ARTICLE

# Macrophages are the primary effector cells in IL-7-induced arthritis

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Synovial macrophages are crucial in the development of joint inflammation and bone damage; however, the pathways that control macrophage remodeling in inflammatory M1 cells or bone-eroding osteoclasts are not fully understood. We determined that elevated IL-7R/CD127 expression is the hallmark of rheumatoid arthritis (RA) M1 macrophages and that these cells are highly responsive to interleukin-7 (IL-7)-driven osteoclastogenesis. We established that lipopolysaccharide (LPS), interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα), the classic M1 macrophage mediators, enhance IL-7R expression in RA and murine macrophages. The local expression of IL-7 provokes arthritis, predominantly through escalating the number of $F480^{+}iNOS^{+}$ cells rather than $CD3^{+}$ T cells. Ectopic LPS injection stabilizes IL-7-induced arthritis by increasing myeloid IL-7R expression, in part via IFNγ induction. Hence, in RAG−/− mice, IL-7-mediated arthritis is suppressed because of the reduction in myeloid IL-7R expression due to the lack of IFNγ. Moreover, the amelioration of IL-7-induced arthritis by anti-TNF therapy is due to a decrease in the number of cells in the unique $F480^{+}iNOS^{+}IL\text{-}7R^{+}CCL5^{+}$ subset, with no impact on the $F480^{+}Arginase^{+}$ cell or $CD3^{+}$ T cell frequency. Consistent with the preclinical findings, the findings of a phase 4 study performed with RA patients following 6 months of anti-TNF therapy revealed that IL-7R expression was reduced without affecting the levels of IL-7. This study shifts the paradigm by discovering that IL-7-induced arthritis is dependent on $F480^{+}iNOS^{+}IL\text{-}7R^{+}CCL5^{+}$ cell function, which activates TH-1 cells to amplify myeloid IL-7R expression and disease severity.

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## INTRODUCTION

Macrophages are a heterogeneous population of myeloid cells that can polarize into different subtypes or be remodeled into bone-resorbing osteoclasts. Macrophages play a central role in rheumatoid arthritis (RA) pathogenesis as key producers of proinflammatory cytokines and precursors of osteoclasts. Hence, effective RA therapy is closely associated with a reduction in the number of synovial tissue (ST) macrophages.[1–3]

Macrophages are plastic cells that are classified into two distinct phenotypes, which are contingent on their microenvironment. Classically activated M1 macrophages express cell surface markers such as CD80 and CD86 and secrete tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1β, C-C motif chemokine ligand 2 (CCL2), IL-8, IL-12, and IL-23 upon activation with interferon-γ (IFNγ), LPS, and granulocyte–macrophage colony-stimulating factor. Nonclassical M2 macrophages are distinguished by the markers CD206, CD209, and dectin1 and produce tumor growth factor-β and IL-10 upon IL-4, IL-13, glucocorticoid, IL-10, and macrophage colony-stimulating factor (M-CSF) stimulation.[4,5]

Earlier studies have shown that the phenotype of RA ST macrophages is heterogeneous and dependent on patient disease activity and response to therapy.[6] More recent studies have shown that macrophages residing in RA synovial fluid (SF) are predominantly of the M1 phenotype and that the induction of interferon regulatory factor 5 activity by anti-citrullinated protein antibodies is partly responsible for M1 polarization.[7] Other studies have demonstrated that fucosyltransferase 1 (FUT1), FUT3, FUT7, and FUT9 are highly expressed in RA SF M1 macrophages ($CD68^{+}CD80^{+}$) and that their expression positively correlates with TNF transcription in RA ST. In collagen-induced arthritis (CIA), the inhibition of fucosylation alleviates arthritis by impairing M1 differentiation.[8]

Osteoclasts are derived from myeloid cells, which fuse to form bone-resorbing cells in the presence of M-CSF and receptor activator of nuclear factor-κB ligand (RANKL) or TNFα.[9–11] It has been shown that the blockade of RANKL function does not impact TNFα-induced osteoclastogenesis. Consistent with this notion, TNFα and IL-1β can substitute for M-CSF and RANKL in the differentiation of RA SF macrophages into fully mature osteoclasts.[3]

Corroborating previous studies,[7,8] we observed that the frequency of M1 macrophages is elevated in RA SF and these

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cells express high levels of IL-7R compared to RA and normal peripheral blood (PB) in vitro differentiated macrophages.[12] Both we and others have shown that the blockade of IL-7 or IL-7R function can relieve CIA joint inflammation and bone erosion.[13,14]

We uncovered for the first time that RA-naive myeloid cells are remodeled into proinflammatory M1 macrophages by IL-7 ligation. We further demonstrated that IL-7-induced M1 macrophages, which express elevated levels of IL-7R, are more responsive to IL-7-mediated osteoclast formation than naive or anti-inflammatory M2 macrophages. We found that the local injection of IL-7 provokes arthritis through escalating the levels of $F480^{+}iNOS^{+}$ cells and their associated factors. Additionally, we showed that LPS and IFNγ potentiate myeloid IL-7R expression and further exacerbate IL-7-mediated joint inflammation and bone erosion by amplifying the number of $F480^{+}iNOS^{+}$ cells, while the frequencies of $F480^{+}Arginase^{+}$ and $CD3^{+}$ cells remain unchanged.

Intriguingly, we discovered a novel population of $F480^{+}iNOS^{+}IL\text{-}7R^{+}CCL5^{+}$ macrophages that are the main instigators of IL-7-driven arthritis. Joint inflammation was reduced but not abrogated in RAG−/− mice or wild-type (WT) mice treated with anti-TNF or anti-IFNγ antibody (Ab) therapy. The suppression of IL-7-mediated arthritis in RAG−/− mice was due to reduced IL-7R expression that resulted from IFNγ deficiency. Similarly, the reduction in the joint IL-7R level was responsible for the amelioration of IL-7-induced arthritis noted in anti-TNF-treated mice. In line with the preclinical findings, the results of a phase 4 study performed with RA patients following 6 months of anti-TNF therapy revealed that IL-7R expression was markedly declined in responsive patients.

We conclude that IL-7 in coordination with other M1 macrophage factors potentiates the expression of joint myeloid IL-7R to further exacerbate arthritis via a unique subset of $F480^{+}iNOS^{+}IL\text{-}7R^{+}CCL5^{+}$ macrophages. Moreover, our results suggest that αIL-7R therapy may be a promising treatment strategy for RA patients whose disease is driven by M1 macrophages and TH-1 cells.

## RESULTS

### IL-7 is a potent inducer of M1 macrophage differentiation

We have previously shown that in vitro-differentiated RA macrophages express higher levels of cell surface IL-7R than RA monocytes.[12] In this study, we documented that IL-7 can further differentiate naive RA macrophages into classical M1 macrophages. Notably, the expression or frequency of M1 markers, such as TNF (3-fold increases in messenger RNA (mRNA) and protein expression), IL-6 (5-fold increase in mRNA and protein expression), CCL2 (2-fold increase in protein expression), CCL5 (3-fold increase in protein expression) and inducible nitric oxide synthase (iNOS) (40%), was markedly enhanced in RA in vitro-differentiated macrophages in response to IL-7 stimulation (Fig. 1a–d). IL-7-induced M1 polarization requires IL-7R (CD127) ligation, as IL-7R expression knockdown cells were responsive to IFNγ/LPS, but incapable of transcribing TNF or IL-6 in response to IL-7 activation (Fig. 1f, g).

In our earlier studies, we found that cell surface IL-7R expression is significantly lower in normal (NL) cells than in RA myeloid cells.[12] Herein, we documented that priming NL-naive macrophages with M1 inducers, namely, IFNγ and/or LPS, can markedly accentuate the frequency of IL-7R surface-expressing cells by 2.5-4-fold (Fig. 1h). Therefore, while NL macrophages primed with M1 inducers responded to IL-7 stimulation and produced TNF and IL-6, unprimed NL cells did not exhibit these properties (Fig. 1i, j).

We next examined murine macrophages and found that, similar to the M1-positive control IFNγ/LPS treatment, IL-7 treatment can remodel mouse bone marrow-derived macrophages (BMMϕs) into the M1 phenotype, resulting in increased levels of TNF (4-fold), IL-6 (24-fold), NOS2/iNOS (5-fold), and CD80 (20%) in the IL-7-treated macrophages compared to the control counterparts (Fig. 2a–c). Moreover, we demonstrated that in IL-7R expression knockdown (Fig. 2e, f) or knockout cells (Fig. 2g–j), IL-7-driven murine M1 polarization is completely abrogated under the unprimed condition. In IFNγ- or LPS-primed BMMϕs (Fig. 2g–j), impairing IL-7R signaling reduced IL-7-induced TNF, IL-6, CCL2, and CCL5 secretion to the baseline levels detected with the priming factor alone. Our results indicate that in human and murine cells, the ligation of IL-7R by IL-7 transforms naive myeloid cells into M1 macrophages. IL-7-mediated M1 differentiation is further potentiated by the elevation of IL-7R expression induced by LPS and IFNγ stimulation.

### Myeloid IL-7R ligation activates RA osteoclast formation

RA PB mononuclear cells (PBMCs; T and myeloid cells) activated with IL-7 can dose-dependently differentiate into mature osteoclasts through a mechanism that was found to be dependent on TNF, RANK, and RANKL (2–3-fold) expression (Fig. 3a–c) and independent of IL-17 (not induced). In addition, we showed that IL-7 can remodel RA monocytes into fully mature osteoclasts in the absence of T cells when the monocytes are cultured under suboptimal conditions (10 ng/ml M-CSF and RANKL; Fig. 3d–f). Consistently, we found that the immunodepletion of IL-7 markedly reduces the ability of RA SF to transform monocytes into RA osteoclasts (Fig. 3g, h). In the absence of T cells, the differentiation of RA monocytes into osteoclasts driven by IL-7 was accompanied by TNF (4-fold), IL-6 (16-fold), and RANK (9-fold) mRNA induction (Fig. 3f). While the transcription of TNF and IL-6 was detectable following IL-7 stimulation in osteoclast precursor cells, the secretion of these factors was shown in cells primed with LPS and/or IFNγ (Fig. 3i, j). In contrast, following IL-7 stimulation, elevated cell surface levels of RANK were observed, and priming was not required for RANK detection (Fig. 3k). Substantiating these findings in RA cells, we also found that the transcription of TNF, IL-6, and RANK is associated with the IL-7-induced differentiation of murine bone marrow progenitor cells into mature osteoclasts (Fig. 3l–o).

Next, experiments were performed to establish whether the blockade of TNF or IL-6R function is capable of nullifying IL-7-induced osteoclastogenesis. Interestingly, while an anti-TNF Ab suppressed osteoclast formation triggered by IL-7, an anti-IL-6R Ab had no impact on this process. Nevertheless, the combination of anti-TNF and IL-6R Abs more potently impaired IL-7-induced osteoclast maturation than the anti-TNF Ab alone (Fig. 4a, b). We conclude that macrophages are the primary cell type contributing to IL-7-modulated osteoclast formation, in part through the upregulation of TNF and RANK expression, in the presence or absence of T cells.

### IL-7-differentiated RA macrophages have a greater potency to mature into osteoclasts than naive RA macrophages

We next asked whether IL-7's ability to polarize naive cells into M1 macrophages is linked to its effect on osteoclastogenesis. Our data showed that IFNγ- and IL-7-primed RA and mouse precursor cells are more responsive to IL-7-mediated osteoclastogenesis than naive or IL-4-pretreated cells (Fig. 4c–f). Regardless of the initial priming factor (IFNγ or IL-7), RA or murine progenitor cells that were differentiated into M1 macrophages had a higher capacity to form osteoclasts in response to IL-7 compared to phosphate-buffered saline (PBS) or IL-4 treated counterparts (Fig. 4c–f). Because TNF played a more intrinsic role in IL-7-driven osteoclast formation than IL-6 (Fig. 4a–d), our results suggested that TNF is of primary importance in the differentiation of M1 macrophages into osteoclasts. Moreover, murine progenitor cells primed with IFNγ may have an additional advantage over cells primed with IL-7, as the IFNγ-primed cells may benefit from M1 macrophage conversion as well as upregulation of myeloid IL-7R that translates into greater IL-7 responsiveness (Fig. 4e, f).

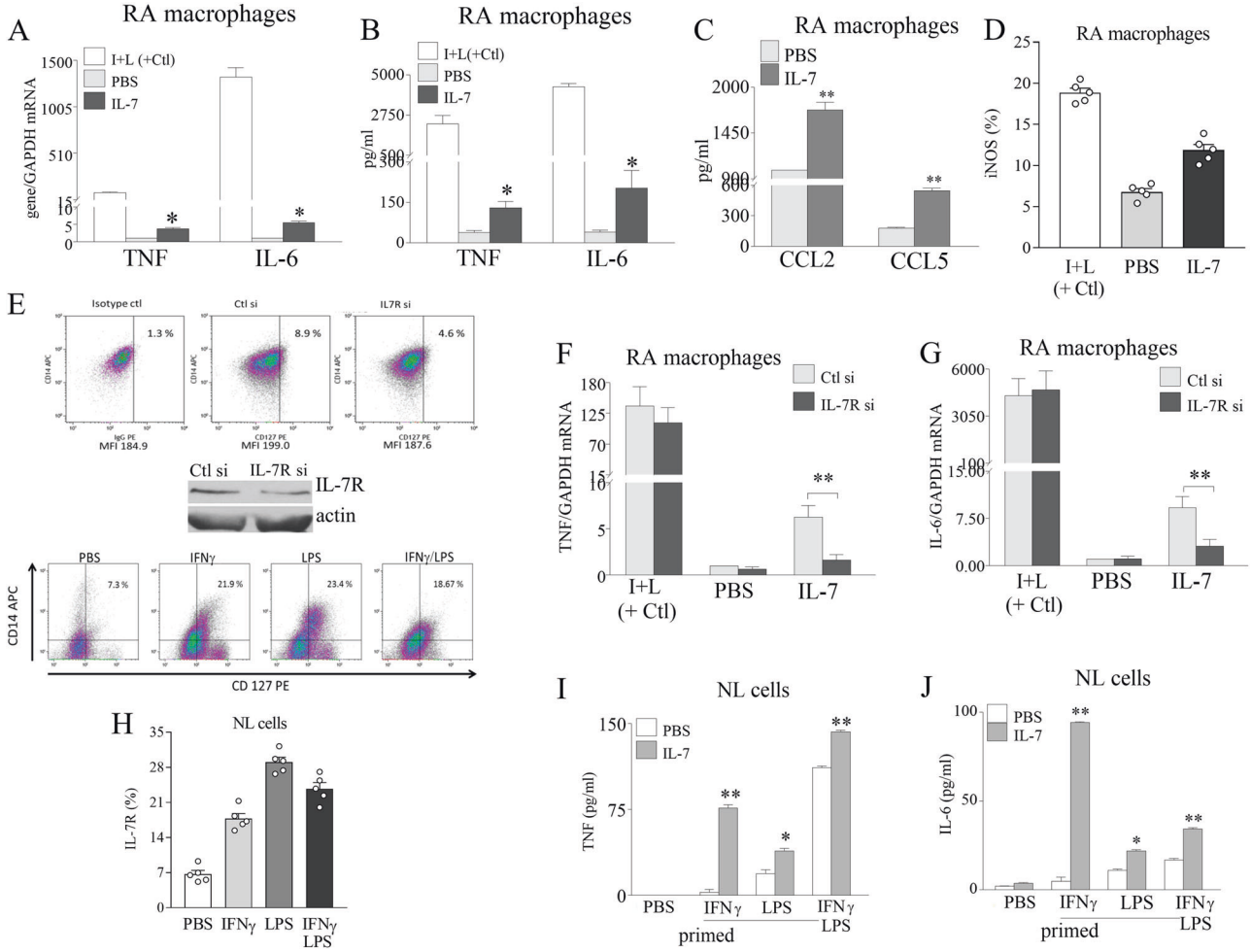


**Fig. 1** Interleukin (IL-7) remodels rheumatoid arthritis (RA) peripheral blood (PB) naive cells into classical M1 macrophages. RA PB in vitro-differentiated macrophages were cultured in the presence of macrophage colony-stimulating factor (M-CSF) (20 ng/ml) for 7 days, and the cells were treated PBS (−control) or with IL-7 (100 ng/ml) and IFNγ/LPS (+control; 100 ng/ml each) for 6 h prior to quantifying the tumor necrosis factor (TNF) and IL-6 messenger RNA (mRNA) levels ($p < 0.05$) by real-time reverse transcription-polymerase chain reaction (RT-PCR), $n = 3$ (**a**), or the cells were treated for 24 h, and the TNF-α and IL-6 ($p \leq 0.05$) (**b**) or C-C motif chemokine ligand 2 (CCL2) and CCL5 ($p < 0.01$) (**c**) protein levels were determined by enzyme-linked immunosorbent assay (ELISA), $n = 5$. **d**. The inducible NO synthase (NOS2/iNOS) frequency was determined by fluorescence-activated cell sorting (FACS) in 7-day RA PB in vitro-differentiated macrophages that were treated with PBS, IL-7 (100 ng/ml) ($p < 0.01$) or IFNγ/LPS (100 ng/ml each), $n = 5$. In vitro-differentiated RA PB myeloid cells were transfected with a control or an IL-7R-specific small interfering RNA (siRNA) (100 nM) on day 4. The cells harvested on day 7 were analyzed for IL-7R expression by Western blotting (1:1000) or FACS analysis (**e**). Transfected cells were treated with IFNγ/LPS (100 ng/ml each), PBS, or IL-7 (100 ng/ml) for 6 h before quantifying the TNF (**f**) and IL-6 ($p < 0.05$ or 0.01) (**g**) transcription levels by real-time RT-PCR, $n = 3$. **h** Seven-day in vitro-differentiated normal (NL) PB macrophages were treated with PBS or 100 ng/ml IFNγ, LPS, or IFNγ/LPS for 24 h, and the frequency of IL-7R$^+$ cells ($p < 0.05$) was quantified by FACS, $n = 5$. Seven-day in vitro-differentiated NL PB macrophages were left untreated (PBS) or primed with 100 ng/ml IFNγ, LPS, or IFNγ/LPS for 24 h before receiving PBS or IL-7 (100 ng/ml) treatment for 48 h. TNF (**i**) and IL-6 ($p < 0.05$ or $p < 0.01$) (**j**) protein expression was determined by enzyme-linked immunosorbent assay (ELISA), $n = 3$. The data are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$

Ectopic expression of IL-7 provokes arthritic bone erosion

We next performed experiments to assess the potential arthritogenicity of IL-7. Local expression of IL-7 increased ankle circumference in mice as early as day 1, and joint inflammation increased progressively until day 6 and plateaued thereafter until day 15 (Fig. 5a, b). Corroborating the clinical data, histological analysis demonstrated that joint inflammation, lining thickness, and bone erosion were markedly aggravated by local IL-7 expression compared to control expression (Fig. 5c, d). We found that the synovial infiltration of F480$^+$ myeloid cells and CD3$^+$ T cells was potentiated in IL-7-treated arthritic mice, with the frequency of the macrophages being 2-fold higher than that of the T cells (Fig. 5e). Moreover, an analysis of joint myeloid cell phenotypes revealed that, although the number of both iNOS$^+$ and Arginase$^+$ cells was increased, IL-7-treated mouse arthritic joints predominately contained iNOS$^+$ M1 macrophages (Fig. 5e, g). Consistent with the dominance of M1 macrophages, factors linked to this phenotype, including TNF-α (mRNA = 790-fold, protein = 2-fold), IL-1β (mRNA = 470-fold, protein = 6.5-fold), CCL2 (mRNA = 770-fold, protein = 5-fold), CCL5 (mRNA = 1500-fold, protein = 18-fold), and NOS2 (mRNA = 1400-fold), were significantly accentuated in the IL-7-treated arthritic mice (Fig. 5h, j). However, there were no differences detected in the IL-6 levels between the IL-7-treated mice and the control mice (Fig. 5h, j). Despite elevated CD3$^+$ T cell infiltration, joint IFNγ and IL-17 levels were not enhanced in the IL-7-treated arthritic mice compared to the control mice harvested on day 15 (Fig. 5i). Joint IL-7R expression was upregulated by 2-fold in the arthritic mice

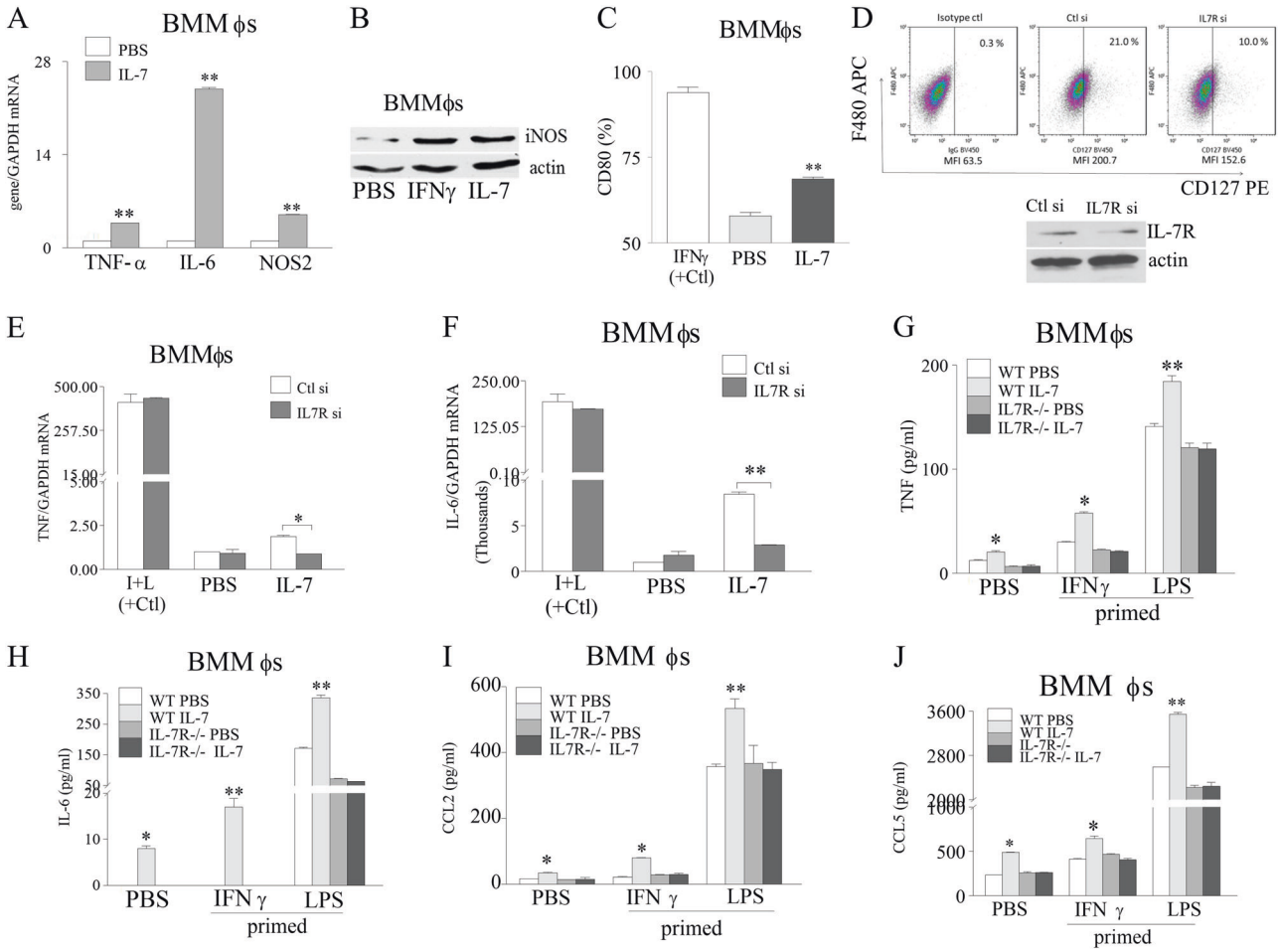


**Fig. 2** Murine bone marrow progenitor cells are transformed into M1 macrophages by interleukin (IL-7) stimulation. **a** Macrophage colony-stimulating factor (M-CSF)-differentiated (20 ng/ml; 7 days) mouse bone marrow macrophages (BMMϕs) were treated with PBS or 100 ng/ml IFNγ/LPS or IL-7 for 6 h, and the tumor necrosis factor (TNF), IL-6, and NOS2 messenger RNA (mRNA) levels ($p < 0.01$) were quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR), $n = 3$. Seven-day M-CSF-differentiated wild-type (WT) BMMϕs were treated with PBS or 100 ng/ml IFNγ (+control) or IL-7 for 24 h prior to detecting inducible NO synthase (iNOS) expression by Western blot analysis (**b**) or cell surface CD80 expression ($p < 0.01$) by fluorescence-activated cell sorting (FACS) (**c**), $n = 3$. Four-day M-CSF-differentiated WT BMMϕs were transfected with 100 nM control or IL-7R-specific small interfering RNA (siRNA) for 3 days. IL-7R expression knockdown cells were validated by Western blot or FACS analysis, $n = 3$ (**d**). Control and IL-7R expression knockdown cells were treated with PBS or 100 ng/ml IFNγ/LPS (+Ctl) or IL-7 for 6 h prior to quantifying the TNF (**e**) and IL-6 (**f**) mRNA levels ($p < 0.05$ or 0.01) by real-time RT-PCR, $n = 3$. M-CSF-differentiated BMMϕs from WT and IL-7R−/− mice were treated with PBS or primed with 100 ng/ml IFNγ or LPS for 24 h prior to being treated with PBS or IL-7 (100 ng/ml) for 48 h. The protein levels of TNF (**g**), IL-6 (**h**), C-C motif chemokine ligand 2 (CCL2) (**i**), and CCL5 (**j**) ($p < 0.05$ or 0.01) were quantified by enzyme-linked immunosorbent assay (ELISA), $n = 3$. The data are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$

compared to the control mice, even though the IFNγ levels were unchanged (Fig. 5i). Moreover, we found that in IL-7 arthritic mice, the number of TRAP+ cells was increased by 5-fold, along with accentuated joint RANK (22×), RANKL (10×) and cathepsin K (CTSK; 4×) transcription levels compared to the control group (Fig. 5k–m). These data highlight the significance of myeloid cell function in IL-7-induced joint inflammation and osteoclastic bone erosion.

## M1-inducing factors increase myeloid IL-7R expression and thereby stabilize IL-7-mediated arthritis

We showed that IL-7R cell surface expression is markedly upregulated by IFNγ and LPS stimulation in blood in vitro-differentiated macrophages (Fig. 1h). We therefore asked whether M1-promoting factors have a similar impact on murine joint macrophages. For this purpose, LPS was intra-articularly (i.a.) injected into WT mice, and harvested ankles were analyzed for $F480^{+}CD127^{+}$ cells. Consistent with the findings in RA cells, LPS i.a. administration increased the frequency of IL-7R (CD127)-expressing cells by 5-fold in the mouse joint macrophage population compared to the control group (Fig. 6a). Next, to stabilize IL-7-mediated arthritis and avoid the booster injections (days 3 and 10) performed in the above-mentioned preclinical studies (Fig. 5a), LPS or saline was i.a. injected into WT mice 48 h prior to an ectopic IL-7 or control injection (Fig. 6b).

The clinical and histological data demonstrated that joint swelling, lining thickness, and bone erosion were markedly higher in mice that received LPS + Adenovirus (Ad)-IL-7 compared to saline + Ad-IL-7, saline + Ad-Ctl, or LPS + Ad-Ctl treatment groups (Fig. 6b–d). In spite of the inferior joint swelling detected in the saline + Ad-IL-7 group relative to the LPS + Ad-IL-7 group, the animals that received the ectopic injection of IL-7 (saline + Ad-IL-7) had greater disease severity than those that were treated with saline + Ad-Ctl or LPS + Ad-Ctl (Fig. 6b–d, significance established on day 4).

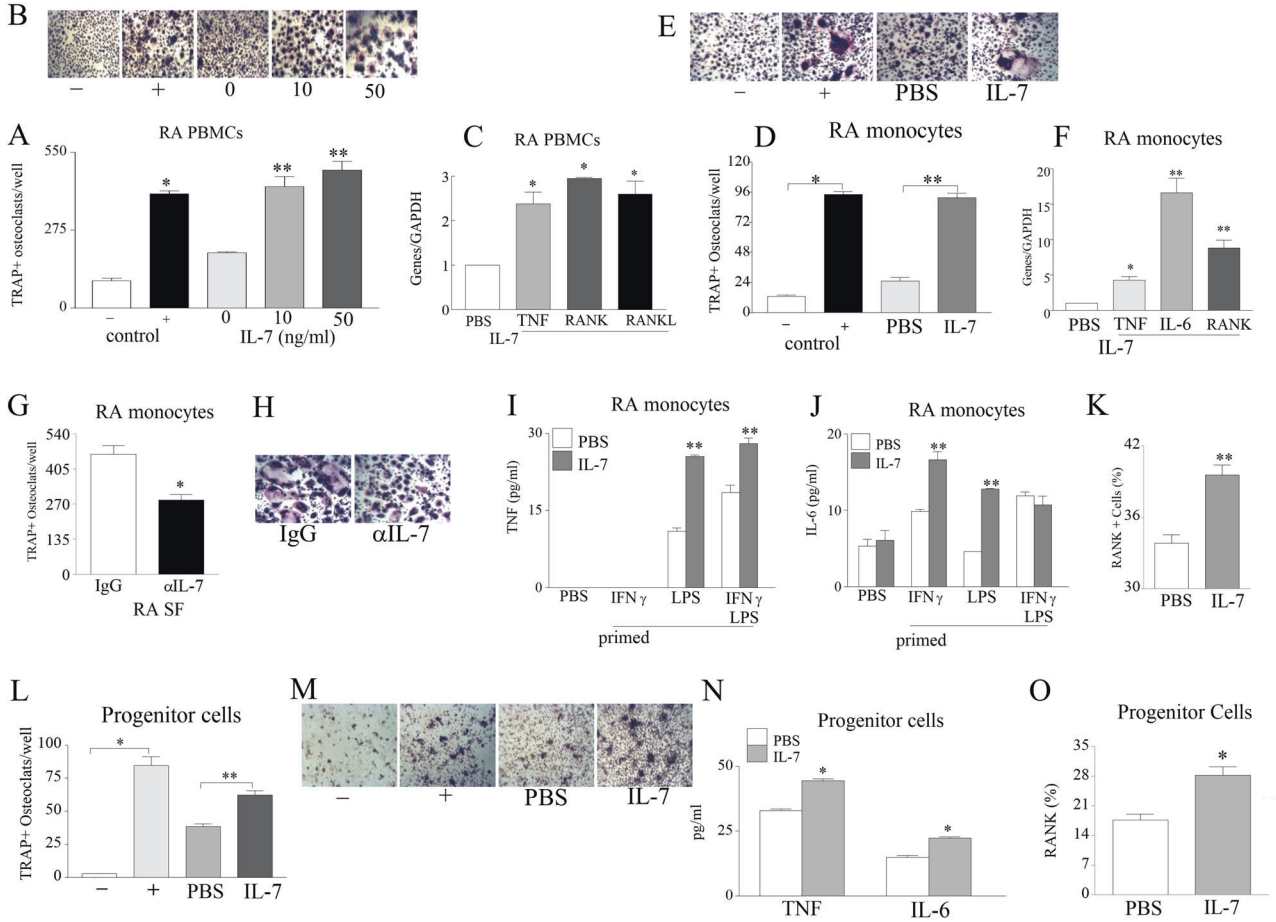


**Fig. 3** Interleukin (IL-7) is a strong promoter of rheumatoid arthritis (RA) osteoclast differentiation, and this manifestation is predominately dependent on myeloid cells. RA cells were cultured in the absence (−control) or presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) (+control; 20 ng/ml). RA peripheral blood mononuclear cells (PBMCs) (0–50 ng/ml) (**a**, **b**) or RA monocytes (100 ng/ml) (**d**, **e**) cultured under suboptimal conditions (10 ng/ml M-CSF/RANKL) were untreated or treated with IL-7 ($p < 0.05$ or 0.01) for 14–21 days before tartrate-resistant acid phosphatase (TRAP) staining, $n = 3$. RA PBMCs (**c**) or RA monocytes (**f**) were cultured under suboptimal conditions for 7 days. Next, the cells primed with IFNγ/LPS (100 ng/ml; 24 h) were either untreated PBS or treated with IL-7 (100 ng/ml) for 6 h before quantifying the tumor necrosis factor (TNF), RANK, and RANKL ($p < 0.05$) (**c**) or TNF, IL-6, and RANK ($p < 0.05$ or 0.01) (**f**) messenger RNA (mRNA) levels by real-time reverse transcription-polymerase chain reaction (RT-PCR), $n = 3$. RA monocytes were cultured with 2% RA synovial fluid (SF) in the presence of IgG or an anti-IL-7 antibody (Ab) (10 μg/ml, R&D Systems, MAB207) for 14–21 days prior to TRAP staining ($p < 0.05$) (**g**, **h**), $n = 5$. RA monocytes cultured for 7 days under suboptimal conditions were primed with 100 ng/ml IFNγ, LPS, or IFNγ/LPS for 24 h. Then, the cells were treated with PBS or IL-7 (100 ng/ml) for 24–48 h prior to quantifying the TNF (**i**) and IL-6 levels (**j**) by enzyme-linked immunosorbent assay (ELISA) or RANK expression ($p < 0.01$) (**k**) by fluorescence-activated cell sorting (FACS), $n = 3$. Unattached mouse bone marrow cells (progenitor cells) were cultured with 30 ng/ml M-CSF for 3 days. Then, the cells were treated with PBS or 100 ng/ml IL-7 under suboptimal conditions (15 ng/ml M-CSF/RANKL) for 7 days before TRAP staining ($p < 0.01$), $n = 3$ (**l**, **m**). Cells cultured in the absence or presence of 30 ng/ml M-CSF/RANKL were considered –control or +control, respectively. Progenitor cells cultured under suboptimal conditions were treated with PBS or 100 ng/ml IL-7 for 24–72 h prior to quantifying the TNF and IL-6 protein levels by ELISA ($p < 0.05$) (**n**) or the $RANK^+$ cell frequency by FACS ($p < 0.05$) (**o**), $n = 3$. The data are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$

Intriguingly, while the frequency of $CD3^+$ T cells was comparable between the LPS + Ad-IL-7 and saline + Ad-IL-7 mice, the number of $F480^+iNOS^+$ cells was increased by LPS priming in the IL-7-treated arthritis mice compared to the saline-treated mice (Fig. 6e–g). Substantiating the data in Fig. 5e, we found that $F480^+iNOS^+$ cells were the main effector cells detected in the saline + Ad-IL-7 and LPS + Ad-IL-7 arthritic mice (Fig. 6e–g). In spite of these findings, we demonstrated that LPS priming did not impact the joint TNF or CCL2 protein levels detected in the IL-7-treated arthritic mice (Fig. 6h). In contrast, the CCL5 protein levels, which were markedly higher than the levels of other M1 markers in the IL-7-treated arthritic mice (saline + Ad-IL-7), were increased 2-fold by LPS priming (LPS + Ad-IL-7) (Fig. 6h). Confirming these observations, we documented that $CD11b^+CCL5^+$ cells were amplified by local IL-7 expression, indicating that these joint cells are a phenotypic marker of IL-7-induced disease progression (Fig. 6i).

The protein expression of T cell-derived cytokines, including IFNγ or IL-17, was undetectable in ankles harvested on day 15 from all treatment groups (Fig. 5i). In contrast, in the mice primed with LPS, the joint IFNγ levels were most significantly elevated 24 h after the local injection of IL-7 (Fig. 6j). The IFNγ concentration was markedly declined after 48 h (15-fold increase above baseline) compared to 24 h (96-fold) following local IL-7 injection in the LPS-primed group. However, these levels were markedly higher than those in the control groups (Fig. 6j). Consistent with the increased joint $F480^+CD11b^+CD127^+$ cell frequency observed following local LPS injection (Fig. 6a), the mice that were treated with LPS + Ad-Ctl exhibited increased IL-7R expression levels (2.5-14-fold increase above the baseline level) at 72

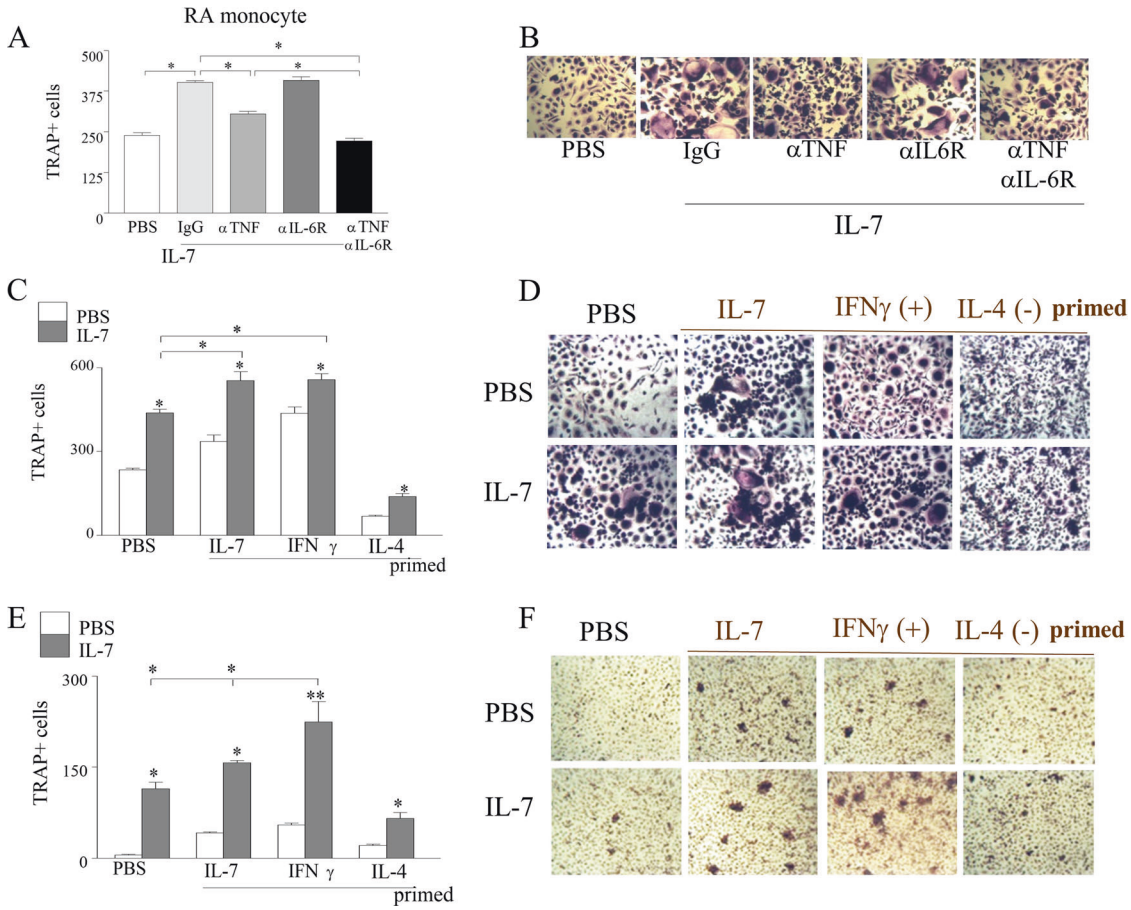


**Fig. 4** Interleukin (IL-7)-differentiated rheumatoid arthritis (RA) macrophages have a greater ability to differentiate into mature osteoclasts than naive RA macrophages. RA monocytes cultured under suboptimal conditions were treated with PBS or IL-7 (100 ng/ml) in the presence of 10 μg/ml IgG or α-tumor necrosis factor (αTNF) (Enbrel (etanercept)), αIL-6R (tocilizumab), or αTNF + αIL-6R antibodies for 14–21 days before tartrate-resistant acid phosphatase (TRAP) staining ($p < 0.05$), $n = 3$ (**a**, **b**). RA monocytes were primed with 100 ng/ml interferon-γ (IFNγ) (+control for M1), IL-4 (+control for M2), or IL-7 for 48 h, and then cultured under suboptimal conditions in the presence or absence of 100 ng/ml IL-7 stimulation for 14–21 days ($p < 0.05$), $n = 3$ (**c**, **d**). Mouse bone marrow progenitor cells cultured under suboptimal conditions for 3 days were primed with 100 ng/ml IFNγ, IL-4, or IL-7 for 24 h. Then, the cells were cultured under suboptimal osteoclast conditions (15 ng/ml M-CSF/receptor activator of nuclear factor-κB ligand (RANKL) in the presence or absence of 100 ng/ml IL-7 for 3–4 days ($p < 0.05$ or 0.01) (**e**, **f**), $n = 3$. The data are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$

and 96 h post LPS injection (Fig. 6k; which is 24–48 h post Ad-Ctl administration). In the LPS + Ad-IL-7 group, the IL-7R levels were more potently increased at 48 h compared to 24 h after Ad-IL-7 injection (65-fold vs. 3.3-fold above the baseline level, respectively) (Fig. 6k). Interestingly, only at 48 h and not at 24 h post IL-7 injection was the IL-7R level markedly higher in the LPS + Ad-IL-7 group than in the LPS + Ad-Ctl group (14-fold vs. 65-fold above the baseline level, respectively) (Fig. 6k). Our findings suggest that the high levels of IFNγ induced by LPS and IL-7 administration may be responsible for the increase in myeloid IL-7R expression (Fig. 6j, k).

We also found that the 30% increase in the frequency of $F480^{+}iNOS^{+}$ cells observed in the LPS + Ad-IL-7 group compared to the saline + Ad-IL-7 group correlated with exacerbated bone erosion and the number of TRAP+ cells (Fig. 6e, l), while the saline or LPS + Ad-Ctl groups had comparable numbers of TRAP+ cells (Fig. 6l). Thus, IL-7R expression may be further amplified by IFNγ produced by the synergistic effect of LPS and IL-7, which translates to potentiated IL-7-mediated joint inflammation and bone destruction. As a result, the increase in the frequency of myeloid cells that transform into a specific macrophage phenotype with high levels of CCL5 secretion may be responsible for the aggravation of disease severity.

IL-7-induced arthritis is suppressed in RAG−/− mice due to a lack of IFNγ and a reduction in myeloid IL-7R levels

To establish that the induction of joint IFNγ expression is involved in LPS + Ad-IL-7-induced arthritis, a neutralizing anti-IFNγ Ab was utilized in this model. We showed that the blockade of IFNγ function was capable of reducing LPS + Ad-IL-7-driven arthritis starting on day 3 and continuing until day 10 when the study was completed (Fig. 7a). To further substantiate the significance of T cells in the IL-7-treated arthritis model, joint inflammation was provoked by LPS priming of IL-7-mediated arthritis in RAG−/− mice. For this purpose, RAG−/− mice received an i.a. injection of LPS or saline 48 h before ectopic IL-7 or control administration. We found that disease severity was markedly higher in the LPS + Ad-IL-7 group (from days 4 to 16) than in the saline + Ad-IL-7, saline + Ad-Ctl and LPS + Ad-Ctl treatment groups (Fig. 7b, significance established on day 4). Ankle circumference was similar between the mice that received saline + Ad-Ctl or LPS + Ad-Ctl, and both groups had lower joint swelling than the group treated with saline + Ad-IL-7 (Fig. 7b, c). The severity of joint inflammation and lining thickness corroborated the dominance of $F480^{+}$ and $iNOS^{+}$ cells, with the highest frequency detected in the LPS + Ad-IL-7 group and a reduced expression pattern observed in the saline + Ad-IL-7 group (Fig. 7b–e).

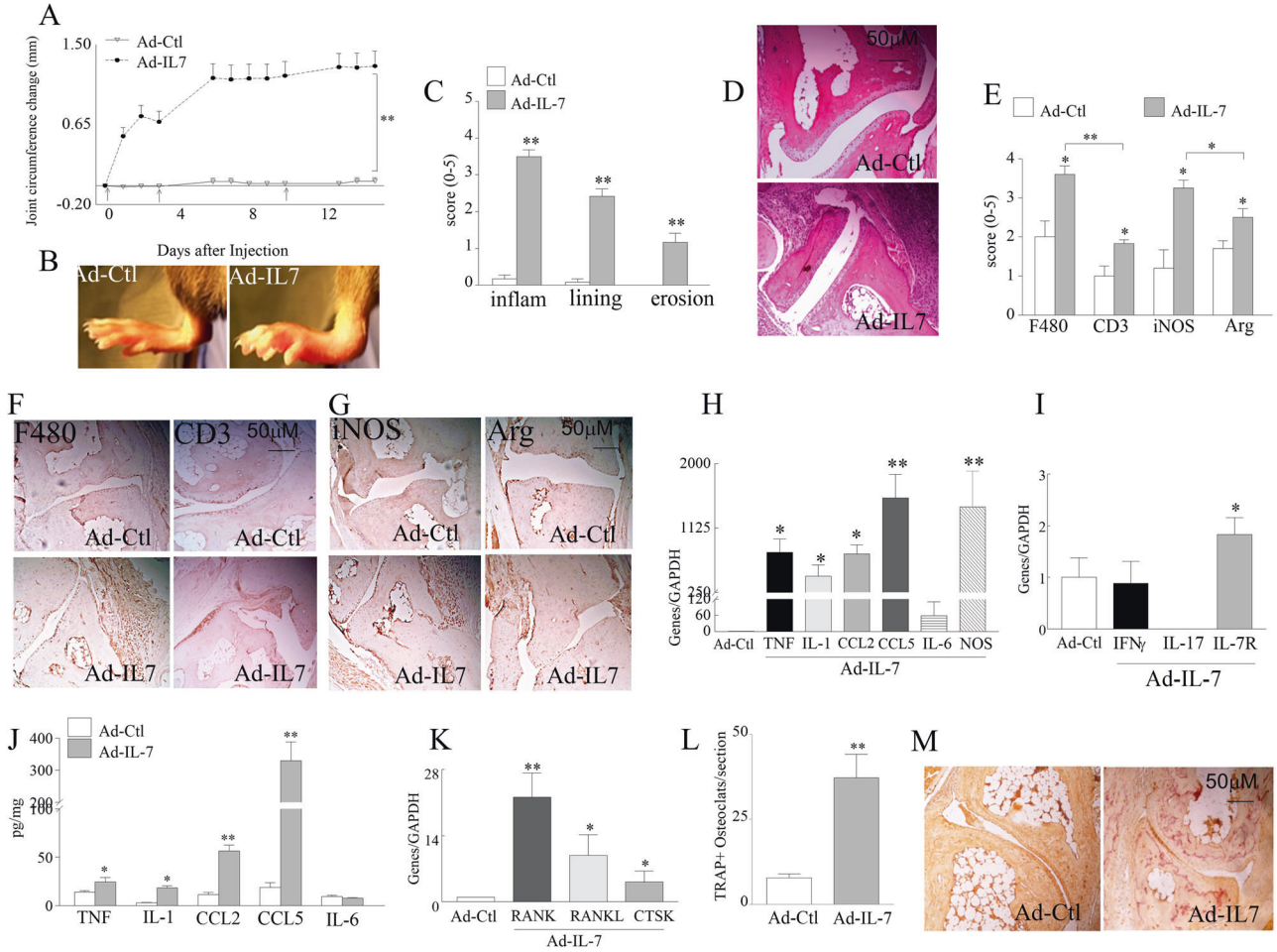


**Fig. 5** Local expression of interleukin (IL-7) provokes erosive arthritis. Wild-type (WT) mice were intra-articularly (i.a.) injected with Ad-Ctl or Ad-IL-7 ($10^7$ pore-forming unit (PFU)) on days 0, 3, and 10. Changes in joint circumference were determined from days 0 to 15 ($p < 0.01$), $n = 16$ ankles (**a**, **b**). Hematoxylin and eosin (H&E)-stained ankles from day 15 were scored for inflammation (inflam), synovial lining thickness, and bone erosion on a 0–5 scale ($p < 0.01$), $n = 8$ ankles (**c**, **d**). Ankles harvested on day 15 were stained with Abs against F480 (1:100), CD3 (1:100) (**e**, **f**), inducible NO synthase (iNOS), and Arginase (1:200) ($p < 0.05$ or 0.01) (**e**, **g**), and the staining was scored on a 0–5 scale, $n = 8$. Ankles harvested from Ad-Ctl and Ad-IL-7 mice were employed to quantify the transcription levels of tumor necrosis factor (TNF), IL-1β, C-C motif chemokine ligand 2 (CCL2), CCL5, IL-6, and NOS2 ($p < 0.05$ or 0.01) (**h**), as well as those of interferon-γ (IFNγ), IL-17, and IL-7R ($p < 0.05$) (**i**) by real-time reverse transcription-polymerase chain reaction (RT-PCR), $n = 5$. **j** Additionally, the protein levels of TNF, IL-1β, CCL2, CCL5, and IL-6 were assessed by enzyme-linked immunosorbent assay (ELISA) ($p < 0.05$ or 0.01), $n = 5$. **k** The transcription levels of RANK, receptor activator of nuclear factor-κB ligand (RANKL) and cathepsin K (CTSK) in ankles harvested from Ad-Ctl- or Ad-IL-7-treated mice were determined by real-time RT-PCR ($p < 0.05$ or 0.01), $n = 5$. The number of tartrate-resistant acid phosphatase-positive (TRAP+) cells in mice that received Ad-Ctl or Ad-IL-7 treatment were counted per section ($p < 0.01$), $n = 6$ (**l**, **m**). Values are shown as the mean ± SE. *$p < 0.05$; **$p < 0.01$

We found that, in RAG−/− mice, the LPS-induced IL-7R expression on $F480^+$ cells was reduced by 3-fold compared with WT mice (Fig. 7f). Supporting the role of IFNγ in amplifying the expression of IL-7R promoted by LPS+ IL-7, we showed that an ectopic injection of IL-7 can potentiate LPS-mediated IL-7R to a greater extent in WT relative to RAG−/− mice (Fig. 7g). Consistently, WT ankles treated with LPS + Ad-IL-7 produced 4.5-fold higher levels of IFNγ than ankles injected with LPS alone (Fig. 7h).

Additionally, we established that IL-7-driven osteoclast formation is further enhanced by LPS; however, the trend was markedly lower in RAG−/− mice than in WT mice (Figs. 6l, 7i–j). When joint monokines were quantified, the levels of TNF, IL-1 and IL-6 were comparable among the various groups. In contrast, CCL2 concentrations were increased in the LPS/saline + Ad-IL-7 groups, and CCL5 levels were only enhanced in the LPS + Ad-IL-7 group compared to the other groups (Fig. 7k). These results indicate that arthritis mediated through IL-7 is dependent on specific M1 macrophages that are $F480^+CD11b^+CD127^+$ and produce high levels of CCL5 (Fig. 6i). We also conclude that T cells participate in IL-7-induced arthritis by upregulating myeloid IL-7R expression through the effect of IFNγ. Hence, joint inflammation and bone erosion provoked by IL-7 are suppressed but not abolished in RAG−/− mice.

## Anti-TNF therapy partially suppresses IL-7-induced arthritis by reducing IL-7R expression

TNF is capable of modulating myeloid IL-7R expression,[12] and it is also produced by RA macrophages in response to IL-7 activation (Fig. 1a, b). Therefore, the impact of anti-TNF therapy on IL-7 and IL-7R expression in RA blood cells was examined. In a phase 4 clinical study (ClinicalTrials.gov Identifier: NCT02451748), RA patients who were not in remission after receiving nonbiologic therapies (disease-modifying anti-rheumatic drugs) received 3 to 6 months of anti-TNF therapy (Fig. 8a). While the expression of IL-7 was not impacted by anti-TNF treatment, the levels of IL-7R were markedly reduced following 6 months (but not 3 months) of therapy (Fig. 8a). Consistently, in vitro studies documented that anti-TNF therapy decreased LPS− and LPS + IFNγ-enhanced IL-7R

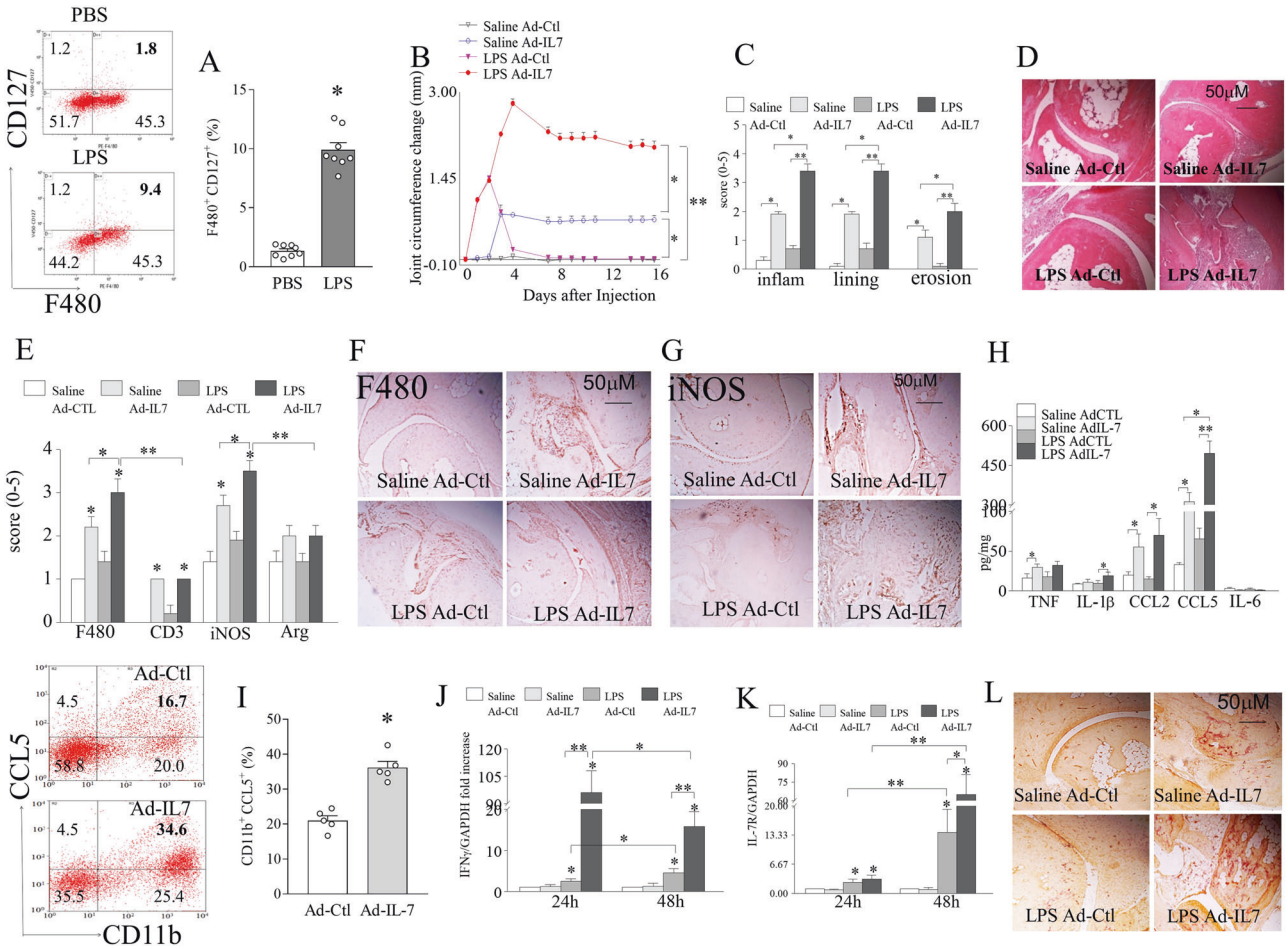


**Fig. 6** LPS enhances joint IL-7R expression and thereby stabilizes interleukin (IL-7)-mediated arthritis. **a** Wild-type (WT) mice were intra-articularly (i.a.) injected with saline or LPS (15 μg/ankle), and ankles harvested after 48 h were analyzed for $F480^+CD127^+$ cells by fluorescence-activated cell sorting (FACS), $n = 8$. **b** On day 0, WT mice were i.a. injected with saline or LPS (15 μg/ankle). Next, on day 2, the mice in the saline and LPS groups received an i.a. injection of $10^7$ pore-forming unit (PFU) Ad-Ctl or Ad-IL-7 ($p < 0.05$ or 0.01). Joint circumference was measured throughout the study, and the mice were sacrificed on day 16, $n = 10$. Ankles harvested from all treatment groups were stained with hematoxylin and eosin (H&E), and inflammation, lining thickness, and erosion were scored on a 0–5 scale ($p < 0.05$ or 0.01), $n = 6$ (**c**, **d**). **e** Ankles harvested from all treatment groups were stained with anti-F480 (1:100), anti-CD3 (1:100), anti-inducible NO synthase (iNOS), and anti-Arginase (1:200) antibodies (Abs) or control IgG, and positive staining was scored on a 0–5 scale ($p < 0.05$ or 0.01), $n = 6$. Images show representative F480 (**f**) and iNOS (**g**) immunostaining, which is quantified in **e**. **h** The protein concentrations of tumor necrosis factor (TNF), IL-1β, C-C motif chemokine ligand 2 (CCL2), CCL5, and IL-6 ($p < 0.05$ or 0.01) were quantified in ankle joints harvested on day 16 and normalized by the joint protein levels, $n = 5$. **i** WT mice were i.a. injected with $10^7$ PFU Ad-Ctl or Ad-IL-7. After 48 h, joint cells were screened to determine the percentage of $CD11b^+CCL5^+$ cells by FACS analysis, $n = 5$. In a different preclinical study, WT mice were i.a. injected with saline or LPS (15 μg/ankle), and after 48 h, the mice received an i.a. injection of $10^7$ PFU Ad-Ctl or Ad-IL-7. Ankles were harvested at 24 or 48 h to quantify the interferon-γ (IFNγ) (**j**) and IL-7R ($p < 0.05$ or 0.01) (**k**) messenger RNA (mRNA) concentrations by real-time reverse transcription-polymerase chain reaction (RT-PCR), $n = 5$. Tartrate-resistant acid phosphatase-positive (TRAP+) cell staining is shown for the ankles harvested from the mice in **b** on day 16, $n = 6$ (**l**). Values are shown as the mean ± SE. *$p < 0.05$; **$p < 0.01$

expression levels (Fig. 8b). We also uncovered that the secretion of IL-6 by RA macrophages was downregulated by anti-TNF therapy at baseline (PBS) and following IL-7 stimulation (Fig. 8c). While anti-IL-7R Ab therapy did not impact baseline IL-6 production, IL-7-induced IL-6 levels were suppressed following Ab therapy (Fig. 8c). These results underscore that a reduction in IL-7R expression is a mechanism by which anti-TNF treatment can suppress the responsiveness of RA cells to IL-7 stimulation.

Based on these observations, we next asked whether anti-TNF therapy can abrogate IL-7-induced arthritis. Joint inflammation, lining thickness and $F480^+iNOS^+$ cell numbers were markedly reduced by anti-TNF therapy relative to control therapy in the LPS + Ad-IL-7 group (Fig. 8d–g). However, anti-TNF treatment was not capable of reducing LPS + Ad-IL-7-mediated joint swelling, pannus formation or the frequency of $F480^+iNOS^+$ cells to the levels detected in the LPS + Ad-Ctl + saline and LPS + Ad-Ctl + anti-TNF groups (Fig. 8d, e, differences in joint circumference was established on day 4). Similar to other M1-inducing factors, TNF can modulate myeloid IL-7R expression in RA cells and IL-7-treated arthritic joints (Fig. 8a, b, g). In contrast to the reduced frequency of $F480^+iNOS^+IL\text{-}7R^+$ cells, the number of $CD3^+$ T cells and $F480^+Arginase^+$ cells was not affected by anti-TNF therapy in the mice that received LPS + Ad-IL-7 (Fig. 8g–j).

Correlating with the reduction in the $F480^+iNOS^+IL\text{-}7R^+$ cell number, joint CCL2 and CCL5 production was decreased by anti-TNF therapy compared to control treatment in the LPS + Ad-IL-7 group (Fig. 8k). Nevertheless, although CCL2 and CCL5 concentrations were downregulated by anti-TNF therapy in the LPS + Ad-IL-7 group, these levels were still 5–6-fold higher than those in the nonarthritic group (Fig. 8k). The preservation of these

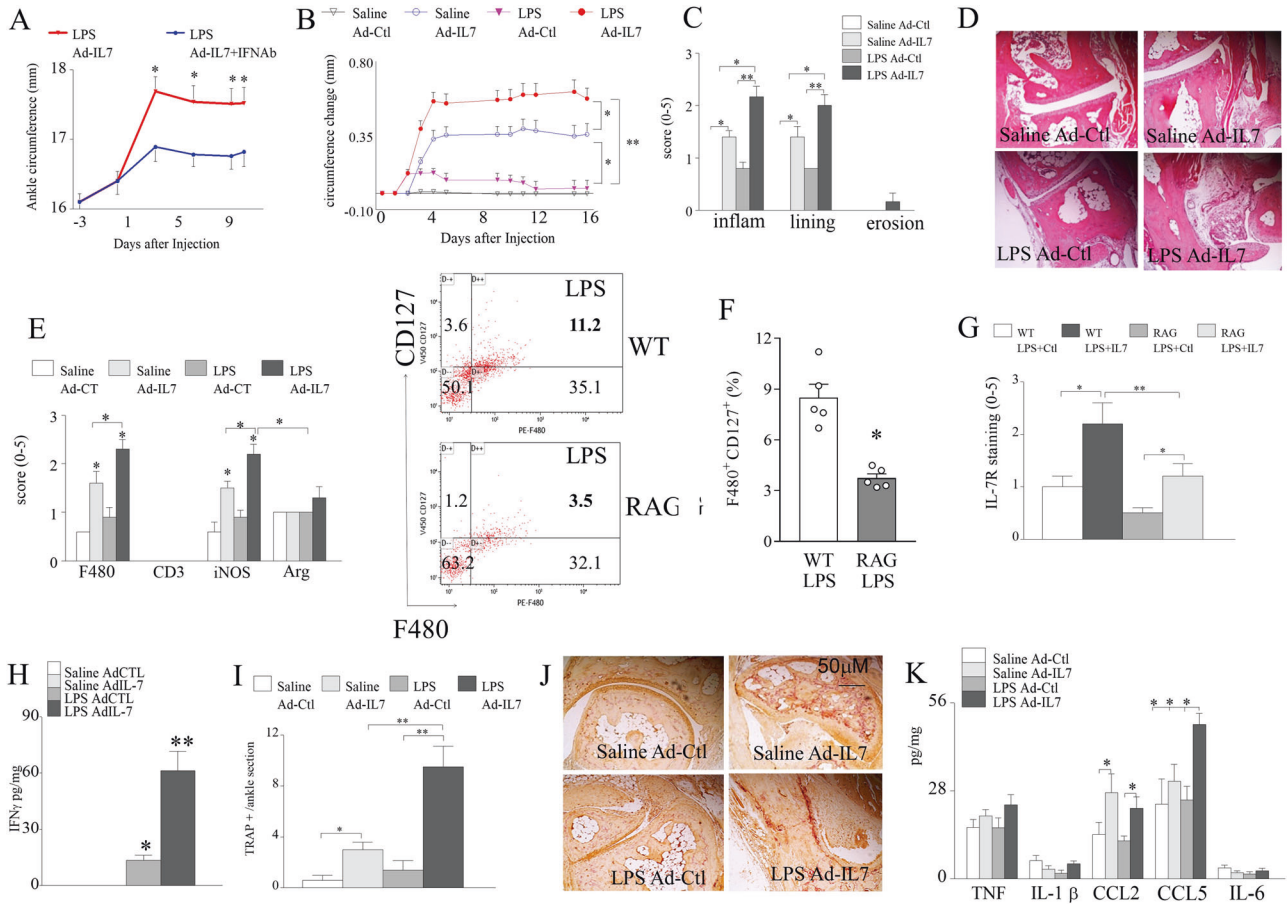


**Fig. 7** Interleukin (IL-7)-mediated arthritis is suppressed in RAG−/− mice due to interferon-γ (IFNγ) deficiency and reduced joint IL-7R expression. **a** Ankle circumference was measured in wild-type (WT) mice that were treated intraperitoneally (i.p.) with an anti-IFNγ antibody (Ab) (100 μg) or control on days −3, 0, 3, 6, and 9. The mice in both groups received an intra-articular (i.a.) injection of LPS (15 μg/ankle) on day 0 and Ad-IL-7 ($10^7$ PFU) on day 2 and were sacrificed on day 10, $n = 10$ ankles. **b**. On day 0, RAG-/- mice were i.a. injected with saline or LPS (15 μg/ankle). On day 2, the mice in the saline and LPS groups received an intra-articular (i.a.) injection of $10^7$ pore-forming unit (PFU) Ad-Ctl or Ad-IL-7. Joint circumference was measured throughout the study, and the mice were sacrificed on day 16 ($p < 0.05$ or 0.01), $n = 10$. Ankles harvested from mice in all four groups were stained with hematoxylin and eosin (H&E), and inflammation, lining thickness, and erosion were scored on a 0–5 scale ($p < 0.05$ or 0.01), $n = 6$ (**c**, **d**). **e** Ankles harvested from all treatment groups were stained with anti-F480 (1:100), anti-CD3 (1:100), anti-inducible NO synthase (iNOS), and anti-Arginase (1:200) Abs or control IgG, and positive staining was scored on a 0–5 scale ($p < 0.05$), $n = 6$. **f** WT and RAG−/− mice were i.a. injected with LPS (15μg/ankle), and ankles harvested after 48 h were screened to determine the percentage of $F480^+CD127^+$ cells by fluorescence-activated cell sorting (FACS) analysis, $n = 5$. **g** In a different experiment, WT or RAG−/− mice were i.a. injected with LPS (15 μg/ankle, day 0), and 48 h later, the mice received an i.a. injection of $10^7$ PFU Ad-Ctl or Ad-IL-7. Ankles harvested on day 16 were stained with an anti-IL-7R Ab (1:100 dilution) and scored on a 0–5 scale ($p < 0.05$ or 0.01), $n = 6$. **h** WT mice i.a. injected with saline or LPS (15 μg/ankle) received an i.a. injection of $10^7$ PFU Ad-Ctl or Ad-IL-7 at 48 h post i.a. injection. Ankles were harvested 48 h post Ad-Ctl or Ad-IL-7 injection to determine the IFNγ protein levels by enzyme-linked immunosorbent assay (ELISA) ($p < 0.05$ or 0.01), $n = 6$. The number of tartrate-resistant acid phosphatase-positive (TRAP+) cells in the ankles harvested from the mice described in **b** were counted per section ($p < 0.05$ or 0.01), $n = 6$ (**i**, **j**). **k** The protein concentrations of tumor necrosis factor (TNF), IL-1β, C-C motif chemokine ligand 2 (CCL2), CCL5, and IL-6 were quantified in ankle joints ($p < 0.05$) harvested on day 16 in the experiments described in **b** and normalized by the joint protein levels ($p < 0.05$), $n = 6$. Values are shown as the mean ± SE. *$p < 0.05$; **$p < 0.01$

M1-associated monokines may in part be due to the fact that $F480^+iNOS^+$ cells were not fully abolished in the IL-7-treated arthritic joints following anti-TNF therapy (Fig. 8g, h, j, k). In short, our data suggest that LPS, IFNγ, and TNF modulate the frequency of myeloid IL-7R expression and ultimately the severity of IL-7-induced arthritis. Nonetheless, the effector cells that control IL-7-induced arthritis are $F480^+iNOS^+CD127^+CCL5^+$ M1 macrophages.

## DISCUSSION

The current study describes a hitherto undefined role for IL-7 and IL-7R in transforming naive monocytes into classical M1 macrophages and mature osteoclasts. Intriguingly, we revealed that the master regulators of the M1 phenotype, LPS and IFNγ, can potentiate myeloid IL-7R expression and IL-7 responsiveness, resulting in the development of inflammatory and bone-erosive cells. We showed that joint inflammation and erosion can be provoked by repeated IL-7 i.a. injections. However, ectopic LPS injection can stabilize IL-7-induced arthritis by enhancing joint myeloid IL-7R expression, in part by inducing IFNγ. Consistent with this notion, in RAG−/− mice, IL-7-mediated arthritis is suppressed because of a reduction in joint myeloid IL-7R expression due to IFNγ deficiency. Additionally, we found that anti-TNF therapy can markedly reduce IL-7R expression without affecting the levels of IL-7 in RA blood cells. Extending these results, we demonstrated that LPS- and/or IFNγ-induced IL-7R expression can be down-regulated by anti-TNF therapy in RA cells and IL-7-treated arthritic mice. We discovered that the predominant cells that manipulate

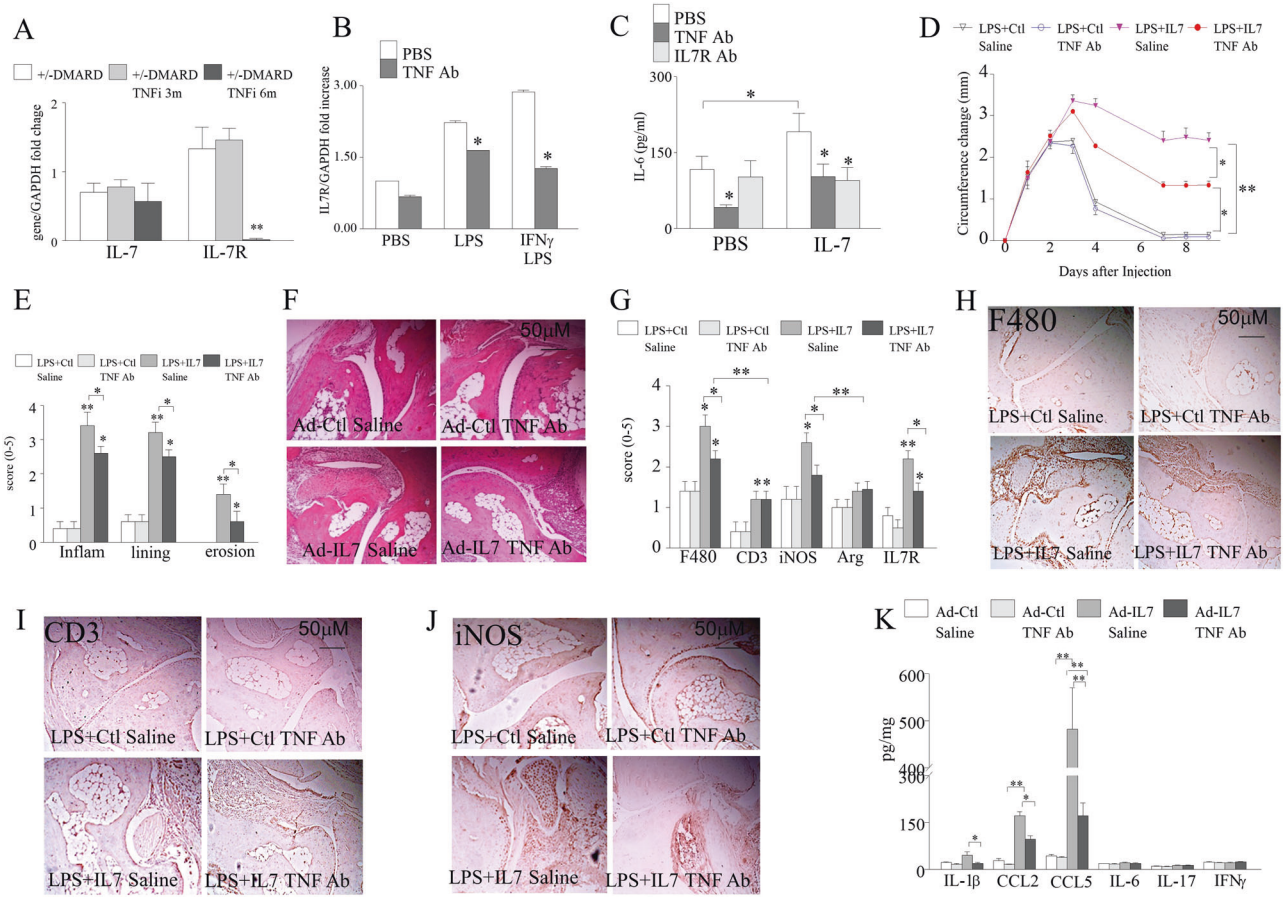


**Fig. 8** Anti-tumor necrosis factor (TNF) therapy reduces IL-7R expression, contributing to the suppression of interleukin (IL-7)-mediated disease severity and the frequency of M1 macrophages. **a** Rheumatoid arthritis (RA) patients who were not treated or treated with disease-modifying anti-rheumatic drugs (DMARDs) at onset ($n = 10$, first visit) received Cimzia (TNFi) therapy for 3 ($n = 10$, second visit) or 6 months ($n = 5$, third visit). RA peripheral blood mononuclear cells (PBMCs) obtained at the onset and after 3 and 6 months of therapy were analyzed for IL-7 and IL-7R expression ($p < 0.01$) by real-time reverse transcription-polymerase chain reaction (RT-PCR), $n = 5$–10. **b** RA in vitro-differentiated macrophages were pretreated with IgG or an anti-TNF antibody (Ab) for 48 h prior to being treated with PBS, 100 ng/ml LPS, or 100 ng/ml LPS/interferon-γ (LPS + IFNγ) for 6 h. Then, IL-7R expression ($p < 0.05$) was quantified by real-time RT-PCR, $n = 4$. **c** RA in vitro-differentiated macrophages were pretreated with IgG, an anti-TNF Ab or an anti-IL-7R Ab for 48 h prior to being treated with PBS or 100 ng/ml IL-7 for 48 h. Then, the IL-6 protein levels ($p < 0.05$) were quantified by enzyme-linked immunosorbent assay (ELISA), $n = 5$. **d** Joint circumference was measured in wild-type (WT) C57BL/6 mice that received an intra-articular (i.a.) injection of LPS (15 μg/ankle) on day 0. Subsequently, on day 2, these mice were ectopically treated with $10^7$ pore-forming unit (PFU) Ad-Ctl or Ad-IL-7. The mice in the Ad-Ctl and Ad-IL-7 groups were also treated intraperitoneally (i.p.) with an αTNF Ab (200 μg) or control ($p < 0.05$ or 0.01) on day 2, and the animals were sacrificed on day 9, $n = 10$. Ankles harvested from all treatment groups were stained with hematoxylin and eosin (H&E), and inflammation, lining thickness, and erosion ($p < 0.05$ or 0.01) were scored on a 0–5 scale, $n = 6$ (**e**, **f**). **g** Ankles harvested from all treatment groups were stained with anti-F480 (1:100), anti-CD3 (1:100), anti-inducible NO synthase (iNOS), anti-Arginase (1:200), and anti-IL-7R (1:100) Abs ($p < 0.05$ or 0.01), and positive staining was scored on a 0–5 scale, $n = 6$. **h**–**j** are representative of **g**, $n = 6$. **k** The protein concentrations of IL-1β, C-C motif chemokine ligand 2 (CCL2), CCL5, IL-6, IL-17, and IFNγ were quantified in ankle joints ($p < 0.05$ or 0.01) harvested on day 9 by ELISA and normalized by the joint protein levels, $n = 5$. Values are shown as the mean ± SE. *$p < 0.05$; **$p < 0.01$

IL-7-induced arthritis are $F480^+iNOS^+CD127^+CCL5^+$ M1 macrophages.

IL-7 is a member of the IL-2/IL-15 family that plays important roles in T cell proliferation, differentiation, and survival.[15] However, IL-7 effects are not restricted to T cells, as IL-7 can differentiate thymic progenitor cells into dendritic cells or macrophages.[16] We and others have shown that IL-7/IL-7R are predominately expressed in RA ST lining and sublining macrophages.[12,17] Additionally, the expression of IL-7 and IL-7R in RA monocytes closely correlates with TNF expression and DAS28 scoring, suggesting that IL-7/IL-7R play a crucial role in the pathogenesis of RA myeloid cells.[13]

Contrary to previous findings that report that cell-to-cell contact between $CD4^+$ and $CD14^+$ cells is required for an inflammatory response to IL-7,[17] we found that IL-7 can remodel RA and mouse-naive cells into classical M1 macrophages that secrete or express elevated levels of TNF, IL-6, CCL2, CCL5, or iNOS. We established that the potency of the IL-7-induced inflammatory response is dependent on the expression of IL-7R by human and murine myeloid cells. As such, we document that NL macrophages that express lower levels of IL-7R than RA macrophages[12] can strongly respond to IL-7 once IL-7R is potentiated by LPS or IFNγ stimulation. Others have also shown that in NL monocytes pretreated with LPS, TNF and IL-1 can amplify IL-7-induced IL-8 production; however, they did not determine the mechanism of action.[18]

T cells, B cells and RA ST fibroblasts can contribute to IL-7-mediated osteoclast maturation through mechanisms dependent on RANKL and TNF induction.[19–21] We reveal that IL-7 can enhance osteoclastogenesis through both utilizing T cells and functioning

independently. IL-7-activated RA PBMCs differentiate into mature osteoclasts due to the induction of RANK, RANKL, and TNF. However, IL-7 promotes the maturation of RA monocytes into osteoclasts via induction of RANK, TNF, and IL-6. Supporting this notion, RA SF ($CD14^+$) macrophages that have highly elevated IL-7R expression[12] are differentiated into mature osteoclasts by IL-7 without T cell involvement.[22]

We noticed that compared to unprimed cells, RA myeloid cells primed with IL-4 form a reduced number of osteoclasts in response to IL-7. This outcome may be due to the polarization of naive myeloid cells into nonclassical M2 macrophages or the inhibitory effect of IL-4 on IL-7R expression. In contrast, IL-7- or IFNγ-primed osteoclast progenitor cells are more responsive to IL-7-induced osteoclastogenesis than PBS-treated cells. It was shown that while IL-7-primed murine progenitor cells are inferior to IFNγ-primed cells, RA cells primed with IL-7 or IFNγ form similar numbers of osteoclasts in response to IL-7. This suggests that in RA cells, enhanced levels of IL-7R are the mechanism by which IL-7 and IFNγ can be equally potent in remodeling M1 macrophages into mature osteoclasts. However, in murine cells, the superior potency of IFNγ over IL-7 may be due to the ability of IFNγ to potentiate IL-7R cell surface expression in addition to activating M1 polarization.

We demonstrated that the ectopic injection of IL-7 promotes arthritis through a mechanism that is dependent on the expansion of joint M1 macrophages ($F480^+iNOS^+$) rather than M2 macrophages ($F480^+Arg^+$). Others have shown that the systemic administration of IL-7 aggravates the severity of collagen-induced arthritis by increasing T cell infiltration into lymph nodes and T cell differentiation into TH-1 and TH-17 cells.[23] However, these studies did not establish that joint T cell infiltration was altered by the systemic administration of IL-7 or an anti-IL-7R Ab in CIA mice.[14,23] In contrast, we were unable to detect increased levels of IL-17 on day 15 post IL-7 local injection in spite of elevated joint $CD3^+$ cell migration. This discrepancy may reflect the inability of IL-7, when expressed locally in the synovial tissue, to influence systemic TH-17 cell differentiation. Corroborating the escalated M1 macrophage frequency, the mRNA and protein levels of monokines, such as TNF, IL-1, CCL2, and CCL5, were increased. We found that the transcription levels of NOS2/iNOS were comparable to those of CCL5, while IL-6 was undetectable, and the induction of joint TNF, IL-1, and CCL2 was 6–18-fold lower than that of CCL5. These results suggest that IL-7 can cultivate a unique M1 macrophage phenotype.

Supporting earlier studies in CIA, we demonstrated that IL-7 amplifies osteoclast differentiation in arthritic mice via RANKL produced by T cells[13,14] in addition to RANK and CTSK expressed by myeloid cells.[13] Substantiating the central role of myeloid cells in this function, we documented that osteoclasts are formed by ectopic IL-7 administration in RAG−/− mice in the absence or presence of LPS priming.

We revealed that the i.a. injection of LPS increases myeloid IL-7R expression and stabilizes IL-7-induced arthritis. We also showed that the local injection of LPS can induce modest levels of IFNγ transcription, which can be further exacerbated by IL-7 administration early in the disease process. In contrast to previous findings that demonstrated that IL-7 alone can differentiate PBMCs into IFNγ-producing TH-1 cells,[24] the ectopic expression of IL-7 alone was incapable of performing this function.

The potentiated IFNγ levels detected in the LPS + Ad-IL-7 group compared to the LPS + Ad-Ctl group led to higher IL-7R expression in WT mice. While authenticating the impact of IFNγ on LPS-induced IL-7R expression, we found that joint IL-7R expression is significantly reduced following LPS or LPS + Ad-IL-7 injection in RAG−/− compared to WT mice. Blockade via anti-IFNγ Ab therapy alleviated LPS + Ad-IL-7-induced arthritis starting on day 3 and throughout the study, which is consistent with the synergistic effect of LPS +Ad-IL-7 on IFNγ transcription (38-fold increase at 72 h post LPS injection) compared to the effect of LPS treatment alone.

Nevertheless, the comparable $CD3^+$ cell frequency and increased $F480^+iNOS^+$ cell population detected in WT mice indicate that M1 macrophages are the effector cells responsible for the LPS-induced exacerbation of IL-7-mediated arthritis. Extending these results, arthritis provoked by IL-7 with or without LPS administration was suppressed but not abolished in RAG−/− compared to WT mice. Our data indicate that T cells participate in IL-7-induced arthritis by modifying myeloid IL-7R expression through the induction of IFNγ.

However, in the absence of T cells, monokines such as CCL2 and CCL5 produced from unique M1 macrophages sustain joint pathology. Arthritis provoked by IL-7 is linked to myeloid cell migration and differentiation; therapeutic treatment with an anti-IL-7 Ab significantly reduces CIA joint monocyte infiltration and M1-associated factors such as TNF, MIP2 (macrophage-inflammatory protein-2), and CCL2.[13] In contrast, anti-IL-7 therapy had no impact on CIA splenic CD3, CD4, TH-1, and TH-17 cells or joint $CD3^+$ cells.[13]

Since the expression of IL-7 and IL-7R on RA PB monocytes closely correlates with DAS28 scoring and TNF transcription,[12] we asked whether anti-TNF therapy can abrogate IL-7/IL-7R-mediated pathology. We revealed that while IL-7 and IL-7R expression was not impacted following 3 months of anti-TNF treatment, the levels of IL-7R were markedly reduced after 6 months of therapy. Consistently, myeloid IL-7R levels enhanced by LPS and IFNγ were downregulated by anti-TNF Ab treatment. Blockade of IL-7R or TNF function reduced RA macrophage responsiveness to IL-7, resulting in comparably lower levels of IL-6 production, suggesting that TNF can modulate myeloid IL-7R expression. In contrast, others have shown that in responsive RA patients, 3 months of anti-TNF therapy decreases IL-7 expression with no effect on the IL-7R concentration.[25] The lack of effect on the IL-7R level may in part be due to the length of treatment. Validating our clinical findings, we demonstrate that in preclinical studies, anti-TNF Ab therapy suppresses IL-7-mediated arthritis through decreasing joint IL-7R levels and reducing $F480^+iNOS^+$ cell differentiation compared to control treatment. In line with these observations, joint CCL2 and CCL5 expression was downregulated by 2-3-fold in response to anti-TNF Ab treatment with no impact on joint T cell infiltration or differentiation. Our results suggest that anti-TNF treatment only partially suppresses IL-7-driven joint inflammation and bone destruction by decreasing IL-7R; therefore, antibodies against IL-7R are now in the pipeline for RA therapy (GSK2618960 phase 1; ClinicalTrials.gov# NCT02293161).

We conclude that RA joint M1 macrophages express elevated levels of IL-7R and are therefore highly responsive to IL-7 stimulation. IL-7 in synergy with the M1-promoting factors secreted in part by TH-1 cells potentiates the expression of joint myeloid IL-7R and can further exacerbate arthritis via a unique subset of M1 $F480^+iNOS^+CD127^+CCL5^+$ cells.

## MATERIALS AND METHODS

### NL and RA cell isolation

Studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors provided written informed consent. RA patients were diagnosed according to the 1987 revised criteria from the ACR.[26] Mononuclear cells were isolated by Histopaque gradient centrifugation, and monocytes were isolated from NL or RA PB using a negative selection kit (Stemcell Technology) according to the manufacturer's instructions.[12,27]

### RA patient population receiving anti-TNF therapy

PB was obtained from patients with RA who were diagnosed according to the 1987 revised criteria of the American College of

Rheumatology.[26] Blood was obtained from eight women and two men (mean age: 60.4 years). On the first visit (onset), prior to Cimzia (certolizumab pegol) therapy, the patients were receiving no treatment ($n = 1$), methotrexate alone ($n = 1$), corticosteroids alone ($n = 2$), methotrexate plus corticosteroid therapy ($n = 4$), triple therapy (leflunomide, hydroxychloroquine, and corticosteroids) ($n = 1$), or quadruple therapy (methotrexate, sulfasalazine, hydroxychloroquine, and corticosteroids) ($n = 1$). During the second visit, PB was obtained from the same RA patients, and this time point was 3 months after the treatment reported at the first visit was combined with Cimzia ($n = 10$). On the third visit, PB was obtained from the RA patients at 6 months post initiation of Cimzia ($n = 5$). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors provided written informed consent (ClinicalTrials.gov# NCT02451748, phase 4).

### Human and mouse M1 differentiation

To assess the role of IL-7 in M1 differentiation, RA macrophages were in vitro-differentiated for 7 days in the presence of M-CSF (20 ng/ml). Cells were untreated (PBS) or treated with IFNγ/LPS (100 ng/ml each, M1 + control) and IL-7 (100 ng/ml) for 6 h or 24 h prior to quantifying the TNF-α, IL-6, CCL2, and CCL5 levels by real-time RT-PCR or enzyme-linked immunosorbent assay (ELISA). To determine whether IL-7R expression knockdown could impair IL-7-induced M1 differentiation, RA PB cells differentiated in the presence of M-CSF (20 ng/ml) were transfected with 100 nM control or IL-7R-specific siRNA (Santa Cruz) on day 4 for 72 h using Lipofectamine RNAiMax (Life Technologies). Thereafter, the cells were either untreated or treated with IFNγ/LPS (100 ng/ml each, M1 + control) and IL-7 (100 ng/ml) for 6 h prior to quantifying the TNF or IL-6 level by real-time RT-PCR. IL-7R/CD127 cell surface levels were determined in PB monocytes differentiated into macrophages in the presence of M-CSF (20 ng/ml) for 7 days and then untreated (PBS) or treated with IFNγ (100 ng/ml), LPS (100 ng/ml), or IFNγ/LPS (100 ng/ml each) for 24 h. Following the described experimental design, cells were washed and treated with PBS or IL-7 (100 ng/ml) for 24 h, and the levels of TNF and IL-6 were determined by ELISA.

In mouse experiments, bone marrow cells were differentiated in the presence of M-CSF (20 ng/ml) for 7 days prior to treating the cells with PBS or IL-7 (100 ng/ml) for 6 h and quantifying the levels of TNFα, IL-6, and NOS2 by real-time RT-PCR. These results were further validated in 7-day M-CSF-differentiated macrophages treated with PBS, IFNγ (100 ng/ml, M1 + control), or IL-7 (100 ng/ml), and the expression of iNOS (Santa Cruz SC-651) or the frequency of $CD80^+$ cells (eBioscience; B7-1) was quantified by Western blotting or FACS analysis, respectively. Significance of IL-7R in IL-7-induced murine M1 differentiation was documented by expression knockdown studies with the same experimental design as the human studies. The expression knockdown studies were further validated in 8-week-old male WT mice compared to IL-7R−/− mice (Jackson Lab; 002295). For this purpose, bone marrow cells from WT and IL-7R−/− mice were differentiated for 7 days in the presence of M-CSF. Thereafter, the cells were untreated or primed with 100 ng/ml IFNγ or LPS for 24 h prior to PBS or IL-7 treatment for 24 h, and then TNF, IL-6, CCL2, or CCL5 expression was measured by ELISA. More details about each experiment are included in the figure legends.

### RA osteoclast differentiation

To generate mature osteoclasts, RA PBMCs or monocytes were cultured in serum-free RPMI medium and allowed to attach for 1 h. Thereafter, the cells were cultured in 10% α-minimum essential medium (αMEM) and were either untreated (−control) or treated with 20 ng/ml human M-CSF and RANKL (+control) for 14–21 days, with the reagents and culture medium replenished every 3–4 days. Osteoclast formation was examined under suboptimal conditions (10 ng/ml M-CSF plus RANKL) in the presence or absence of the test compound for 14–21 days. To quantify osteoclast formation, TRAP staining was performed using the Acid Phosphatase Leukocyte Kit (Sigma-Aldrich) with cells or mouse ankles according to the manufacturer's instructions. In vitro experiments were performed in triplicate, and the total number of osteoclasts was determined by counting the TRAP+ multinuclear (>4 nuclei) cells in each well.

### Mouse osteoclast differentiation

To determine the impact of IL-7 on mouse osteoclast formation, o/n unattached mouse bone marrow cells were cultured in the presence of 30 ng/ml M-CSF for 3 days. Subsequently, the cells were cultured in 15 ng/ml M-CSF and RANKL (suboptimal condition) and treated with PBS or IL-7 (100 ng/ml) for 72 h before TRAP staining. Alternatively, the TNF, IL-6, or RANK level was quantified after 24 h of treatment. Untreated cells were considered the −control group, and cells grown in the presence of 30 ng/ml M-CSF and RANKL were considered the +control group.

### Western blot analysis

To examine whether IL-7R expression knockdown could impair IL-7 activation, RA PB in vitro-differentiated macrophages or day 4 M-CSF-differentiated bone marrow macrophages were transfected with 100 nM control or IL-7R-specific siRNA (Santa Cruz) for 72 h using Lipofectamine RNAiMax (Life Technologies). IL-7R expression knockdown was examined by Western blot analysis, and blots were probed with anti-IL-7R [(1:50; R&D Systems, MAB306 (human) or Santa Cruz, SC-514445 (mouse)] or anti-actin (1:3000; Santa Cruz, SC-47778) Abs. The activation of iNOS was examined in 7-day M-CSF-differentiated bone marrow macrophages that were untreated or treated with 100 ng/ml IFNγ or IL-7 for 24 h; thereafter, the cell lysates were probed with an anti-iNOS Ab (1:1000; Santa Cruz, SC-651).

### Flow cytometry analysis

To document that IL-7 transforms naive cells into M1 macrophages, RA PB differentiated macrophages were untreated or treated with IFNγ/LPS (100 ng/ml each) and IL-7 (100 ng/ml) for 24 h prior to determining the frequency of cells expressing iNOS/NOS2 (Santa Cruz; C-11, SC-727) by FACS analysis. To demonstrate that IL-7 promotes M1 polarization in murine myeloid cells, 7-day M-CSF-differentiated WT bone marrow macrophages were untreated or treated with 100 ng/ml IFNγ or IL-7 for 24 h prior to quantifying the frequency of $CD80^+$ cells (eBioscience; B7-1) by FACS analysis. To validate IL-7-mediated osteoclastogenesis, RANK (Abcam; ab13918) cell surface expression was determined in RA monocytes and M-CSF-differentiated mouse bone marrow cells cultured under suboptimal conditions and treated with PBS and IL-7 (100 ng/ml) for 24 h. Additionally, the frequencies of $F480^+$ (eBioscience; 123116), $CD127^+$ (BD Bioscience; 561205), $CD11b^+$ (BioLegend; 101216), and $CCL5^+$ (BioLegend; 149103) cells were determined in mouse ankle joints.

### Preclinical studies

All animal studies were approved by the University of Illinois at Chicago Animal Care and Use Committee. Eight-week-old WT C57BL/6 mice were i.a. injected with $10^7$ pore-forming unit (PFU) Ad-Ctl or Ad-IL-7 (Welgen Inc.; $n = 16$ ankles) on days 0 (onset), 3, and 10, and joint circumference was measured[13,28,29] from days 0 to 15 when the mice were sacrificed. These experiments were independently repeated at least twice.

On day 0, 6- to 8-week-old male WT C57BL/6 or RAG1−/− mice (Jackson; 002216) were i.a. injected with saline or LPS (15 μg/ankle). Next, on day 2, the WT and RAG1−/− mice in the saline or LPS group were i.a. injected with $10^7$ PFU Ad-Ctl or Ad-IL-7 ($n = 10$ ankles). Joint circumference was measured with calipers from day 0 to day 16 when the mice were sacrificed.

To demonstrate that IFNγ is partially responsible for the arthritis mediated by IL-7, 6- to 8-week-old male WT C57BL/6 mice were injected i.p. with an anti-IFNγ Ab (100 μg; BioLegend, 505802) on days −3, 0, 3, 6, and 9 in addition to receiving i.a. injections of LPS (15 μg/ankle) on day 0 and Ad-IL-7 ($10^7$ PFU) on day 2 ($n = 10$ ankles). Joint circumference was measured throughout the study, and the mice were sacrificed on day 10. In a different experiment, WT C57BL/6 mice received an i.a. injection of LPS (15 μg/ankle) on day 0; subsequently, on day 2, these mice were ectopically treated with $10^7$ PFU Ad-Ctl or Ad-IL-7. The mice in the Ad-Ctl and Ad-IL-7 groups were also systemically treated with an αTNF Ab (200 μg; Enbrel (etanercept)) or a control substance on day 2 ($n = 10$ ankles). Joint circumference was measured from day 0 to day 9 when the mice were sacrificed. These experiments were independently repeated at least twice.

### Immunohistochemistry

Ankles were decalcified, formalin fixed, paraffin embedded, and sectioned. Briefly, slides were deparaffinized in xylene, and antigens were unmasked by incubating the slides in a Proteinase K digestion buffer (Dako, CA, USA). The ankles were stained with anti-F480 (1:100; Serotec CI: A31, MCA497R), anti-iNOS (1:200; Santa Cruz N-20, SC-651), anti-Arginase (1:200; Santa Cruz N-20, SC-18351), anti-CD3 (1:100, Abcam CD3-12, ab11089), anti-IL-7R (1:100, Santa Cruz C-20, SC662), or control IgG Abs. Hematoxylin and eosin-stained ankles were scored for inflammation, synovial lining thickness, bone erosion, myeloid or T cell recruitment into the joint, M1 (iNOS) or M2 (Arginase) macrophage differentiation, and IL-7R staining on a 0–5 scale[12] by two blinded observers. Images were captured by using the Zeiss microscope 1156-700, the Axiocam ICc1 camera 1069-415, and the Zeiss Zen imaging software.

### Statistical analysis

For comparisons among multiple groups, one-way analysis of variance followed by the Bonferroni post hoc test was utilized using the GraphPad Prism version 7 software. The data were also analyzed using a two-tailed Student's *t* test for paired or unpaired comparisons between two groups. Values of $p < 0.05$ were considered significant.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. S.S. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: S.K. and S.S. Acquisition of data: S.K., M.V.V., K.P., S.U., K.V., R.Z., and S.S. Analysis and interpretation of data: S.K., H.C., K.P., S.U., K.V., J.C., M.M., D.F., and S.S. Providing reagents and involved in the phase 4 study: H.C., A.C., S.V., S.A., A.M., and N.S.

## ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

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