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Ligation of TLR5 Promotes Myeloid Cell Infiltration and Differentiation into Mature Osteoclasts in Rheumatoid Arthritis and Experimental Arthritis

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Our aim was to examine the impact of TLR5 ligation in rheumatoid arthritis (RA) and experimental arthritis pathology. Studies were conducted to investigate the role of TLR5 ligation on RA and mouse myeloid cell chemotaxis or osteoclast formation, and in addition, to uncover the significance of TNF-α function in TLR5-mediated pathogenesis. Next, the in vivo mechanism of action was determined in collagen-induced arthritis (CIA) and local joint TLR5 ligation models. Last, to evaluate the importance of TLR5 function in RA, we used anti-TLR5 Ab therapy in CIA mice. We show that TLR5 agonist, flagellin, can promote monocyte infiltration and osteoclast maturation directly through myeloid TLR5 ligation and indirectly via TNF-α production from RA and mouse cells. These two identified TLR5 functions are potentiated by TNF-α, because inhibition of both pathways can more strongly impair RA synovial fluid–driven monocyte migration and osteoclast differentiation compared with each factor alone. In preclinical studies, flagellin postonset treatment in CIA and local TLR5 ligation in vivo provoke homing and osteoclastic development of myeloid cells, which are associated with the TNF-α cascade. Conversely, CIA joint inflammation and bone erosion are alleviated when TLR5 function is blocked. We found that TLR5 and TNF-α pathways are interconnected, because TNF-α is produced by TLR5 ligation in RA myeloid cells, and anti–TNF-α therapy can markedly suppress TLR5 expression in RA monocytes. Our novel findings demonstrate that a direct and an indirect mechanism are involved in TLR5-driven RA inflammation and bone destruction. The Journal of Immunology, 2014, 193:000–000.

Rheumatoid arthritis (RA) is a chronic autoimmune disorder in which the numbers of monocyte-derived macrophages are greater than in normal (NL) joints and are well correlated with radiological damage, joint pain, and inflammation (1, 2). Yet, the mechanism that derives RA myeloid cell trafficking and further facilitates their maturation to osteoclasts is incompletely understood.

Osteoclasts are multinucleated bone-resorbing cells differentiated from the myeloid lineage found in RA peripheral blood (PB) and synovial tissue (ST) (3–5). In RA, dominance of proinflammatory factors such as TNF-α, IL-1β, IL-6, and IL-17 can promote osteoclast maturation through enhancing myeloid receptor activator for NF-κB receptors. This is suggested by TNF-α receptor activator for NF-κB (RANK) expression, as well as RANK ligand (RANKL) production from RA ST fibroblasts and T cells (4, 6–9). Interestingly, ligation of TNF-α to myeloid TNFR1 and TNFR2 can directly facilitate osteoclast differentiation through a mechanism that is independent of RANK/RANKL cascade (10, 11). Confirming this notion, others have shown that RA synovial fluid (SF) macrophages can transform into mature osteoclasts in the presence of M-CSF in combination with RANKL or TNF-α/-IL-1β, suggesting that proinflammatory factors such as TNF-α/-IL-1β can replace the function of RANKL (12), whereas TNF-α and IL-17 are known to be responsible for joint myeloid cell retention directly through myeloid cell ligation and indirectly via induction of monocyte chemokines (13–15).

TLRs belong to the family of pattern recognition receptors, and TLR2 and TLR4 are abundantly expressed in RA PB monocytes, RA SF, and ST macrophages (16–19). Identification of TLR2 and TLR4 endogenous ligands in RA synovium has triggered an interest in discovering the role of these receptors in the RA pathogenesis (20). Hence the impact of TLR2 and TLR4 ligation has been extensively studied on bone degradation, primarily in mouse bone marrow cells (21–23) with a few studies performed in NL human myeloid cells (24). Despite these comprehensive in vitro investigations, the obtained results are controversial and the effect of TLR4 ligation on osteoclast differentiation is greatly dependent on the treatment time point, the cell type used, and the concentration of reagents used (21, 24, 25). However, the in vivo studies consistently support the significance of TLR4 activation in experimental...
arthritis bone loss (26–28). Unlike TLR2 and TLR4, the role of TLR5 in RA and murine models of RA is undefined.

In our recent article, we uncovered for the first time, to our knowledge, that the TLR5 expression is markedly accentuated in RA compared with NL ST and PB myeloid cells (29). We also found that ligation of myeloid TLR5 to potential endogenous ligands in the RA joint can modulate SF TNF-α transcription (29). Notably, expression of myeloid TLR5 closely correlates with RA disease activity and TNF-α levels (29), suggesting that ligation of TLR5 in RA myeloid cells contributes to disease progression. Therefore, the significance of the TLR5 cascade was investigated in monocyte cell function using RA PB monocytes and mouse PB and bone marrow cells, as well as in acute and chronic experimental arthritis models.

In this study, we demonstrate that the TLR5 agonist, flagellin, can dose-dependently promote monocyte migration and osteoclast maturation through its direct effect on monocyte cell function and indirectly via TNF-α production from RA and mouse myeloid cells or collagen-induced arthritis (CIA) ankle joints. Conversely, anti-TLR5 Ab therapy attenuates CIA joint myeloid cell homing and bone erosion. Consistent with our findings in RA, flagellin treatment can strongly transform mouse bone marrow progenitor cells into mature osteoclasts through a TNF-α-dependent and IFN-β-independent mechanism. In conclusion, a strong positive feedback regulation exists between TLR5 and TNF-α pathways in attracting the circulating monocytes and further remodeling the newly recruited cells into mature osteoclasts; therefore, disruption of TLR5 ligation can dysregulate both functions in preclinical arthritis models.

**Materials and Methods**

**Monocyte chemotaxis**

Experiments were performed to determine the effect of flagellin on monocyte chemotaxis. Mononuclear cells were isolated by Histopaque (GE Healthcare Bio-Sciences, Pittsburgh, PA) gradient centrifugation, and monocytes were isolated from NL or RA PB using negative selection kit (Stem Cell Technology, Vancouver, BC) according to the manufacturer’s instruction (30, 31). Chemotaxis was performed in a Boyden chamber (Neuro Probe, Gaithersburg, MD) using NL monocytes for 2 h with varying concentrations (0.001–100 ng/ml) of flagellin (Ultrapure; endotoxin level <50 endotoxin units/mg; Invivogen, San Diego, CA), fMLF (f; 10 nM) and EG2 (1:1000 dilution; Cell Signaling) and IκB Ab (1:3000 dilution; Santa Cruz) or probed with AKT, ERK, p38, or actin Abs (1:3000 dilution; Cell Signaling or Sigma).

**Flagellin signaling in monocytes**

NL monocytes were untreated or treated with flagellin (100 ng/ml) for 15–65 min. Cell lysates were examined by Western blot analysis (14, 15). Biorec were probed with p-AKT1, p-ERK, p-p38, p-JNK, p-paxillin, and p-FAK (1:1000 dilution; Cell Signaling) and IκB Ab (1:3000 dilution; Santa Cruz) or probed with AKT, ERK, p38, or actin Abs (1:3000 dilution; Cell Signaling or Sigma).

**RA patient population**

These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. RA patients were diagnosed according to the 1987 revised criteria from the American College of Rheumatology (32). PB was obtained from 68 patients (64 women and 4 men; mean age 48±6.1 years) at the time of evaluation, patients were either on disease-modifying antirheumatic drugs (DMARDs; n = 34, 3 men and 31 women, mean age 51.6±16.2) or treatment with anti-TNF therapy (n = 34, 1 man and 33 women, mean age 45.7±12.4). Treatment with DMARDs (n = 34) consisted of DMARDs alone (methotrexate, lefunomide, sulfasalazine, azathiprine, hydroxychloroquine, or minocycline; n = 27), of which two were on hydroxychloroquine only, or treatment with DMARD plus prednisone (n = 7). Patients treated with anti-TNF therapy (n = 34) were either on anti-TNF therapy alone (n = 7), anti-TNF plus prednisone (n = 1), anti-TNF therapy plus DMARD (n = 21), or anti-TNF with DMARD and prednisone (n = 5).

**Quantification of tartrate-resistant acid phosphatase**

To generate mature osteoclasts, we cultured NL or RA PBMCs or monocytes in 0% RPMI media and allowed them to attach for 1 h. Thereafter, cells were cultured in 10% α-MEM and were either untreated (negative control) or treated with 20 ng/ml human M-CSF and RANKL (positive control; ProSpec, Brunswick, NJ) for 14–21 d with the reagents and the culture media replenished every 3 d. The ability of test reagents to differentiate human precursor cells to fully mature osteoclasts was examined in the suboptimal culture conditions that consisted of 10 ng/ml human M-CSF and RANKL (R&D Systems) plus anti–TNF-α Ab (B (Bay 11-7085) (EMD Millipore, Billerica, MA) for 19701A; Stem Cell Technology) according to the manufacturer’s instruction (30, 31). Chemotaxis was performed in a Boyden chamber (Neuro Probe, Gaithersburg, MD) using NL monocytes for 2 h with varying concentrations (0.001–100 ng/ml) of flagellin (Ultrapure; endotoxin level <50 endotoxin units/mg; Invivogen, San Diego, CA), fMLF (f; 10 nM) was used as positive control, and PBS was used as negative control (14, 15). Cell culture media, PBS, culture plates, and all reagents were used for endotoxin contamination.

To demonstrate the RA SF-mediated monocyte trafficking involves TLR5 ligation, we incubated cells with anti-TLR5 (10 μg/ml; Invivogen) or IgG Abs for 1 h before performing monocyte chemotaxis in response to eight different RA SFs (20% dilution). To show that TLR5 and TNF-α pathways are interconnected in facilitating monocyte migration, we incubated RA SFs (20%) with IgG or anti–TNF-α (10 μg/ml; R&D Systems), and monocytes were immunoneutralized by either anti-TLR5 or IgG Abs (10 μg/ml) before performing monocyte chemotaxis.

To examine the signaling pathways associated with flagellin-induced monocyte chemotaxis, we treated monocytes with DMSO or 1 and 5 μM inhibitors to PI3K (LY294002), ERK (PD98059), JNK (SP600125), and NF-κB (Bay 11-7085) (EMD Millipore, Billerica, MA) for 1 h. Subsequently, monocyte chemotaxis was performed in response to 100 ng/ml flagellin.

To document that flagellin and TNF-α synergistically contribute to monocyte chemotaxis, we examined monocyte migration in response to various concentrations of flagellin (0.1 and 10 ng/ml) or TNF-α (0.1 and 5 ng/ml) alone or in combination.

To determine whether T cell function plays a critical role in flagellin-mediated osteoclastogenesis, we cultured negatively selected RA myeloid cells in suboptimal conditions in the presence or absence of flagellin (10 ng/ml) before TRAP staining.

To show that flagellin-mediated osteoclast maturation is due to TLR5 ligation and TNF-α induction, we incubated RA PBMCs cultured in suboptimal conditions with 10 μg/ml IgG, anti–TNF-α, or anti–TLR5-α before flagellin (10 ng/ml) treatment and followed by TRAP staining.

To determine whether TLR5 ligation on osteoclasts, we exposed NL and RA PBMCs to varying concentrations of flagellin (0.001–100 ng/ml) in suboptimal culture conditions (10 ng/ml human M-CSF and RANKL) before TRAP staining.

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mouse 10 ng/ml M-CSF and 100 ng/ml RANKL were regarded as positive control.

To determine that TLR5 ligation contributes to bone erosion in acute and chronic models of arthritis, we TRAP-stained ankles ectopically treated with flagellin or vehicle, as well as ankle joints harvested from CIA postonset treatment of flagellin or PBS. To document that blockade of TLR5 ligation can impair osteoclast differentiation, we performed TRAP staining on CIA ankle joints harvested from IgG or anti–TLR5 Ab therapy. Number of osteoclasts stained in mouse ankles was quantified by counting the number of TRAP+ cells in each section (33).

**FACS analysis**

Cells were blocked with 50% human serum in 0.5% BSA to determine the percentage of TLR5+ T cells in NL and RA PBMCs. Subsequently, cells were stained with PE-conjugated anti-TLR5 (Imgenex, San Diego, CA) and FITC-labeled anti-CD3 (BD Pharmingen, Franklin Lakes, NJ) or IgG Abs (BD Pharmingen).

**Real-time RT-PCR**

Total cellular RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA), and relative gene expression was determined by real-time RT-PCR using the $\Delta\Delta C_t$ method (29–31). To determine the mechanism by which TLR5 ligation promotes osteoclast formation, we cultured RA PBMCs for 7 d at suboptimal conditions (10 ng/ml M-CSF and RANKL). Thereafter, cells cultured in α-MEM in the absence of M-CSF and RANKL were untreated or stimulated with flagellin (100 ng/ml) for 6 h, and the expression of RANK, RANKL, TNF-α, and IFN-β was quantified.

TNF-α and IFN-β real-time RT-PCR was performed on mouse osteoclasts precursor cells from day 4 cultured in 10 ng/ml mouse M-CSF plus 25 ng/ml mouse RANKL treated with or without flagellin (100 ng/ml) for 6, 24, 48, and 72 h. In addition, transcription of calcitonin receptor (CTR), cathepsin K (CTSK), RANKL, and IFN-β was determined in CIA ankles treated with flagellin (20 μg) or PBS. TLR5 expression was quantified in RA monocytes treated with DMARDs or anti–TNF-α with or without DMARDs.

**Cytokine quantification**

Mouse TNF-α protein concentration was determined by ELISA (R&D Systems) according to the manufacturer’s instructions in CIA mouse ankles treated with PBS or flagellin (20 μg), as well as in day 4 mouse bone marrow precursor cells untreated (PBS) or treated with flagellin (100 ng/ml) plus IgG (10 μg/ml) versus flagellin (100 ng/ml) plus anti–TLR5 (10 μg/ml) for 24 h. Joint IL-6 and CCL2 protein levels were determined in CIA mice treated with IgG or anti–TLR5 Ab.

**Study protocol for animal models**

Eight-week-old DBA/1 mice were immunized with collagen type II (Chondrex, Redmond, WA) on days 0 and 21 (34, 35). Flagellin (20 μg, n = 10; Invivogen) or PBS (n = 10) was injected i.p. on day 33 after CIA induction, mice were sacrificed on day 57, and experiments were repeated twice. Ankle circumference was determined by Caliper using the following formula: Circumference = $2B \times (\sqrt{a^2 + b^2/2})$, where a and b represent the diameters (34–36). In a different experiment, C57BL/6 mice were injected intra-articularly (i.a.) on day 0 with 20 μg flagellin or PBS, mice were sacrificed on day 10, and ankle circumference was determined by Caliper (34–36). To validate that the loss of TLR5 function can critically impact joint inflammation and bone erosion, after CIA induction (34, 35), we treated mice i.p. with IgG or anti–TLR5 Ab.

**FIGURE 1.**

TLR5 ligation promotes monocyte migration through activation of AKT1/P3K, JNK, and NF-κB pathways. (A) Flagellin monocyte chemotaxis was performed in a Boyden chemotaxis chamber with varying concentration (0.001–100 ng/ml), n = 3. (B) Monocytes were incubated with anti–TLR5 Ab (10 μg/ml) or control IgG for 1 h; thereafter chemotaxis was performed in response to 20% RA SF (n = 8). (C) Monocytes were stimulated with 100 ng/ml flagellin for 0–65 min, and the cell lysates were probed for p-AKT1, p-ERK, p-p38, p-JNK, p-FAK, p-paxillin, and degradation of IκB (n = 3). (D) Cells were preincubated with DMSO (D) or 1 and 5 μM inhibitors to P3K (LY294002), ERK (PD98059), p38 (SB203580), JNK (SP600125), and NF-κB (Bay 11-7085) for 1 h. Subsequently, monocyte chemotaxis was performed in response to 100 ng/ml flagellin for 2 h (n = 3). For all experiments, PBS and fMLF (f) served as negative and positive controls. Values demonstrate mean ± SE. *p < 0.05.
monoclonal rat anti-mouse TLR5 Ab (100 μg/injection; Invivogen) on days 23, 27, 30, 34, 37, 41, 44, and 48. Animals were sacrificed on day 49 after induction.

Immunohistochemistry

Mouse ankles were decalcified, formalin fixed, paraffin embedded, and sectioned. In brief, slides were deparaffinized in xylene, and Ags were unmasked by incubating slides in Proteinase K digestion buffer (Dako, Carpinteria, CA). Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako). CIA ankles were stained with P480 (1:100 dilution; Serotec), iNOS (1:200 dilution; Santa Cruz), or control IgG Abs (Beckman Coulter). Joint myeloid cell or M1 macrophage staining was scored on a 0-5 scale by two blinded observers.

Statistical analysis

One-way ANOVA was used for comparison among multiple groups followed by post hoc two-tailed Student t test. The data were also analyzed using two-tailed Student t test for paired or unpaired comparisons between two groups. The p values < 0.05 were considered significant.

Results

Activation of PI3K/AKT1, JNK, and NF-κB pathways contributes to TLR5-induced monocyte chemotaxis

Because TLR5+ monocyte-derived macrophages are elevated in RA compared with NL ST, we asked whether circulating monocytes can migrate into the RA joint where TLR5 endogenous ligands are expressed (29). We found that flagellin is chemotactic for monocytes at concentrations ranging from 1 to 100 ng/ml (Fig. 1A). Further blockade of myeloid TLR5 suppressed RA SF-mediated monocyte chemotaxis, suggesting that the potential TLR5 endogenous ligands can contribute to joint monocyte homing (Fig. 1B). We next demonstrate that monocytes stimulated with flagellin phosphorylate AKT1, ERK, p38, JNK, paxillin, and degrade IκB pathways; in contrast, FAK was not activated by TLR5 ligation (Fig. 1C). Interestingly, although inhibition of ERK and p38 was ineffective, suppression of PI3K, JNK, and NF-κB pathways markedly reduced flagellin-mediated chemotaxis starting at 1 μM (Fig. 1D). These results suggest that ligation of TLR5

FIGURE 2. TLR5-mediated monocyte trafficking is interconnected to TNF-α pathway, and ligation of myeloid TLR5 is a strong promoter of RA osteoclast differentiation. (A) Expression of TLR5 was quantified in RA monocytes treated with DMARDs (n = 34) or with anti–TNF-α in the presence or absence of DMARDs (n = 34). (B) Monocyte migration was examined in response to various concentrations of flagellin (0.1 and 10 ng/ml) or TNF-α (0.1 and 5 ng/ml) alone or in combination. The combined doses were compared with the same doses treated alone (n = 3). (C) Twenty percent RA SFs were incubated with 10 μg/ml IgG or anti–TNF-α, and monocytes were either immunoneutralized by 10 μg/ml anti-TLR5 or IgG control before performing chemotaxis (n = 7). (D) RA PBMCs were differentiated to fully mature osteoclasts in the presence 20 ng/ml M-CSF and RANKL, whereas suboptimal conditions consisted of 10 ng/ml M-CSF and RANKL (n = 4). NL (n = 3) (E) and RA PBMCs (n = 4) (F) were exposed to varying concentrations of flagellin (0.001–100 ng/ml) in the presence of 10 ng/ml human M-CSF and RANKL (suboptimal condition) before TRAP staining, and significance is compared with no flagellin treatment (0) in the suboptimal condition in (E) and (F). (G) Representative TRAP staining (original magnification ×200) of (F). Negative (−) and positive (+) controls consisted of untreated cells or cells treated with 20 ng/ml M-CSF and RANKL. Values demonstrate mean ± SE. *p < 0.05.
by SF endogenous ligands can modulate monocyte homing through activation of PI3K/AKT1, JNK, and NF-κB pathways.

**TLR5 interconnects with TNF-α in mediating monocyte chemotaxis**

Ligation of TLR5 strongly induces production of TNF-α in RA monocytes and macrophages (29); thus, we asked whether TLR5 expression can be affected by anti–TNF-α therapy. Interestingly, myeloid TLR5 expression was 2.5-fold higher in RA patients treated with DMARDs compared with those treated with anti–TNF-α agents (Fig. 2A), suggesting that these two pathways are cross regulated. Consistently, we demonstrate that TNF-α and flagellin can synergistically induce myeloid cell migration (Fig. 2B), and hence blockade of both cascades can more potently suppress RA SF-mediated monocyte chemotaxis compared with each pathway alone (Fig. 2C). These results indicate that in the RA joint, expression of TNF-α triggered by myeloid TLR5 ligation can further enhance TLR5-driven myeloid cell infiltration.

**Myeloid TLR5 ligation activates RA osteoclast formation**

Because TLR5 ligation contributes to RA joint myeloid cell chemotaxis, we asked whether TLR5 activation could transform the recruited myeloid cells into mature osteoclasts. Experiments performed established that PBMCs could be differentiated into fully mature osteoclasts in the presence 20 ng/ml M-CSF and RANKL, whereas the suboptimal conditions consisted of 10 ng/ml M-CSF and RANKL in RA PBMCs (Fig. 2D). We next demonstrated that TLR5 ligation in NL and RA PBMCs could dose-dependently contribute to osteoclast formation when cultured in suboptimal conditions (Fig. 2E–G), suggesting that flagellin can promote transcription of essential osteoclastogenic factors.

We found that in RA PBMCs, flagellin treatment upregulates RANK, RANKL, and TNF-α expression levels by 2- to 8-fold (Fig. 3A–C). In contrast with our findings, others have shown that TLR5 ligation inhibits mouse bone marrow cell differentiation to mature osteoclast through IFN-β induction (37); therefore, transcription of IFN-β was also assessed in our culture system. Interestingly, we document that there was an insignificant higher trend of IFN-β expression in RA cells treated with flagellin compared with the PBS treatment (Fig. 3D).

Because RANKL is produced from RA T cells and fibroblasts, we asked whether TLR5 is expressed on T cells and whether these cells are critical for flagellin-mediated osteoclastogenesis. Although the cell-surface TLR5 levels were not significantly higher, there was, however, a greater expression trend in RA compared with NL CD3+ T cells (Fig. 3E, 3F). We show that ligation of TLR5 could differentiate osteoclast precursor cells into fully mature osteoclasts in the absence of T cells when monocytes were

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**FIGURE 3.** Ligation of TLR5 in RA PBMCs drives the transcription of pro-osteoclastogenic factors, and TLR5 ligation promotes RA myeloid cells to form mature osteoclasts in suboptimal culture conditions. Using real-time RT-PCR, we quantified RANK (A), RANKL (B), TNF-α (C), and IFN-β (D) mRNA levels in RA osteoclast precursor cells that were cultured in suboptimal condition (10 ng/ml M-CSF and RANKL) for 7 d before being treated with PBS or 10 ng/ml flagellin for 6 h in the absence of M-CSF and RANKL (n = 7). Results are shown as fold increase above the PBS group and are normalized to GAPDH. (E) NL and RA PBMCs were immunostained with FITC-labeled anti-CD3 Ab and PE-conjugated anti-TLR5 to determine the percentage of CD3+ and TLR5+ cells (n = 3); (F) a representative flow cytometry histogram of (E). (G) Negatively selected RA monocytes were cultured in the suboptimal condition and were either untreated (PBS) or treated with 10 ng/ml flagellin before TRAP staining. (H) Representative TRAP staining (original magnification ×200) of (G). Negative and positive control consisted of untreated cells or cells treated with 20 ng/ml M-CSF and RANKL. Values demonstrate mean ± SE. *p < 0.05.
cultured in a suboptimal condition (Fig. 3G, 3H). Taken together, these results suggest that monocytes are the effector cells in TLR5-mediated osteoclastogenesis, and flagellin can facilitate osteoclast formation by increasing RANK expression and allowing the cells to be more responsive to RANKL binding, resulting in less RANKL being required for this process. In addition, it is possible that the pro-osteoclastogenic factors counterbalance the inhibitory effect of IFN-β in part because flagellin-induced TNF-α transcription can potentiate RANK/RANKL cascade whereas suppressing the IFN-β transcription (38).

**TLR5 links with TNF-α in enhancing joint osteoclastogenesis**

Next, experiments were performed to document whether TNF-α is capable of potentiating TLR5-mediated osteoclast formation. We show that TLR5-mediated osteoclastogenesis is in part due to TNF-α produced from RA myeloid cells (Fig. 4A, 4B). However, because osteoclast differentiation driven by TLR5 and TNF-α is dose dependent, supplementing the flagellin-treated cells with exogenous TNF-α further increased the number of TRAP+ osteoclasts by 2-fold (Fig. 4C, 4D). Confirming this notion, we show that in RA SF, TLR5 endogenous ligand(s) connect with TNF-α in promoting osteoclastogenesis, because inhibition of both pathways can suppress RA SF-mediated osteoclast formation more potently compared with each pathway alone (Fig. 4E). Our results indicate that TLR5 and TNF-α pathways are linked in fostering RA myeloid cell recruitment and osteoclast differentiation.

**Flagellin fosters osteoclastogenesis in mouse PB and bone marrow cells via TNF-α, which is distinct from IFN-β pathway**

In contrast with previous findings (37), we document that ligation of TLR5 plays a critical role in osteoclastogenesis as determined in vitro in RA PB myeloid cells and in vivo in acute and chronic models of experimental arthritis. Therefore, experiments were performed for the first time, to our knowledge, in mouse PB myeloid cells, as well as in bone marrow cells, to address the data discrepancy. We show that flagellin activation transforms negatively selected mouse PB monocytes cultured in suboptimal conditions into multinuclei mature osteoclasts (Fig. 5A, 5B). Because of limited access to mouse PB monocytes (blood from 20 mice was used to obtain adequate mouse PB myeloid cells for 4 wells/conditions), mouse bone marrow cells were used to determine the mechanism by which flagellin ligation to TLR5 promotes osteoclastogenesis. Consistent with our results in RA cells, we demonstrate that blockade of TLR5 or TNF-α in mouse bone marrow osteoclast precursor cells significantly reduces flagellin-induced

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**FIGURE 4.** In RA joint, TLR5- and TNF-α-mediated osteoclastogenesis is interconnected. (A) RA PBMCs cultured in suboptimal conditions (10 ng/ml M-CSF and RANKL) were untreated or pretreated with 10 μg/ml IgG, anti–TNF-α, and anti–TLR5 Abs before being stimulated with 10 ng/ml flagellin, followed by TRAP staining. (B) A representative image of TRAP+ cells (original magnification ×200) from (A), n = 3. (C) RA PBMCs were cultured in suboptimal condition and were either untreated or treated with 1 ng/ml flagellin, 1 ng/ml TNF-α, or both before TRAP staining. (D) A representative image of TRAP staining (original magnification ×200) from (C), n = 3. (E) RA PBMCs cultured in suboptimal condition were immunoneutralized by 10 μg/ml IgG control or anti-TLR5 Abs, and cells were then incubated with 2% RA SF plus IgG or 2% RA SF plus anti–TNF-α (10 μg/ml) subsequent to TRAP staining (n = 4). Negative and positive control consisted of untreated cells or cells treated with 20 ng/ml M-CSF and RANKL. Values demonstrate mean ± SEM. *p < 0.05.
osteoclast maturation, in part, by inhibiting TNF-α production (Fig. 5C–E). In mouse bone marrow osteoclast precursor cells, flagellin treatment elevates TNF-α transcription by 4-fold at 6 h, and these levels remain constantly 2-fold higher from 24 to 72 h of stimulation, whereas IFN-β mRNA levels are not significantly accentuated at 6 h stimulation and are further reduced after 24–72 h of flagellin activation (Fig. 5F). These results suggest that similar to RA myeloid cells, ligation of murine TLR5 cells by flagellin can strongly promote osteoclast formation, in part, through production of TNF-α and dysregulation of IFN-β cascade.

**Postonset treatment of CIA with flagellin contributes to elevated joint myeloid cell infiltration and osteoclastogenesis**

We next asked whether acute and/or chronic arthritis driven by TLR5 ligation is due to homing and differentiation of myeloid cells into mature osteoclasts. We document that when CIA mice were therapeutically treated with TLR5 agonist, joint swelling was markedly greater in mice that received flagellin treatment compared with PBS control (Fig. 6A, 6B). Similar to our in vitro studies, we found that postonset treatment with flagellin could strongly facilitate myeloid cell recruitment into the CIA ankle joints (Fig. 6C, 6D). Further, joint TNF-α production levels were 5.5-fold higher, whereas transcription of IFN-β was unchanged in CIA mice that received postonset treatment of flagellin compared with the control group (Fig. 6E, 6F). We also show that the number of TRAP⁺ cells (Fig. 7A, 7B) and the concentration of bone erosion markers CTR, CTSK, and RANKL (Fig. 7C) were lower in the control ankles compared with the flagellin-treated CIA joints. These results indicate that the elevated joint myeloid cell migration and their differentiation to osteoclasts may be caused by TLR5 ligation, as well as TNF-α production, or it is possible that both mechanisms of action contribute to the detected observations.

Because CIA ankle swelling and bone erosion were exacerbated by flagellin postonset treatment, we next asked whether local in-
jection of flagellin alone could drive joint inflammation and osteoclastogenesis. We demonstrate that ectopic TLR5 ligation elevates ankle circumference from days 0 to 2, and subsequently joint inflammation plateaus until day 8; however, swelling remains consistently higher than the PBS group (Fig. 7D, 7E). Corroborating with the CIA data, i.a. injection with flagellin resulted in 10-fold greater mature osteoclasts compared with the control group (Fig. 7F, 7G). Hence, consistent with our in vitro studies, data generated in acute and chronic animal models suggest that flagellin ligation to joint TLR5 contributes directly, as well as indirectly, to myeloid cell infiltration and osteoclast maturation.

Blockade of TLR5 function impairs CIA joint monocyte infiltration and osteoclast formation

To document the critical role of TLR5 in RA pathogenesis, we systemically treated CIA mice with IgG or anti-TLR5 Ab. Results from these experiments demonstrate that CIA mice treated with anti-TLR5 Ab have markedly reduced joint swelling starting on day 44 until day 48 compared with the IgG control group (Fig. 8A–C). When the underlying mechanism of function was examined, we found that blockade of TLR5 inhibited F480CD80+iNOS+ M1 macrophage differentiation in CIA ankle joints (Fig. 8D, 8E) which resulted in significantly lower production of joint IL-6 and CCL2 protein levels compared with the IgG control group (Fig. 8F). In addition, we show that joint myeloid cell migration and their remodeling to mature osteoclasts were compromised in the anti-TLR5 Ab group compared with the control group (Fig. 8G, 8H). Because blockade of TLR5 function ameliorates joint myeloid cell trafficking and their transformation into M1 macrophages and/or fully mature osteoclasts, these results suggest that TLR5 can be used as a target for RA therapy.

Discussion

In this study, we demonstrate for the first time, to our knowledge, that myeloid TLR5 ligation to endogenous ligands expressed in the RA joint strongly promotes monocyte trafficking and osteoclast formation. We document that TLR5 and TNF-α pathways are cross regulated, because ligation of TLR5 in RA and mouse osteoclast precursor cells activates TNF-α production, and anti–TNF-α therapy markedly reduces RA myeloid TLR5 expression. Flagellin treatment in mouse PB and bone marrow cells, as well as in CIA ankle joints, reveals that joint TLR5 ligation contributes to elevated osteoclast maturation through a TNF-α–dependent and IFN-β–independent mechanism. Finally, alleviation of joint inflammation and bone destruction by anti-TLR5 Ab therapy in CIA further establishes TLR5 as a novel RA therapeutic target.

Our initial observation that TLR5 expression is elevated in RA myeloid cells and has a close correlation with disease activity and myeloid TNF-α concentration (29) triggered our interest in unraveling how ligation of myeloid TLR5 impacts RA pathogenesis. Because increase in the number of joint myeloid cells can be caused by increased chemotaxis, reduced efflux, or cell death, we examined the role of TLR5 in monocyte chemotaxis. We demonstrate that RA SF TLR5 endogenous ligand(s) participate in

FIGURE 6. Flagellin postonset treatment in CIA contributes to joint inflammation, elevated joint myeloid cell trafficking, and TNF-α production. (A) Changes in joint circumference were determined in CIA mice that were treated i.p. with PBS or flagellin (20 μg) on day 33 (n = 10). (B) Ankles were harvested on day 57 from CIA mice treated with PBS or flagellin and were H&E stained (original magnification ×200; n = 7). (C) STs from CIA mice treated with PBS or flagellin were harvested on day 57 and were immunostained with anti-F480 Ab (original magnification ×200). Arrows demonstrate F480+ cells. (D) Macrophage staining was quantified on a 0–5 scale (n = 7). (E) TNF-α protein levels (pg/ml) were quantified by ELISA in ankle homogenates from CIA mice treated with PBS or flagellin (n = 7). (F) Transcription of IFN-β was determined by real-time RT-PCR in CIA ankles that had received postonset treatment of flagellin or control, and the data are shown as fold increase above PBS group and are normalized to GAPDH (n = 5). Values are mean ± SE. *p < 0.05.
joint monocyte homing through activation of PI3K/AKT, JNK, and NF-κB, which is distinct from the pathway used by classical monocyte chemoattractants such as CCL2/MCP-1, CCL5/RANTES, CCL3/MIP-1α, fMLF, and IL-17 that use p38 cascade (14, 39). We further demonstrate that TLR5-induced monocyte migration is potentiated by TNF-α, as blockade of both pathways can more efficiently reduce RA SF-mediated myeloid cell recruitment. TNF-α is shown to be a potent in vitro (13) and in vivo (40) monocyte chemoattractant that facilitates monocyte transendothelial migration, in part, via enhancing endothelial adhesion molecules ICAM-1 and CD44 (41). Consistently, RA responders to anti–TNF-α therapy have significantly reduced numbers of RA ST CD68+ sublining macrophages (42). Previous studies demonstrate that TLR4 ligation can promote osteoclast formation in RANKL pretreated mouse bone marrow cells through a TNF-α–related cascade (21). However, when mouse preosteoclasts were simultaneously treated with LPS, RANKL, and M-CSF, osteoclast maturation was suppressed by TLR4 ligation despite elevated TNF-α production (21, 23). It was shown that the inhibitory effect of TLR4 ligation on osteoclastogenesis was due to downregulation of RANK, as well as elevated expression of osteoprotegerin or IFN-γ (22, 24). Others have shown that similar to TLR4, ligation of TLR5 inhibits transformation of mouse bone marrow cells to differentiated osteoclasts through IFN-β induction (37). In contrast with the previous studies (37), we document that in RA and murine cells, as well as in CIA ankles, flagellin treatment was unable to enhance IFN-β transcription, and this may be, in part, because of the robust TNF-α transcription, because earlier findings demonstrate that TNF-α signaling can counterbalance type I (IFN-β and other isoforms) and type II (IFN-γ) IFN function (38, 46–48).

 Much like our findings in RA (29), earlier studies demonstrate that TLR5 is elevated in cancer compared with NL lingual epithelium and may be a novel predictive marker for tongue cancer.
recurrence (49). Notably, although flagellin can strongly facilitate migration and invasion of the salivary gland adenocarcinoma cell line, LPS and Pam3CSK4 did not have any effect on these functions (50), suggesting that TLR5 shows some unique characteristics that do not overlap with other TLRs both in cancer and RA.

Despite extensive studies, controversial results were obtained when the effect of TLR4 ligation was examined on osteoclastogenesis of mouse bone marrow cells because the outcome was heavily dependent on the cell treatment conditions (21–23). Therefore, in this study, the pathogenic role of TLR5 was assessed in RA and mouse cells, as well as in experimental arthritis models. Unlike results generated in mouse bone marrow studies, ligation of TLR2 and TLR4 in RA ST fibroblasts cocultured with myeloid cells could strongly promote osteoclast formation through induction of IL-1β and RANKL (51). In contrast with TLR2- and TLR4-mediated osteoclastogenesis, which is driven by IL-1β (51), our data demonstrate a strong connection between TLR5 and TNF-α in joint bone erosion.

Notably, it was demonstrated by two independent groups that ligation of TNFR1 and TNFR2 by TNF-α could strongly drive osteoclast differentiation, and this process was unaffected by blockade of RANKL via osteoprotegerin (10, 11). Confirming this finding, others show that macrophages from RA SF can differentiate to fully mature osteoclasts in the presence of M-CSF with RANKL or TNF-α/TNF-β, suggesting that factors such as TNF-α and IL-β can substitute RANKL’s function in the RA joint (12).

The same group found that osteoclast formation was more pronounced in RA SF that contained higher TNF-α levels (52). We document that transcription of RA SF TNF-α is modulated by SF TLR5 endogenous ligands (29), and that TLR5 interconnects with TNF-α in enhancing joint osteoclastogenesis. Therefore, these observations indicate that SF with most abundant TNF-α may be a result of elevated TLR5 endogenous ligands that contribute to markedly enhanced bone degradation.

Interestingly, a number of endogenous ligands have been identified that can activate TLR4 function (including fibrinogen, surfactant protein-A, fibronectin extra domain A, heparan sulfate, soluble hyaluronan, and defensin 2), whereas other endogenous ligands can trigger both TLR2 and TLR4 pathways (such as heat shock proteins [HSPs; HSP60, 70, and 96] and HMGB1) (53, 54). Most recently, RNA extracted from RA SF was shown to be a TLR7 endogenous ligand that could dose-dependently modulate RA SF TNF-α transcription (55). Earlier studies demonstrate that stimulation with HSP70 can further potentiate flagellin-mediated NF-κB luciferase activity in TLR5 transfected HEK 293T reporter cells, but because this effect was not detected with HSP70 treatment alone, these results suggest that HSP70 may operate as a chaperone protein for TLR5 endogenous ligands (56). There is still a possibility that other HSPs can serve as a potential TLR5 endogenous ligand in the RA joint; therefore, experiments are currently being conducted to identify these TLR5 endogenous ligands in RA SFs and STs.

Although gain of TLR5 function promotes myeloid cell recruitment and osteoclast formation partly through potentiating the

**FIGURE 8.** Anti-TLR5 Ab treatment alleviates CIA joint swelling and bone resorption. (A) Changes in joint circumference were recorded for CIA mice that were treated i.p. with IgG or anti-TLR5 Ab (100 μg/mouse) on days 23, 27, 30, 34, 41, 44, and 48, and mice were sacrificed on day 49 after induction, n = 6 mice (12 ankles). (B) Effect of anti-TLR5 Ab treatment on inflammation, lining thickness, and bone erosion was scored on a 0–5 scale (n = 6). (C) Representative ankle H&E staining (original magnification ×200) of (B). (D) STs from CIA mice treated with IgG or anti-TLR5 Ab were harvested on day 49 and immunostained with anti-F480 (1:200 dilution) or iNOS Abs (1:200 dilution) (original magnification ×200). Joint myeloid cells and iNOS+ M1 macrophage staining were quantified on a 0–5 scale (n = 6). (E) Representative F480 and iNOS immunostaining (original magnification ×200) of (D). (F) Changes in IL-6 and CCL2 protein levels in ankle homogenates from CIA mice treated with IgG control or anti-TLR5 Ab were determined by ELISA (n = 6). (G) Number of TRAP+ cells were counted per section in CIA mice treated with IgG or anti-TLR5 Ab (n = 6). (H) Representative ankle TRAP staining (original magnification ×200) of (G). Values are mean ± SE. *p < 0.05.
joint TNF-α production, loss of TLR5 function reverses both mechanism of function, in part, by suppressing the proinflammatory M1 macrophage differentiation process in CIA ankle joints. In contrast, joint inflammation and bone erosion mediated by local injection of TNF-α was unaffected in TLR2- and TLR4-deficient mice, suggesting that TNF-α-driven joint pathology is independent of TLR2 and TLR4 function (26). In contrast, severity of IL-1-induced bone erosion and cartilage destruction was markedly suppressed in TLR4−/− mice. Similarly, TLR4-induced calvarial bone resorption was significantly reduced in IL-1R

...importance of this receptor in RA disease. Clinical evidence obtained from TLR5 loss of function highlight the and both functions are interconnected to joint TNF-α cross-regulation in immune-mediated inflammatory disease: basic

References

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.


