IL-17-/- mice were established by crossing KO1 with TNFα-deficient and IL-17-deficient B6 mice, respectively. The incidence and severity of RA, cartilage and bone destruction, immunological abnormalities, and transcription levels of receptor activator of NF-κB lig and (RANKL)/osteoprotegerin (OPG) and inflammatory cytokines/chemokines in ankle joints were compared among KO1, KO1.TNFα-/-, and KO1.IL-17-/- mice.

Results The development of RA was completely inhibited in KO1.TNFα-/- mice. In contrast, KO1.IL-17-/- mice unexpectedly developed severe RA comparable to KO1. Compared with those in KO1 and KO1.IL-17-/- mice, frequencies of peripheral monocytes, known to be containing osteoclast precursors, were significantly decreased in KO1.TNFα-/- mice. Intriguingly, while RANKL expression levels in ankle joints did not differ among the three strains, OPG expression levels were drastically decreased in arthritis-prone, but not arthritis-free, mice. The expression levels of inflammatory cytokines/chemokines, such as MCP-1, IL-6, and TNFα, were up-regulated in arthritis-prone mice.

Conclusion TNFα is indispensable while IL-17 is dispensable in the pathogenesis of RA in KO1 mice. In this model, TNFα may contribute to the development of arthritis, through mediating the increase in frequencies of osteoclast precursors in circulation and their migration into the joints, and the decrease in OPG expression, leading to the up-regulated osteoclastogenesis associated with severe cartilage and bone destruction.
**Introduction**

Rheumatoid arthritis (RA) is one of the most serious systemic autoimmune diseases, characterized by marked synovial hyperplasia associated with inflammatory cell infiltration in multiple synovial joints, followed by the progressive destruction of cartilage and bone. Accumulating evidence shows that the generation and activation of osteoclasts in inflamed joint tissues are essential for bone loss in RA. Osteoclasts are multinucleated giant cells positive for tartrate-resistant acid phosphatase (TRAP) and cathepsin K, and resorb bone matrix [1, 2]. These cells differentiate from osteoclast precursors of monocyte/macrophage lineage cells in the bone marrow [3] and in peripheral blood [4]. The process of osteoclastogenesis is controlled by the interaction of receptor activator of NF-κB (RANK) expressed on osteoclast precursors with its ligand RANKL expressed on synovial fibroblasts, osteoblasts, and Th17 cells [5-7]. RANKL-mediated osteoclastogenesis is counterbalanced by the physiologically expressed decoy receptor, osteoprotegerin (OPG) [8], and also by several cytokines induced by activated immune cells, such as IFNγ [9].

In the last decade, TNFα has been shown to play a key role in the pathogenesis of RA. Clinical trails have shown that blockade of TNFα is successful in the treatment of
RA [10-12] and is able to modulate the RANKL/OPG system in favor of bone formation [13]. However, the therapies are not always effective in all patients [14]. Experimentally, anti-TNFα therapy has been shown to be effective in type II collagen-induced arthritis (CIA) mouse model [15, 16]. Furthermore, TNFα-deficient mice showed a significantly reduced degree of CIA arthritis; however, severe disease still developed in some individuals [17]. In the K/BxN serum transfer arthritis model, a proportion of TNFα-deficient mice were reported to develop robust disease [18]. Intriguingly, the TNFα deficiency did not affect disease development at all in arthritis observed in an RA model of human T-cell leukemia type I (HTLV-1) transgenic mice [19].

Recently, the cytokine IL-17 has attracted attention in relation to the pathogenesis of RA. IL-17 is a proinflammatory cytokine, produced by a variety of immune cells, such as Th17 cells, macrophages, NK cells, NKT cells and γδ-T cells [20]. Since the discovery of IL-17, many studies have shown that, while IL-17 plays a role in host defense in many infectious diseases [21], it promotes the inflammatory process in several autoimmune diseases, such as RA, systemic lupus erythematosus (SLE), inflammatory bowel disease and experimental autoimmune encephalomyelitis [22].
Among these, RA has been intensively studied because Th17 cells can express RANKL and induce osteoclastogenesis [7]. Early studies showed that high levels of IL-17 are detected in the synovial tissues from RA patients, and that the culture medium of RA synovial tissues induces osteoclast generation in vitro [23].

To confirm the roles of IL-17 in RA pathogenesis, the genetic deficiency of IL-17 was introduced into several murine RA models. The development of arthritis observed in RA models of IL-1 receptor antagonist-deficient mice [24] and ZAP-70-mutant SKG mice [25] was completely inhibited, and the arthritis in HTLV-1 transgenic mice [19] and CIA mice [26] was moderately inhibited. In contrast, in the case of human cartilage proteoglycan-induced arthritis, both inflammation and bone erosion were not suppressed in IL-17 deficiency [27].

Since RA is a multifactorial disease under the control of multiple susceptibility genes and environmental factors [28] and since the same RA phenotype may appear under different mechanisms due to different sets of susceptibility genes and environmental factors, further studies are required to clarify the mechanism of the effects of RA-related proinflammatory cytokines using several kinds of RA mouse models. We recently found that a C57BL/6 (B6) mouse strain genetically deficient in
inhibitory IgG Fc receptor IIB (FcγRIIB), designated KO1, spontaneously develops severe RA closely resembling human RA [29]. Our model is unique, because another FcγRIIB-deficient B6 strain of mice has been reported to develop severe SLE but not RA [30, 31]. The mechanism responsible for this disease phenotype difference remains undetermined; however, it is likely that these two sublines have the shared and disease-specific predisposition toward RA and SLE. Indeed, we found that the introduction of autoimmune susceptible Yaa mutation into KO1 mice causes the disease phenotype conversion from RA to SLE [32]. In the present study, we examined the effects of IL-17 and TNFα on RA using our unique arthritis-prone mouse model by introducing genetic deficiency of IL-17 and TNFα.

**Materials and methods**

**Mice**

Arthritis-prone KO1 is an FcγRIIB-deficient B6 congenic line [29], obtained by backcrossing the originally constructed FcγRIIB-deficient mice on a hybrid (129 x B6) background [33] into a B6 background for over 12 generations. KO1.IL-17−/− mice were established by crossing KO1 with IL-17−/− mice on a B6 background [34], a kind gift
from Dr. Nakae, the University of Tokyo. KO1.TNFα−/− mice were established by
crossing KO1 with TNFα−/− mice on a B6 background [35], obtained from Dr. Iwakura,
Tokyo University of Science. Female mice were analyzed in the present study. All mice
were housed under identical specific pathogen-free conditions, and all experiments were
performed in accordance with our institutional guidelines.

**Scoring of arthritis**

Ankle joint swelling was examined by inspection and arbitrarily scored as follows: 0, no
swelling; 1, mild swelling; 2, moderate swelling; and 3, severe swelling. Scores for both
ankle joints were summed for each mouse, and mice with the score 2 or over were
considered positive for arthritis.

**Histopathology and tissue immunofluorescence**

For histological examination, joint tissues were decalcified in 10% EDTA in 0.1 M Tris
buffer (pH 7.4), and fixed in 4% paraformaldehyde and embedded in paraffin. Tissue
sections were stained with hematoxylin/eosin, and also stained for TRAP using
TRAP/ALP stain kit (Wako Pure Chemical Industries Ltd.). For immunofluorescence of
spleen, tissues were embedded in Tissue-Tek OCT compound and frozen in liquid
nitrogen. Frozen sections were three-color stained for 30 min at room temperature with
Alexa 488-labeled anti-CD4 and anti-CD8 mAbs, Alexa 647-labeled anti-B220 mAb and Alexa 546-labeled PNA. Antibodies and PNA were purchased from BD Pharmingen and Vector Laboratories Inc. (Burlingame, CA, USA), respectively, and the labeling of these reagents was carried out in our laboratory. Color images were obtained using laser scanning microscopy (LSM510META Ver. 3.2, Carl Zeiss Co., Ltd., Germany).

**Serum levels of antibodies**

Serum levels of IgG class anti-cyclic citrullinated peptide (CCP) antibodies and rheumatoid factor (RF) were measured employing commercially available kits (Cosmic Corporation and Shibayagi, respectively), and are expressed as optical density.

Serum levels of IgG antibodies against type II collagen (CII) and double-stranded (ds) DNA were measured using an ELISA plate pre-coated with 10 µg/ml bovine CII (Sigma-Aldrich) and 5 µg/ml bovine dsDNA (Sigma-Aldrich). CII-binding activities are expressed as optical density. DNA-binding activities are expressed in units, as previously described [29].

**Flow cytometric analysis**

Spleen cells were four-color stained with the following mAbs: anti-B220, anti-CD69, anti-CD138, anti-CD4, anti-ICOS, and ant-PD1, and with peanut agglutinin (PNA). For
peripheral monocyte staining, peripheral leukocytes were four-color stained with the following mAbs: anti-CD11b, anti-Gr1, anti-FcγRIV (9E9), anti-CCR2, and anti-CX3CR1. Stained cells were analyzed using a FACSARia cytometer and FlowJo software (Tree Star Inc.).

**Quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was isolated from ankle joints using QIAGEN RNeasy Lipid Tissue Minikit (Cat. No. 74804). Briefly, ~25 mg of ankle joint tissue was added in 500 µl of QIAzol lysis reagent in a 2 ml tube containing 5-mm-diameter zirconia beads (Hirasawa YTZ-5) and homogenized on TissueLyser (Qiagen) for 1 min at 30 Hz. Total RNA was extracted from homogenized materials using Minikit according to the manufacturer’s instructions, and the single-stranded cDNA was synthesized using an oligo(dT)-primer with Superscript II First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). The cDNA product was used for qRT-PCR. The data were normalized to β-actin reference. The primer pairs used and the length of PCR products are shown in Table 1. The quantity was normalized using the formula of the $2^{-\Delta\Delta CT}$ method. Values of B6 mice were designated as 1, and values of KO1, KO1.IL-17$^{-/-}$ and KO1.TNFα$^{-/-}$ mice are evaluated as fold change compared with the values in B6 mice.
Statistics

Statistical analysis was carried out using Mann-Whitney’s U test for antibody levels and qRT-PCR analysis, and Student’s t-test for flow cytometric analysis. A value of \( P < 0.05 \) was considered as statistically significant.

Results

Comparison of disease incidence and severity among KO1, KO1.IL-17\(^{+/−}\), and KO1.TNF\(\alpha\)^\(^{−/−}\) mice

Fig. 1a compares the cumulative incidence and severity of arthritis in ankle joints among the three strains. KO1 mice spontaneously developed severe arthritis with swelling and limited mobility of ankle joints symmetrically after 5 months of age, and severity of arthritis increased with aging. The arthritis observed in KO1 mice was almost completely inhibited in KO1.TNF\(\alpha\)^\(^{+/−}\) mice and there was no evidence of arthritis even by 12 months of age. In contrast, KO1.IL-17\(^{+/−}\) mice unexpectedly developed severe arthritis with comparable incidence and severity to that observed in KO1 mice. Fig. 1b shows representative macroscopic findings of hind paws of KO1, KO1.IL-17\(^{+/−}\), and KO1.TNF\(\alpha\)^\(^{−/−}\) mice at 10 months of age. Histological examination revealed severe
synovitis with destruction of cartilage and bone due to remarkable pannus formation in KO1 and KO1.IL-17\(^{-/-}\), but not KO1.TNF\(\alpha\)^{-/-}, mice (Fig. 1c upper panel). TRAP staining showed an increase in the number of TRAP-positive osteoclasts at the erosion site of cartilage and bone in KO1 and KO1.IL-17\(^{-/-}\) mice (Fig. 1c lower panel).

**Activation status of lymphocytes and serum autoantibody levels**

Fig. 2a shows immunohistochemical findings of spleen sections. Age-associated germinal center (GC) formation was found in KO1 and KO1.IL-17\(^{-/-}\) mice. In contrast, spontaneous GC formation was not observed in KO1.TNF\(\alpha\)^{-/-} mice, a finding consistent with a previous report [35]. Table 2 compares the spleen weight and the frequencies of lymphocyte subsets among the three strains. KO1 mice developed splenomegaly, and the spleen weight was significantly suppressed in KO1.IL-17\(^{-/-}\) and KO1.TNF\(\alpha\)^{-/-} mice. As for B-cell subsets, while there was no difference in the frequencies of total B cells among the three strains, the frequencies of CD69\(^{+}\) activated B cells and PNA\(^{+}\) GC B cells per total B cells were significantly lower in KO1.TNF\(\alpha\)^{-/-} mice than those in KO1 mice. These differences were not observed between KO1 and KO1.IL-17\(^{-/-}\) mice. There was no difference in the frequencies of CD138\(^{+}\) plasma cells, total T cells, CD69\(^{+}\) activated CD4\(^{+}\) T cells and ICOS\(^{+}\)PD1\(^{+}\)-phenotype CD4\(^{+}\) T cells among the three
strains.

Fig. 2b compares serum levels of IgG class RF and IgG class autoantibodies against CCP, CII and dsDNA at 8 months of age. Levels of all autoantibodies in KO1, KO1.IL-17^{−/−} and KO1.TNFα^{−/−} mice were significantly higher than those in normal B6 mice. There was no significant difference in these autoantibody levels among KO1, KO1.IL-17^{−/−} and KO1.TNFα^{−/−} mice, indicating that there was no association between the levels of these autoantibodies and the development of RA in our mouse models.

**Frequencies of peripheral monocytes**

To clarify the mechanism involved in the disease suppression in KO1.TNFα^{−/−} mice, we then examined the frequency of peripheral CD11b^{+} monocytes, since peripheral monocytes contain osteoclast precursors [4]. As shown in Fig. 3a, flow cytometric analysis revealed that the frequencies of CD11b^{+} monocytes of especially Gr1^{-} subsets were increased in KO1 and KO1.IL-17^{−/−} mice at 8~9 months of age; however, this was not observed in KO1.TNFα^{−/−} mice. Fig. 3b showed the significant difference in the frequencies of CD11b^{+} monocytes between arthritis-prone and arthritis-free mice. Fig. 3c shows the expression levels of stimulatory IgG Fc receptor, FcγRIV, and chemokine receptors, CCR2 and CX3CR1, in the CD11b^{+} gated cell population, indicating that
Gr1\(^+\) monocytes were Fc\(\gamma\)RIV\(^+\)CCR2\(^+\)CX3CR1\(^{\text{dull}}\), while Gr1\(^-\) monocytes were Fc\(\gamma\)RIV\(^+\)CCR2\(^-\)CX3CR1\(^{\text{high}}\). These two phenotypically different subsets correspond to the previously reported monocyte subsets; the former is the “inflammatory” subset that has the capacity to migrate to the inflamed tissues because of the high expression of CCR2 (MCP-1 receptor), and the latter is the “resident” subset that is recruited to the non-inflamed tissues [4, 36].

**qRT-PCR analysis of RANKL/OPG and cytokine/chemokine mRNA expression levels in ankle joints**

To evaluate the mRNA expression levels, qRT-PCR analysis was performed using mRNA extracted from ankle joint tissues of each of the KO1, KO1.IL-17\(^{-/-}\) and KO1.TNF\(\alpha\)^\(-/-\) mice, as well as normal B6 mice as a relative control. We first examined the relationship between RANKL/OPG expression levels and the development of RA. The expression levels in KO1, KO1.IL-17\(^{-/-}\) and KO1.TNF\(\alpha\)^\(-/-\) mice were evaluated as fold change compared with the level in B6 mice tentatively designated as 1. As shown in Fig. 4a, while the RANKL expression levels in the three strains were similar to that in normal B6 mice and there was no strain difference in RANKL expression levels, OPG expression level was significantly suppressed in KO1 and KO1.IL-17\(^{-/-}\) mice with
severe arthritis, but not in arthritis-free KO1.TNFα^{−/−} mice.

As MCP-1 and RANTES are known to be the major chemokines involved in the pathogenesis of RA [37], we then compared the expression levels of MCP-1 and RANTES in ankle joints. As shown in Fig. 4b, the MCP-1 expression level was markedly up-regulated in arthritis-prone KO1 and KO1.IL-17^{−/−} mice, compared with the finding in arthritis-free KO1.TNFα^{−/−} mice. In contrast, there was no difference in RANTES expression levels among the three strains of mice.

Fig. 4C compares the expression levels of IL-6, IL-10, IFNγ and TNFα among the three strains of mice. IL-6 expression level was up-regulated in arthritis-prone KO1 and KO1.IL-17^{−/−} mice, but the level in KO1.TNFα^{−/−} mice was similar to that in normal B6 mice. IL-10 expression levels in the three strains were higher than that in B6 mice; however, there was no difference in expression levels among the three strains. Intriguingly, IFNγ expression level was up-regulated in arthritis-free KO1.TNFα^{−/−} mice. The levels of TNFα were up-regulated in arthritis-prone KO1 and KO1.IL-17^{−/−} mice. As for IL-17 expression, there was no detectable level in ankle joint tissues from all the mouse strains examined.
Discussion

Disease features of RA were completely inhibited in KO1 mice with TNFα deficiency, indicating a pivotal role of TNFα in the pathogenesis of RA in KO1 mice. In contrast, RA features were seldom suppressed in IL-17-deficient KO1 mice. On the basis of studies using several kinds of the arthritis mouse model, it has been shown that IL-17 plays a central role in the development of RA through activating synoviocytes, fibroblasts, endothelial cells and macrophages to induce TNFα, which subsequently induces inflammation and osteoclast differentiation in multiple synovial joints [14, 22]. However, the present study showed that severe RA developed in KO1 mice in the absence of IL-17, showing that IL-17 is dispensable in the pathogenesis of RA in this model.

qRT-PCR analysis revealed that there was no detectable level of IL-17 mRNA expression in arthritic joints of arthritis-prone KO1 and non-arthritis KO1.TNFα−/− mice. This is consistent with our earlier findings in flow cytometric analysis of cytokine-producing CD4+ T cells in inflamed synovial tissues from KO1 mice, revealing that nearly 30% of the cells were positive for TNFα, while IL-17-producing cells were seldom observed [29]. The current studies clearly indicated that several different mechanisms underlie the processes of joint inflammation and osteoclastogenesis in
clinically similar RA individuals. Recent phase II clinical studies of RA patients under anti-IL-17 antibody treatments showed variable results [38], suggesting the presence of different disease mechanisms underlying in individual patients.

Recently, Pisitkun et al. [39] reported that the IL-17 deficiency protected SLE-prone FcγRIIB-deficient B6 mice from kidney damage, even though this did not prevent the spontaneously developing GC formation and autoantibody production. The protection from kidney disease was suggested to be due to the decrease in inflammatory cell infiltration in the kidney. Consistent finding in our model is no influence on the spontaneous formation of GCs and the production of autoantibodies; however, the IL-17 deficiency did not protect KO1 mice from RA with respect to its incidence, severity, and histopathology of the joint disease. Taken collectively, it is clear that the level of IL-17 production shows differential effects between SLE and RA seen in the two different sublines of FcγRIIB-deficient B6 mice. As these two sublines of FcγRIIB-deficient B6 mice appear to carry similar genetic background, different environmental factors may underlie the difference in their disease phenotypes. Intriguingly, Ivanov et al. [40] reported that B6 mice obtained from different commercial vendors may have different commensal intestinal bacteria, and that this difference affects the immune system, most
strikingly, IL-17 production, which may possibly affect the specificity and severity of autoimmune diseases.

Discrepancy between the levels of autoantibody production and GC formation in the spleen was observed in KO1.TNFα–/– mice. KO1.TNFα–/– mice had no GC formation, in keeping with previous reports [35, 41]. Despite the lack of GC formation, KO1.TNFα–/– mice showed comparable serum levels of autoantibodies to those found in KO1 and KO1.IL-17–/– mice. These findings suggest that the production of autoantibodies could be performed by extrafollicular B cells, as reported elsewhere [42]. TNFα stimulates the vascular endothelium to express adhesion molecules and to accelerate the inflammatory cell migration from circulation into the inflammatory site [19]. Thus, KO1.TNFα–/– mice did not develop arthritis because of the lack of inflammatory cell infiltration, irrespective of the deposition of ICs in the joint tissue.

FcγRIIB molecules are expressed not only on B cells but also on monocytes/macrophages, and negatively regulate their activation mediated by stimulatory FcγRs and cytokine/chemokine production [43, 44]. Analysis of mice deficient in stimulatory FcγRs demonstrated that monocyte frequencies are increased as a consequence of IC-triggered activation through stimulatory FcγRs [45]. Lack of FcγRIIB
expression may strengthen IC-triggered activation signals from stimulatory FcγRs and accelerate monocytosis. In the present study, age-associated monocytosis was evident in KO1 and KO1.TIL-17−/−, but not KO1.TNFα−/−, mice. It has been shown that monocytosis is one of the characteristic features in SLE-prone mice and well associates with autoantibody production and lupus nephritis [46]. Our present study revealed that monocytosis is also associated with the development of RA, suggesting that peripheral monocytes might be a potential therapeutic target for RA. Monocytosis did not develop in KO1.TNFα−/− mice, irrespective of the lack of FcγRIIB expression and of comparable serum levels of autoantibodies as those found in KO1 and KO1.IL-17−/− mice. Yao et al. [47] reported that the administration of TNFα induces an increase in circulating CD11b+ monocytes. This event is reversible, and anti-TNF therapy reduces monocytosis, owing to inhibition of monocyte mobilization from the bone marrow. Thus, it appears that, in KO1.TNFα−/− mice, monocytosis does not develop even in the presence of circulating ICs.

It has been shown that monocytes in peripheral blood contain osteoclast precursors [4]. Peripheral monocytes are composed of heterogeneous populations and could be subdivided into two phenotypically and functionally distinct subsets in mice, namely a CD11b+CX3CR1lowCCR2+Gr1+ “inflammatory” subset that is actively recruited to
inflamed tissues and a CD11b^CXCR3^{high} CCR2^-Gr1^- “resident” subset that is recruited to non-inflamed tissues [4, 36]. Intriguingly, this latter subset is selectively expanded in lupus-prone mice and expresses the stimulatory FcγRII, suggesting that this subset may be involved in IgG-mediated kidney damage [45]. Furthermore, there is a report that the “resident” subset gives rise to specialized cell types, including osteoclasts [47]. Thus, the originally classified Gr1^- “resident” subset may contain functionally different cell types, one responsible for lupus nephritis and the other osteoclastogenesis in joints. However, further studies are required for the role of the CCR2^-Gr1^+ “inflammatory” subset in osteoclastogenesis, since MCP-1, the CCR2 ligand, is shown to play a role in promoting the fusion of CCR2^+ osteoclast precursors, resulting in an acceleration of multinucleated osteoclast generation [37].

An additional important finding in the present study is that the development of arthritis is associated with OPG, but not RANKL, expression levels. There was no difference in RANKL expression levels among KO1, KO1.IL-17^-/- and KO1.TNFα^-/- mice, while OPG expression levels in arthritis joints in KO1 and KO1.IL-17^-/- mice were markedly decreased, but those in KO1.TNFα^-/- mice were at normal levels comparable to those in B6 mice. These findings suggest that the balance
between RANKL and OPG determines the degree of osteoclastogenesis, in keeping with the findings reported in studies of RA patients under anti-TNF therapy [13].

OPG is produced by a variety of cells including bone marrow stromal cells, B cells, dendritic cells and vascular endothelial cells [48, 49]. Marked decrease in OPG expression in arthritic joints is suggested to be due to the exhaustion of OPG production over extended periods of chronic stimulation by TNFα [49]. In addition, although the precise mechanism for up-regulated expression of IFNγ in joint tissues in KO1.TNFα−/− mice remains unknown, this may also contribute to the inhibitory effect of osteoclastogenesis, since IFNγ inhibits RANK signaling by accelerating the degradation of TRAF6 through activation of the ubiquitin/proteasome system [9]. Thus, TNFα may contribute to the development of arthritis in KO1 mice, through mediating the increase in frequencies of osteoclast precursors in circulation and their migration into the joints, and the decrease in OPG expression, leading to the up-regulation of osteoclastogenesis.

Taken collectively, our RA mouse model is useful for obtaining a more thorough understanding of the complex disease process of RA, and for obtaining precise therapeutic targets for tailor-made therapy of such a complex disease.
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Conflict of interest None.

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Figure legends

**Fig. 1** Comparisons of arthritic changes. **a** The cumulative incidence of arthritis and arthritis score (mean and SE) in KO1 (n=33), KO1.IL-17−/− (n=21) and KO1.TNFα−/− (n=19) mice. **b** Representative macroscopic findings of hind paws in KO1, KO1.IL-17−/−
and KO1.TNFα−/− mice at 10 months of age. KO1 and KO1.IL-17+/−, but not KO1.TNFα−/−, mice developed marked swelling and stiffness of the ankle joints. e Representative histopathological changes in finger joints in KO1, KO1.IL-17+/− and KO1.TNFα−/− mice at 10 months of age. KO1 and KO1.IL-17+/− mice show marked synovitis with inflammatory cell infiltration and the destruction of bone due to pannus formation (Pn) and TRAP-positive osteoclast generation (arrowhead). There are no such changes in KO1.TNFα−/− mice. Representative results obtained from six female mice in each strain. Hematoxylin/eosin and TRAP staining. Bars = 50 µm.

**Fig. 2** Comparisons of spontaneous germinal center formation and serum levels of autoantibodies among KO1, KO1.IL-17+/− and KO1.TNFα−/− mice. a Frozen spleen sections of 10-month-old mice were triple stained with a mixture of anti-CD4 and anti-CD8 mAbs, anti-B220 mAb and PNA to examine the extent of germinal center formation. Representative results obtained from six female mice in each strain. b Serum levels of IgG class RF, anti-CCP, -CII and -dsDNA antibodies were compared at 8 months of age. The horizontal bar represents the mean level. Statistical significance is shown (* P<0.05, ** P<0.01, ***P<0.001).
**Fig. 3** Comparisons of peripheral monocyte frequencies and cell surface phenotypes of monocytes among KO1, KO1.IL-17−/− and KO1.TNFα−/− mice at 8–9 months of age. **a** Peripheral mononuclear cells were double stained with anti-CD11b and -Gr1 mAbs, and frequencies of Gr1⁺CD11b⁺ and Gr1⁻CD11b⁺ monocytes are shown. **b** CD11b⁺ monocyte frequencies are compared (**P<0.01**). **c** Peripheral mononuclear cells were stained with anti-CD11b, -Gr1, -FcγRIIV, -CCR2 and -CX3CR1 mAbs, and cell surface phenotypes were examined using CD11b⁺-gated cells. Results were obtained from at least four mice in each group.

**Fig. 4** Comparisons of mRNA expression levels of **a** RANKL and OPG, **b** MCP-1 and RANTES, and **c** IL-16, IL-10, IFNγ and TNFα in ankle joints by quantitative real-time PCR analysis among KO1, KO1.IL-17−/− and KO1.TNFα−/− mice at 8–9 months of age. The value of normal B6 mice was designated as 1, and values of each of the KO1, KO1.IL-17−/− and KO1.TNFα−/− mice were evaluated as fold change compared with the value in B6 mice. Data are shown as mean ± SEM of four mice for each strain and representative of three experiments performed. Statistical significance was shown...
(**P<0.01, *** P<0.001).
a) KO1, KO1.IL-17⁻/⁻, KO1.TNFα⁻/⁻

B220/PNA/CD4/8

b) Graphs showing:
- RF (OD₄⁵₀)
- Anti-CCP (OD₄⁵₀)
- Anti-CII (OD₄⁵₀)
- Anti-dsDNA Abs. (x10⁻³)

Comparisons:
- B6 vs KO1
- KO1 vs KO1.IL-17⁺
- KO1 vs KO1.TNFα⁺

Significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
(a) Flow cytometry plots showing the percentage of Gr1 and CD11b expression in KO1, KO1.IL17−/−, and KO1.TNFα−/− cells.

(b) Graph showing the percentage of monocytes in KO1, KO1.IL17−/−, and KO1.TNFα−/− cells. The graph indicates statistical significance (**).

(c) Additional flow cytometry plots showing Gr1, FcγRIV, CCR2, and CX3CR1 expression in KO1, KO1.IL17−/−, and KO1.TNFα−/− cells.
Table 2  Spleen weight and splenic lymphocyte subpopulations in KO1, KO1.IL-17\(-/-\) and KO1.TNF\(\alpha\)-/- mice\(^a\)

<table>
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<tr>
<th></th>
<th>KO1</th>
<th>KO1.IL-17(-/-)</th>
<th>KO1.TNF(\alpha)-/-</th>
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<tbody>
<tr>
<td>Spleen weight (gm)</td>
<td>0.28 ± 0.03</td>
<td>0.20 ± 0.02(^b)</td>
<td>0.17 ± 0.02(^b)</td>
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<td>Spleen cell populations</td>
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<tr>
<td>B220(^+) B cells/total cells</td>
<td>60.2 ± 2.8</td>
<td>47.7 ± 3.3</td>
<td>57.4 ± 2.7</td>
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<tr>
<td>CD69(^+)B220(^+) B cells/total B cells</td>
<td>7.4 ± 0.5</td>
<td>8.0 ± 0.9</td>
<td>5.0 ± 0.8(^c)</td>
</tr>
<tr>
<td>PNA(^+)B220(^+) B cells/total B cells</td>
<td>4.5 ± 0.9</td>
<td>3.9 ± 0.5</td>
<td>2.0 ± 0.4(^c)</td>
</tr>
<tr>
<td>CD138(^+) plasma cells/total cells</td>
<td>1.0 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>CD4(^+) T cells/total cells</td>
<td>17.3 ± 2.2</td>
<td>17.3 ± 2.8</td>
<td>13.3 ± 2.0</td>
</tr>
<tr>
<td>CD69(^+)CD4(^+) T cells/total T cells</td>
<td>27.7 ± 1.4</td>
<td>23.0 ± 2.6</td>
<td>27.1 ± 6.8</td>
</tr>
<tr>
<td>CD4(^+)ICOS(^+)PD1(^+) T cells/total T cell</td>
<td>3.2 ± 1.3</td>
<td>2.2 ± 0.6</td>
<td>2.8 ± 1.0</td>
</tr>
</tbody>
</table>

\(^a\)Values are the mean ± SEM of at least 6 female mice aged 9-10 months.

\(^b\)Differences were statistically significant versus KO1 (\(P < 0.01\)).

\(^c\)Differences were statistically significant versus KO1 (\(P < 0.05\)).