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12/15-Lipoxygenase Counteracts Inflammation and Tissue Damage in Arthritis

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Eicosanoids are essential mediators of the inflammatory response and contribute both to the initiation and the resolution of inflammation. Leukocyte-type 12/15-lipoxygenase (12/15-LO) represents a major enzyme involved in the generation of a subclass of eicosanoids, including the anti-inflammatory lipoxin A₄ (LXA₄). Nevertheless, the impact of 12/15-LO on chronic inflammatory diseases such as arthritis has remained elusive. By using two experimental models of arthritis, the K/BxN serum-transfer and a TNF transgenic mouse model, we show that deletion of 12/15-LO leads to uncontrolled inflammation and tissue damage. Consistent with these findings, 12/15-LO-deficient mice showed enhanced inflammatory gene expression and decreased levels of LXA₄ within their inflamed synovia. In isolated macrophages, the addition of 12/15-LO-derived eicosanoids blocked both phosphorylation of p38MAPK and expression of a subset of proinflammatory genes. Conversely, 12/15-LO-deficient macrophages displayed significantly reduced levels of LXA₄, which correlated with increased activation of p38MAPK and an enhanced inflammatory gene expression after stimulation with TNF-α. Taken together, these results support an anti-inflammatory and tissue-protective role of 12/15-LO and its products during chronic inflammatory disorders such as arthritis. The Journal of Immunology, 2009, 183: 3383–3389.

Both the initiation and resolution of inflammation are strictly controlled by regulatory feedback mechanisms to promote rapid clearance of the causative agent and limit tissue damage by reactive oxygen species and matrix-degrading enzymes. Improper regulation of this process has been implicated in the development of chronic inflammatory diseases such as arthritis and atherosclerosis (1).

Bioactive lipid-mediators were shown to act as key modulators of inflammation (2, 3). During the initial phase of the inflammatory response, cyclooxygenase (COX)²⁻² and 5-lipoxygenase (LO) catalyze the generation of prostaglandins and leukotrienes, respectively. These two groups of eicosanoids facilitate the inflammatory reaction by promoting edema formation and leukocyte influx (2). Inhibition of COX-2 and 5-LO, as well as genetic deletion of their encoding genes, was shown to ameliorate the disease progression in different mouse models of rheumatoid arthritis (RA) and atherosclerosis (4–8). Other arachidonic acid-metabolising enzymes, including human 15-LO-1 and its murine ortholog the 12/15-LO, have been implicated in the generation of anti-inflammatory lipid mediators such as lipoxin A₄ (LXA₄; Ref. 9). Though lipoxins were shown to exert multiple biologic effects in vitro and in vivo, the potential role of 12/15-LO during the resolution of inflammation is incompletely understood. Importantly, this enzyme displays additional properties, including the oxidation of low-density lipoproteins (10), allowing it to contribute to vascular inflammation and development of atherosclerosis (11–14). Hence, and despite the potential anti-inflammatory action of 12/15-LO, animal models studying the role of 12/15-LO in the pathogenesis of atherosclerosis delivered conflicting results (15–19).

The involvement of 12/15-LO in the pathogenesis of arthritis as a prototype of an inflammatory disease has not yet been investigated. In the present study, we determined the impact of 12/15-LO-deficiency in two mouse models of inflammatory arthritis. Interestingly and consistent with an anti-inflammatory role of this enzyme, we observed a dramatic exacerbation of arthritis and inflammatory joint destruction in 12/15-LO⁻/⁻ mice. This correlated with reduced levels of anti-inflammatory 12/15-LO-derived LXA₄ in synovial extracts from 12/15-LO-deficient mice. In vitro studies showed that LXA₄ was able to inhibit TNF-α-induced phosphorylation of p38MAPK and expression of a subset of inflammatory genes in macrophages. Conversely, the reduction of endogenous LXA₄-levels in 12/15-LO⁻/⁻ macrophages correlated with an enhanced activation of p38MAPK and an increased inflammatory gene expression. These data demonstrate a prominent anti-inflammatory role of 12/15-LO and 12/15-LO-derived lipid mediators during chronic inflammation and highlights the 12/15-LO-LXA₄ axis as an important endogenous feedback loop during inflammatory diseases such as arthritis.

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Abbreviations used in this paper: COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; LXA₄, lipoxin A₄; RA, rheumatoid arthritis; tg, transgenic; WT, wild type; KC, keratinocyte chemoattractant.

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Materials and Methods

Materials

Murine 12/15-LO Ab, 12-hydroxyeicosatetraenoic acid (HETE), and 15-HETE were obtained from Cayman Chemicals. LXA₄ was bought at Sigma-Aldrich, while the Abs against p-p38MAPK, p-AKT and p-IκB were purchased from Cell Signaling Technology. 12/15-LO-deficient mice were purchased from The Jackson Laboratory. Mice suffered from no apparent health defects or morphologic alterations such as hepatosplenomegaly (maximum age of used mice was 10 wk).

Lipid quantification

Competitive ELISA techniques were used to determine levels of LXA₄ (Oxford Biomedical Research), 13-S-hydroxyoctadecadienoic acid (HODE), and PGE₂ (both kits from Assay Designs). For each sample, synovial tissue from knee joints (three mice per group) was homogenized and the respective eicosanoids were isolated following the protocol provided by the manufacturer using solid phase columns.

TNF transgenic (tg) mice and K/BxN serum transfer.

Scoring of clinical signs of arthritis in TNF tg mice and measurement of body weight was done in all mice once weekly starting from 6- to 10-wk of age. Arthritis was evaluated in a blinded manner as described previously (20). K/BxN serum transfer and quantification of arthritis was performed as previously described (21) using 10-wk-old male C57/B6 mice. After i.p. application of 200 μl of K/BxN serum, mice were killed at day 11 and tissue was prepared for gene expression and histology. Histology and histomorphometric analyses were performed as previously described (20). For the experiments presented, male mice were used. Similar results were obtained with female mice (data not shown).

Quantitative real-time PCR

Real-time PCR was performed as previously described (22). RNA was isolated using TRIzol Reagent (Invitrogen). Nine hundred nanograms of total RNA were reverse transcribed with human leukemia virus reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems) and oligo(dT) primers. mRNA levels were normalized to β-actin expression. The following primer-sequences were used: 12/15-LO: CTCTCAAG

Arthritis exacerbates in 12/15-LO-deficient animals

12/15-LO deficiency significantly aggravates the clinical course of arthritis and causes accelerated and pronounced joint swelling (Fig. 2A), which indicates an anti-inflammatory role of 12/15-LO in arthritis. Clinical arthritis scores reached a plateau at day 11 after serum stimulation. Histological analysis of the hind paws of arthritis mice at day 11 revealed increased destruction of the joint architecture in 12/15-LO−/− animals (Fig. 2B). This, thus, histomorphometric analysis showed a significant increase in bone erosions and in the number of bone-resorbing osteoclasts in 12/15-LO-deficient mice (Fig. 2C). Furthermore, real-time PCR-based analysis of the gene expression pattern revealed a significant increase in the mRNA levels of a subset of proinflammatory genes, including IL-6 and IL-1β in the synovial tissue of 12/15-LO−/− mice (Fig. 2D).

Abnormalities of 12/15-LO aggravates arthritis of TNF tg mice

Based on the conflicting data on the pro- vs anti-inflammatory role of 12/15-LO in the pathogenesis of atherosclerosis, we sought to confirm our data using a second model of arthritis. Therefore, we interbred 12/15-LO-deficient animals with TNF tg mice, which are characterized by a severely destructive form of arthritis. Strikingly, TNF tg animals lacking 12/15-LO displayed a reduced survival rate and did not achieve appropriate weight gain (Fig. 3A). Moreover, and in accordance with the data obtained from the K/BxN serum-transfer model, 12/15-LO-deficiency caused a much severer form of arthritis in the TNF tg model (Fig. 3A). Histological analysis showed increased destruction of the hind paws in 12/15-LO−/− TNF tg mice in comparison with 12/15-LO+/+ TNF tg mice (Fig. 3B). This was evident by significantly enlarged inflammatory infiltrates, increased size of bone erosions, and an increased number of osteoclasts (Fig. 3C). Similarly, real-time PCR analysis showed a significantly enhanced gene expression of IL-1β and IL-6 in joints of 12/15-LO−/− TNF tg as compared with 12/15-LO+/+ TNF tg mice (Fig. 3D).

Importantly, the deregulated inflammatory gene expression in 12/15-LO−/− TNF tg mice was not confined to the inflamed joints. Systemically we observed profound alterations in the expression of inflammatory marker genes in various tissues (Fig. 3E). This pronounced systemic inflammation might additionally explain the

Results

12/15-LO is expressed in experimental murine arthritis

To address the role of 12/15-LO in arthritis, we performed the K/BxN serum-transfer model (21) in wild-type (WT) and 12/15-LO-deficient mice. Immunohistochemical analysis of joints of arthritic WT mice revealed strong expression of 12/15-LO in inflammatory synovial tissue (Fig. 1A). Likewise, the quantitative real-time PCR showed a prominent expression of 12/25-LO mRNA in synovial tissue after induction of the serum-transfer arthritis (Fig. 1B).

Arthritis exacerbates in 12/15-LO-deficient animals

12/15-LO deficiency significantly aggravates the clinical course of arthritis and causes accelerated and pronounced joint swelling (Fig. 2A), which indicates an anti-inflammatory role of 12/15-LO in arthritis. Clinical arthritis scores reached a plateau at day 11 after serum stimulation. Histological analysis of the hind paws of

FIGURE 1. 12/15 is expressed in murine experimental arthritis. A, Hind paws of WT and 12/15-LO−/− mice were immunohistochemically stained for 12/15-LO expression at day 11 after stimulation with K/BxN serum. B, Quantification of 12/15-LO mRNA expression levels in synovial tissue from knee joints of the indicated group of animals (n = 3 per group). KO, Knock out; rel., relative; N.D., non-detectable.
progressive symptoms of wasting observed in 12/15-LO−/− TNF tg mice and suggests a role of 12/15-LO in the inhibition of local as well as of systemic inflammatory responses. Furthermore, 12/15-LO appears to be essential to limit inflammation-associated tissue damage during chronic inflammatory arthritis.

12/15-LO-deficient mice display reduced levels of anti-inflammatory LXA4 in their synovial tissue

12/15-LO activity has the potential to oxygenate different fatty acids, including linoleic and arachidonic acid, thereby participating in the generation of different lipid mediators, including LXA4. Because this eicosanoid has been implicated in anti-inflammatory feedback loops promoting the resolution of inflammation, we sought to quantify levels of different lipid mediators in the inflamed synovium of arthritic mice. Consistent with the proposed actions of 12/15-LO, this analysis revealed strong alterations in the eicosanoid profile in synovial extracts in 12/15-LO−/− animals. The levels of the abundant linoleic acid-derived 12/15-LO product 13-hydroxyoctadecadienoic acid were drastically reduced within the synovia of 12/15-LO−/− animals (Fig. 4, top left). Moreover, and in line with a prominent role of 12/15-LO during lipoxin biosynthesis (9, 18), these mice displayed decreased levels of LXA4 (Fig. 4, top right). In contrast, the levels of COX-derived PGE2 were not different among WT and 12/15-LO−/− mice in the K/BxN serum-transfer arthritis model (Fig. 4, bottom left), while PGE2 levels were increased rather than decreased in 12/15-LO−/− TNF tg mice as compared with their 12/15-LO+/+ TNF tg littermates. This observation might additionally reflect the overwhelming inflammatory response in these animals (Fig. 4C).

12/15-LO-derived eicosanoids differentially modulate the inflammatory response

To further elucidate mechanisms involved in the anti-inflammatory effects of 12/15-LO and 12/15-LO-derived lipid mediators, we evaluated the effects of single 12/15-LO-derived lipid oxidation products on the inflammatory response in isolated macrophages. Gene expression analyses revealed expression of both FPRL1 and FPR-RS2 as potential receptors for LXA4 (23) in murine peritoneal macrophages (Fig. 5A). ELISA-based measurement of the cytokine production in TNF-α-stimulated cells showed that the 12/15-LO-derived eicosanoids LXA4, 12-HETE, and 15-HETE differentially inhibited the inflammatory response (Fig. 5B). Although IL-6 secretion was almost completely blocked by these lipid-mediators, only LXA4 significantly inhibited keratinocyte chemoattractant (KC) expression. In contrast, none of these eicosanoids altered secretion of IFNγ. Quantification of mRNA transcripts by real-time PCR confirmed these observations on mRNA expression level (Fig. 5C). 12/15-LO-derived mediators such as LXA4 were shown to negatively regulate different proinflammatory signaling pathways, including the p38MAPK and NFκB pathways, in several cell-types (24–27). The analysis of TNF-α-induced signaling events in macrophages showed that the addition of the different 12/15-LO-derived eicosanoids did not influence TNF-α-induced activation of the proximal NFκB or PI3K pathway, although they potently blocked TNF-induced phosphorylation of p38MAPK (Fig. 5D).
12/15-LO-deficient macrophages display an increased inflammatory response

We studied the effects of TNF-α on macrophages from WT and 12/15-LO−/− mice to delineate the role of endogenous 12/15-LO activity on the inflammatory response. In accordance with our in vivo findings and in line with previous data from other groups (18), the quantification of eicosanoids in cellular extracts of 12/15-LO−/− macrophages revealed significantly reduced levels of LXA4 (Fig. 6A). In parallel with the reduction in the content of this major anti-inflammatory lipid mediator, these cells exhibited an increased inflammatory response. ELISA-based measurement of different inflammatory cytokines showed that 12/15-LO−/− macrophages released significantly more IL-6 and KC after stimulation with TNF-α (Fig. 6B). Again, this effect was restricted to a subset of TNF-α-induced genes, as we did not observe an increase in the TNF-α-induced release of IFNγ. This enhanced production of proinflammatory cytokines was also evident on the mRNA expression level, which reflected the differential effect of 12/15-LO activity on inflammatory gene expression (Fig. 6C) and revealed a prolonged inflammatory response in 12/15-LO−/− macrophages (Fig. 6D).

Based on the observed effects of 12/15-LO-derived eicosanoids on TNF-α-induced signaling events, we analyzed activation of various signaling pathways in WT and 12/15-LO-deficient macrophages. Again, immunoblot analysis revealed no difference in the phosphorylation state of IκB after stimulation with TNF-α (Fig. 6E). Although the basal phosphorylation of AKT was clearly reduced in 12/15-LO-deficient macrophages, we did not observe alterations in the TNF-α-induced levels of phospho-AKT among WT and 12/15-LO−/− macrophages (Fig. 6E). In line with the effects of the 12/15-LO-derived products on p38MAPK activity, the determination of the p38MAPK phosphorylation state revealed a striking increase in basal and TNF-α-induced activation of p38MAPK in macrophages from 12/15-LO−/− mice (Fig. 6E). To further confirm this 12/15-LO-mediated regulation of p38MAPK activation in vivo, we determined the phosphorylation of p38MAPK in synovial tissue extracts after induction of KxB/N serum-transfer arthritis. In accordance with our in vitro observations, we found increased levels of phosphorylated p38MAPK in 12/15-LO−/− mice (Fig. 6F), suggesting a major role of 12/15-LO and its products in the regulation of p38MAPK activity under chronic inflammatory conditions.

Discussion

Inflammatory arthritides such as RA constitute a major disease burden worldwide. This group of chronic inflammatory diseases leads to progressive destruction of cartilage and bone. Although...
attention has so far mainly focused on factors contributing to the progression of the disease, it is equally important to understand counter-regulatory mechanisms involved in the termination of the inflammatory response and limitation of associated tissue injury. Resolution of inflammation can be regarded as an active process governed by multiple factors including arachidonic acid-derived metabolites such as lipoxins, which are considered key anti-inflammatory players. LXA₄ blocks proinflammatory signals and initiates resolving mechanisms, including apoptosis and phagocytosis of neutrophils (9). Application of lipoxins and their stable analogues was shown to ameliorate the course of inflammatory diseases, including inflammatory bowel disease, arthritis, and asthma (28–30). Studies using a mouse transgenic for the human lipoxin receptor (ALX) have additionally provided genetic evidence for a role of the lipoxin receptor FPRL-1 (ALX) in the resolution of inflammation (31). Nevertheless, little is known about the role of endogenously produced lipoxins during the pathogenesis of chronic inflammatory disorders such as RA. This group of eicosanoids is generated via a biosynthetic process involving both 12/15-LO and 5-LO activity (9). Although 5-LO itself mainly contributes to the propagation of inflammation by an abundant generation of proinflammatory leukotrienes, the expression of human 15-LO and its murine homologue 12/15-LO follows the expression of 5-LO and COX-2 and shows a peak expression during the resolution phase of inflammation (32). Nevertheless, the exact role of this enzyme during chronic inflammatory disease has remained elusive. 12/15-LO activity has been initially implicated in the oxidation of low density lipoprotein particles, thereby generating proinflammatory oxidized low density lipoprotein. Deletion of 12/15-LO was shown to reduce lipid peroxidation in vivo as well as to ameliorate the formation of atherosclerotic lesions in murine models of atherosclerosis (33). A recent study, however, which included data from animals deficient for 12/15-LO and from animals overexpressing a 12/15-LO transgene, indicated a more complex role of this enzyme during vascular inflammation and pointed toward additional anti-inflammatory properties, which mainly seem to involve the generation of lipoxins (18). This study supports previous findings showing that 15-LO tg rabbits display reduced atherosclerotic lesions (17), which probably indicates a context-dependent role of 12/15-LO in the modulation of the inflammatory response.

Using two models of inflammatory arthritis, we now present clear evidence that 12/15-LO is a major anti-inflammatory enzyme during the course of inflammatory joint disease. In both models, the reduced levels of LXA₄ correlated with an increased expression of proinflammatory cytokines and increased tissue damage. Additional support for a protective role of LXA₄ during arthritis comes from a recent study demonstrating beneficial effects of an LXA₄ agonist on murine collagen-induced arthritis (29). Moreover, overexpression of 15-LO in rabbits was shown to ameliorate inflammation, tissue damage, and bone loss in a model of acute periodontitis (34). Expression of 15-LO as well as the level of LXA₄ is increased in the inflamed synovium of RA patients (35), which strongly indicates an important role of this enzyme in human RA as well. Despite the absence of 12/15-LO, we still detected LXA₄ in synovial tissue of 12/15-LO−/− mice. Alternative
pathways involving platelet 12-LO, which can additionally contribute to lipoxin generation in vivo, might explain this fact. 12/15-LO/H11002/TNF tg mice displayed increased levels of COX-derived PGE2 in their synovial tissue. Interestingly, the PGE2-producing enzyme PGE2 synthase was shown to be regulated by proinflammatory cytokines in rheumatoid synoviocytes (36). Consistent with previous studies, we observed strong anti-inflammatory effects of LXA4 in vitro (24, 25). Although LXA4 was the most potent 12/15-LO-derived eicosanoid tested, 12-HETE and 15-HETE also displayed anti-inflammatory activity, which might involve conversion of these compounds to lipoxins by endogenous macrophage-derived 5-LO activity. LXA4 was shown to act in a SOCS-2-dependant manner (37). In splenocytes, the anti-inflammatory effects of LXA4 seem to involve the induction of TRAF6-degradation leading to a blockade of both p38MAPK and the NFκB pathway (38). Moreover, LXA4 was shown to induce expression of the transcriptional corepressor NAB1 in human neutrophils, indicating that inhibitory effects of lipoxins might be mediated by de novo synthesized proteins (39). Although we observed a clear inhibition of the phosphorylation of p38MAPK by 12/15-LO-derived eicosanoids in peritoneal macrophages, activation of the NFκB and PI3K pathways was not affected. Similarly, expression of the corepressor NAB1 was not changed after LXA4 treatment (data not shown). In line with these effects exerted by the addition of 12/15-LO-derived eicosanoids, 12/15-LO /-/ macrophages exhibited an increased activation of p38MAPK with no alteration in the activation level of NFκB.

Previously it was shown that with an increase in age, 12/15-LO-deficient animals can develop a myeloproliferative disorder involving deregulation of the PI3K pathway (40). Interestingly, we observed that the basal phosphorylation of AKT was diminished in 12/15-LO /-/ macrophages. This might indicate pathway-specific differences in the effects of LXA4 in certain cell types. In parallel with the modulation of the p38MAPK pathway, we observed LXA4-mediated inhibition of IL-6 and KC expression. Again, TNF-α-induced expression of these cytokines was increased in 12/15-LO /-/ macrophages. This data is supported by a recent study demonstrating increased expression levels of IL-1 in 12/15-LO /-/ macrophages after challenge with bacterial products (41). Interestingly, the TNF-α-induced expression of IFNγ was altered neither by addition of LXA4 nor by the absence of 12/15-LO, which indicates a differential regulation of inflammatory cytokines by the 12/15-LOX-LXA4-axis. This observation is in accordance with a necessity for p38MAPK activity in the transcriptional induction of KC and IL-6 expression by NFκB, which involves p38-mediated histone H3 phosphorylation (42).

Taken together, our present study presents evidence supporting a prominent role of 12/15-LO and 12/15-LO-derived mediators in a regulatory feedback loop, which counteracts the exacerbation of inflammation and associated tissue damage. These insights in

FIGURE 6. 12/15-LO-deficient macrophages exhibit an increased inflammatory response. A, Quantification of LXA4 levels in extracts from isolated peritoneal macrophages by competitive ELISA technique. (B) ELISA-based measurement of IL-6, KC, and IFNγ protein levels in supernatants and (C and D) quantification of mRNA levels by real-time PCR after stimulation of peritoneal macrophages with TNF (10 ng/ml for 6 h). Western blot analysis of the indicated signaling pathways in (E) extracts of peritoneal macrophages isolated from WT and 12/15-LO /-/ mice after stimulation with TNF (10 ng/ml) or (F) extracts of knee joints of the indicated group of mice. Data shown in A–E is representative for at least three independent experiments.
mechanisms underlying the resolution of inflammation are useful to develop novel tools to control inflammation and to efficiently treat chronic inflammatory diseases such as arthritis.

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Disclosures
The authors have no financial conflict of interest.

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