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Atf3 negatively regulates Ptgs2/Cox2 expression during acute inflammation

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Abstract

By generating prostaglandins, cyclooxygenase-2 (Cox-2/Ptgs2) plays a critical role in regulating inflammatory responses. While several inflammatory stimuli have been shown to increase *Ptgs2* expression, less is known about how the transcription of this gene is terminated. Here we show that stimulation of macrophages with yeast zymosan, a TLR2/6 and dectin-1 agonist, causes a transient increase in the expression of *Ptgs2* accompanied by a simultaneous increase in the expression of the transcriptional repressor, Activating transcription factor-3 (*Atf3*). The expression of *Ptgs2* was significantly higher in resident peritoneal macrophages isolated from $Atf3^{-/-}$ mice than that from $Atf3^{+/+}$ mice and was associated with higher prostaglandin production upon stimulation with zymosan. In activated macrophages, Atf3 accumulated in the nucleus and chromatin-immunoprecipitation analysis showed that Atf3 is recruited to the *Ptgs2* promoter region. In acute peritonitis and in cutaneous wounds, there was increased leukocyte accumulation and higher levels of prostaglandins (PGE_2/PGD_2) in inflammatory exudates of $Atf3^{-/-}$ mice compared with WT mice. Collectively, these results demonstrate that during acute inflammation Atf3 negatively regulates *Ptgs2* and therefore dysregulation of this axis could potentially contribute to aberrant Ptgs2 expression in chronic inflammatory diseases. Moreover, this axis

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Authorship contributions

J.H., Y.T., and M.J.Z. carried out experiments, analyzed data and contributed to the writing of the manuscript; A.B. and S.S. contributed to the planning of the project and writing the manuscript; T. H. provided the *Atf3*-deficient mice and contributed to the writing of the manuscript. M.S. planned the project, analyzed data and wrote the manuscript.

Disclosure of Conflicts of Interest

None

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could be a new therapeutic target for suppressing *Ptgs2* expression and the resultant inflammatory responses.

Keywords

Inflammation; prostaglandins; lipid mediators

Introduction

Cyclooxygenases/prostaglandin endoperoxide synthases (Cox/Ptgs 1 & 2) generate prostaglandins that play well-documented roles in regulating vascular tone, thrombosis, inflammation and pain [1–3]. Because of their critical role in regulating inflammation, cyclooxygenase enzymes are therapeutic targets of one of the most widely used classes of drugs, namely, the non-steroidal anti-inflammatory drugs (NSAIDs) [4]. While *Ptgs1* is constitutively expressed, *Ptgs2* is induced to high levels during inflammation [5, 6]. Previous studies have shown that *Ptgs2* transcription is initiated by several transcription factors associated with inflammatory signaling cascades, including NF-κB, AP-1, Sp1 and C/EBP [6]. Less is known about how the transcription of *Ptgs2* is repressed, although repression elements in the promoter region of the *Ptgs2* gene have been identified, and some negative regulatory factors including nuclear receptor co-repressor (NCoR), the poly (ADP-ribose) polymerase-1 (Parp-1) and sumoylated CEBPB have been shown to repress *Ptgs2* expression in cervical cancer cells, pancreatic β-cells and carcinoma cells, respectively [6–9]. Nevertheless, it is not known how during resolution of acute inflammation, *Ptgs2* is repressed in tissues and immune cells.

The Activating transcription factor 3 (Atf3) is a member of the ATF/CREB family of basic leucine zipper (bZip) transcription factors. It forms homodimers with itself or heterodimers with other bZip proteins, and the resulting dimers can function as a transcriptional activator or repressor [10]. Atf3 is induced by a variety of cellular stressors, such as ischemia and endoplasmic reticulum stress, and there is accumulating evidence indicating that Atf3 negatively regulates toll-like receptor (TLR)-response genes including interleukin 6 (*Il-6*) and *Il-12b* [10–12]. Computational approaches predict that because of proximity of ATF/CRE sites to NF-κB and AP-1 binding sites in the *Ptgs2* promoter, Atf3 could also be a potential regulator of *Ptgs2*. Studies showing that *Ptgs2* is one of the genes upregulated in *Atf3*-deficient cardiomyocytes [13] supports this possibility, although regulation of *Ptgs2* by Atf3 per se has not been extensively studied in immune cells. Here, we provide direct evidence that, during the development and resolution of acute inflammation, Atf3 negatively regulates *Ptgs2* in leukocytes.

Materials and Methods

Acute peritonitis and cutaneous wounds

To induce peritonitis, male 8–12 week old WT (C57/BL6J; Jackson laboratories) and age-matched *Atf3*-deficient mice (C57/BL6J background) [14] were administered yeast zymosan (0.04mg/g; *i.p.*; Sigma) and inflammatory exudates were collected 4h after injection [15]. In

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some experiments, mice received prostanoid receptor antagonist, AH6809 (10ng; i.p.), or COX-2 inhibitor, NS-398 (100ng; i.p.), 30 minutes prior to zymosan administration. All procedures were approved by the University of Louisville IACUC. Inflammatory exudates were obtained by lavaging the peritoneum with 5ml DPBS$^{-/-}$ and leukocytes were enumerated and identified by flow cytometry using anti-Ly6G (polymorphonuclear neutrophils; PMN) and anti-F4/80 (macrophages) antibodies (Biolegend). Expression of *Atf3* and *Ptgs2* mRNA was evaluated in cell pellets obtained from inflammatory exudates (see below). Cytokines and chemokines were measured in cell-free exudates (Aushon biosystems and Ebioscience Flowcytomix). Cutaneous wounds were created as described previously [16]. Briefly, the dorsal skin was shaved and treated with depilatory cream after anesthesia. The skin was rinsed and two circular, full thickness wounds (skin and panniculus carnosus) were created using a 5-mm biopsy punch. Wounds were covered by a semipermeable polyurethane dressing and wound tissue was collected 24h post-wounding. Wound tissue was formalin fixed, paraffin embedded and sectioned. Accumulation of PMN was evaluated in deparaffinized sections of wound tissue using anti-myeloperoxidase (MPO) antibodies and an UltraVision detection system (Thermo Scientific, Labvision).

In vitro macrophage incubations

Resident peritoneal macrophages were isolated from WT or *Atf3*-deficient mice in DPBS$^{-/-}$ and incubated for 1h in 24-well plates at 37°C in DMEM containing 10% FBS. Non-adherent cells were removed and the macrophages were either left untreated or stimulated with yeast zymosan (50μg/ml) for 3 or 6h. Supernatants were collected for LC-MS/MS analysis (see below) and cell pellets were used for gene expression analysis or Western blot. For this, RNA was extracted using the RNeasy mini kit (Qiagen), followed by cDNA synthesis. RT-PCR amplification was performed with SYBR-green qPCR master mix (SA Biosciences) using a 7900HT fast system (Applied Biosystems) and commercially available primers for murine *Atf3* (IDT), *Ptgs2, Ptgs1*, microsomal prostaglandin E synthase 1 (*mPges1*), hematopoietic prostaglandin D synthase (*hPGDS*), arachidonate 5 lipoxygenase (*Alox5*), *Alox5* activating protein (*ap*) and interleukin 1 beta (*Il-1*β) (SA biosciences). Relative expression was determined by the $2^{-\ \ C_T}$ method after internal normalization to *Hprt*. To assess COX-2 protein expression, macrophages treated without or with zymosan for 6h and lysates were prepared. Expression of COX-2 was determined using an anti-COX-2 antibody (Thermo Scientific) and was normalized for loading using an anti-β-actin antibody (Sigma).

LC-MS/MS analysis of lipid mediators

Macrophage supernatants or inflammatory exudates were collected for targeted LC-MS/MS analysis of leukotriene B_4 (LTB_4) and prostaglandins E_2/D_2 (PGE_2/D_2) [15]. Upon sample collection, 2 volumes of cold methanol containing deuterium-labeled (d4) PGE_2 was added and samples were placed at −80°C to allow for protein precipitation. Lipid mediators were extracted using solid-phase (C18) columns and methyl formate fractions were collected and taken to dryness under a stream of N_2 gas. After resuspending in methanol, samples were analyzed using an HPLC system (Shimadzu prominence) equipped with a C18 reverse-phase column (4.6 mm × 50 mm) coupled to a triple quadrupole mass spectrometer (AB Sciex; API2000). The instrument was operated in negative ionization mode and the mobile phase

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consisted of methanol:water:acetic acid (60:40:0.01, vol/vol/vol), which was ramped to 80:20:0.01 over 3 min and to 95:5:0.01 in the next 14 min at a constant flow rate of 400μL per min. Lipid mediators were identified and quantified using multiple reaction monitoring (MRM) and transitions for LTB_4 (335>195) and PGE_2/PGD_2 (351>189). We note that under these LC conditions, PGE_2 and PGD_2 are not readily separated and are therefore presented as "PGE_2/D_2". Extraction recovery was determined using internal d4-PGE_2, while all lipid mediators were quantified based on external calibration curves using authentic standards (Cayman chemical).

Nuclear localization of Atf3

RAW 264.7 (ATCC) macrophages were incubated with zymosan (50μg/ml) for 6h. The NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) was used to obtain nuclear extracts that were analyzed by immunoblotting with anti-Atf3 (Santa Cruz) and anti-Hdac1 (Cell Signaling) antibodies. Immunoblots were developed using Luminata Forte Western HRP Substrate (Millipore), detected using a Typhoon 9400 variable mode imager (Amersham Biosciences), and quantified with ImageQuant TL.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out according to the manufacturers' instructions (Santa Cruz) and essentially as described in [17]. Briefly, RAW 264.7 macrophages were stimulated with zymosan (50μg/ml) for 6h and the cells were fixed with 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated with glycine (0.125M). Cell lysis was carried out in ice-cold lysis buffer and crude nuclear extracts were obtained by centrifugation. Pellets were resuspended in high salt lysis buffer on ice and DNA was sheared by sonication. The resulting chromatin solution was precleared with salmon sperm/protein-A agarose and a sample of input DNA (3%) was collected. Immunoprecipitation was carried out overnight at 4°C with anti-Atf3 antibody (200 μg/0.1 ml) or normal rabbit serum (NRS). Antibody–protein–DNA complexes were captured, beads were resuspended in elution buffer and cross-links were reversed. The supernatant was incubated at 67°C overnight and DNA was extracted with a QIAquick PCR Purification kit (QIAGEN). PCR was conducted using promoter specific primers (see Fig. 2) for murine *Ptgs-2*; sense 5′-CGCAACTCACTGAAGCAGAG -3′ and antisense 5′- TCCTTCGTGAGCAGAGTCCT-3′. PCR products were separated on 2% agarose gels and bands captured under UV illumination.

Statistical analysis

Experimental results are mean ± SEM. Statistical significance ($P<0.05$) was determined using an unpaired two-tailed Student's *t* test or one-way ANOVA, followed by Tukey's multiple comparisons post-test, as appropriate.

Results

To determine the temporal relationship between induction of *Ptgs2* and *Atf3*, we stimulated macrophages with the TLR2/6 and dectin-1 agonist, zymosan [18, 19]. A robust induction of *Ptgs2* was observed after 3h, and its levels declined significantly (~50%) after 6h (Fig. 1A),

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suggesting termination of transcription or an increase in degradation. In addition, zymosan stimulation of macrophages led to robust induction of *Atf3* mRNA, with a time course similar to the induction of *Ptgs2* (Fig. 1B). Given this similarity, we assessed whether *Atf3* deficiency would alter *Ptgs2* expression. We found that in comparison with WT mice, levels of *Ptgs2* mRNA were higher in resident peritoneal macrophages isolated from *Atf3*$^{-/-}$ mice (Fig. 1C). The regulation of *Ptgs2* by Atf3 was dynamic, as the difference in *Ptgs2* mRNA in unstimulated macrophages was lost after 3h of stimulation with zymosan, whereas *Ptgs2* was again elevated in *Atf3*$^{-/-}$ macrophages after 6h of zymosan stimulation (Fig. 1D). This time course mirrored the induction and repression of *Ptgs2* in WT macrophages (Fig. 1A). This increase in *Ptgs2* mRNA translated to increased COX-2 protein expression in *Atf3*-deficient macrophages stimulated with zymosan relative to WT macrophages (Fig. 1E). To determine whether the induced Ptgs2 was catalytically active, we measured the level of its products—prostaglandins E_2 and D_2 (PGE_2/D_2), which are generated by downstream synthases in macrophages from the common biosynthetic intermediate, PGH_2. Indeed, we found that the higher levels of Ptgs2 in *Atf3*-deficient macrophages correlated with higher levels of PGE_2/D_2 in supernatants of macrophages stimulated with zymosan for 6h, while no differences were observed without stimulation (Fig. 1F). Although mRNA levels of *Ptgs1* and PG synthases, *mPges1* and *hPgds*, were elevated in *Atf3*-deficient macrophages at baseline, there were no significant differences in expression of these genes in cells stimulated with zymosan (Supplemental Figure 1). Taken together, these observations suggest that Atf3 negatively regulates *Ptgs2* expression in activated macrophages.

We next tested whether Atf3 regulates *Ptgs2* gene expression via direct recruitment to the *Ptgs2* promoter. In addition to inducing *Atf3* transcription (Fig. 1B), zymosan stimulation of macrophages increased nuclear accumulation of Atf3 protein (Fig. 2A & B). As noted, the *Ptgs2* promoter contains consensus ATF/CRE binding sites, which are in close proximity to both AP-1 and NF-κB binding sites [11]. Hence, we used chromatin-immunoprecipitation (ChIP) to determine whether Atf3 is recruited to the *Ptgs2* promoter in cells stimulated with zymosan. PCR amplification of DNA bound to immunoprecipitated Atf3 (using primers flanking an ATF/CRE binding site −56/−52 upstream of the transcription start site; Fig. 2C) showed that Atf3 is recruited to the *Ptgs2* promoter (Fig. 2D). No amplification was observed in samples immunoprecipitated with non-immune rabbit serum (NRS).

To test the *in vivo* relevance of our findings, we used an acute model of peritonitis with zymosan as a model stimulus [15]. As expected, *i.p.* administration of zymosan led to a robust leukocyte infiltrate, most of which were identified as PMN (Ly6G^{+}F4/80^{-}) at this 4h time point (Fig. 3A). Co-administration of an E and D-prostanoid receptor antagonist, AH6809 [20], led to a significant decrease in PMN infiltration in response to zymosan, (Fig. 3A), confirming the role of prostanoids in mediating leukocyte trafficking [1, 5, 21]. In addition, the selective COX-2 inhibitor, NS-398, also significantly decreased PMN infiltration in response to zymosan (Fig. 3B). Measurement of *Atf3* showed that there was a significant increase in the gene expression of this transcription factor in exudate leukocytes 4h post-zymosan, when compared with resident, unstimulated leukocytes (Fig. 3C). To assess the significance of this increase in *Atf3* and to determine its role in leukocyte trafficking, we measured the levels of PMN and macrophages (F4/80^{+}/Ly6G^{-}) in WT and

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Atf3-deficient mice. We found that PMN recruitment was significantly higher in *Atf3*-deficient mice than their WT controls (Fig. 3D). Importantly, levels of peritoneal macrophages in WT and *Atf3*-deficient mice were similar; this is true for both the resident macrophages from naïve mice (Fig. 3E) and recruited macrophages from zymosan-treated mice (Fig. 3D). Because resident macrophages play an important role in PMN recruitment in this acute peritoneal inflammation model [22], the increased PMN in *Atf3*-deficient mice (at 4 hours after zymosan injection) is not due to the differences in resident macrophage numbers. However, it is possible that the bioactivity of these macrophages (such as expression of genes important for PMN recruitment) is different (see below Fig. 4), contributing to the increased PMN recruitment. To test whether the increased PMN recruitment in *Atf3*-deficient mice is limited to this peritonitis model or not, we examined PMN recruitment in a different inflammation model: cutaneous wounds. As shown in Fig 3F, PMN levels were consistently higher in the *Atf3*-deficient wounds than WT wounds, indicating that Atf3 negatively regulates PMN infiltration in the context of sterile tissue injury. Collectively, these data suggest that Atf3 plays an important role in negatively regulating leukocyte trafficking during acute inflammation.

As results showed that inhibition of prostanoid signaling significantly dampens the development of peritonitis and that loss of *Atf3* has the opposite effect, we sought to determine how the Ptgs2 pathway was modulated in *Atf3*-deficient mice during peritonitis. Consistent with our *in vitro* studies showing that loss of *Atf3* increases *Ptgs2* levels, we found a significant increase in *Ptgs2* mRNA expression in exudate leukocytes obtained from *Atf3*-deficient mice compared with WT mice (Fig. 4A). This increase in *Ptgs2* translated into a significant elevation of PGE_2/D_2 levels in exudates isolated from *Atf3*-deficient mice (Fig. 4B). As enhanced acute leukocyte trafficking in *Atf3*-deficient mice could involve other lipid mediators or chemokines, we measured pro-inflammatory cytokines and chemokines that have either been previously shown to be regulated by Atf3 (i.e., IL-6), or those which have well-documented roles in regulating PMN chemotaxis during acute inflammation. Consistent with previous reports [11], we found that the loss of *Atf3* increased levels of IL-6 in inflammatory exudates of mice undergoing peritonitis (Fig. 4B). Levels of IL-1β were also significantly increased. In contrast, levels of PMN chemokine, Cxcl1, were not significantly affected by *Atf3* deficiency. Similarly, other leukocyte chemokines, including Ccl3 and Ccl4, were also not different between WT and *Atf3*-deficient mice, demonstrating selective regulation of inflammatory signaling by Atf3 (Fig. 4B). We also measured exudate levels of leukotriene B_4 (LTB_4), as this lipid mediator is rapidly generated during acute inflammation and plays a critical role in PMN chemotaxis [23]. As shown in Fig. 4B, levels of LTB_4 were significantly elevated in inflammatory exudates of *Atf3*-deficient mice compared with WT mice. Overall, these results confirm our *in vitro* studies demonstrating that loss of *Atf3* increases *Ptgs2* and its downstream products and that *Atf3*-deficiency increases leukocyte infiltration without altering the generation of classic PMN chemokine, Cxcl1.

Based on our results showing that *Atf3*-deficient mice have elevated PMN infiltration during acute inflammation and that this is correlated with increased levels of IL-6, IL-1β, LTB_4 and PGE_2/D_2, we next sought to determine how Atf3 regulates the production of these

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mediators. Previous studies demonstrated that Atf3 down-regulates *Il-6* transcription [24]. Our results here indicate that Atf3 is recruited to the *Ptgs2* promoter (Fig. 2D); its correlation with the reduced *Ptgs2* steady-state mRNA level also suggests that Atf3 down-regulates *Ptgs2* gene. To determine whether levels of IL-1β and LTB_4 were elevated because of their regulation by Atf3 in macrophages upon exposure to zymosan, or whether they were simply increased as a consequence of increased PMN levels in the peritoneal cavity (which can produce both IL-1β, and LTB_4), we assessed the expression of *Il-1*β and the enzymes required for LTB_4 biosynthesis in isolated resident peritoneal macrophages. This analysis showed that expression of arachidonate-5 lipoxygenase (*Alox5*) and Alox5 activating protein (*alox5 ap*) was not significantly different in resident peritoneal macrophages isolated from WT or *Atf3*-deficient mice and stimulated with zymosan (Fig. 4C). Moreover, expression of *Il-1*β was similar between WT and *Atf3*-deficient macrophages, suggesting that increased levels of IL-1β and LTB_4 *in vivo* were likely a result of increased PMN levels. Collectively, these results suggest that loss of *Atf3* increases PMN infiltration during acute inflammation and that, consistent with the *in vitro* studies described above, *Atf3* deficiency is associated with increased prostanoid production.

Several previous studies have shown that prostaglandins, particularly PGE_2, increase PMN infiltration during acute inflammation and that these effects are dependent in part by the downstream production of IL-17, which can promote PMN recruitment to sites of inflammation [21, 25–29]. Indeed, congruent with elevated levels of prostanoids in inflammatory exudates of *Atf3*-deficient mice (Fig. 4B), there was an increase in exudate levels of IL-17 in *Atf3*-deficient mice compared with WT mice (Fig. 4D). Overall, the enhanced recruitment of PMN during acute inflammation in *Atf3*-deficient mice is consistent with amplification of the prostaglandin/IL-17 axis.

Discussion

The results of this study demonstrate that Atf3 negatively regulates *Ptgs2* expression during acute inflammation through recruitment to the *Ptgs2* promoter. This role of Atf3 is supported by the observation that mice lacking *Atf3* had higher PMN infiltration in two distinct models of acute inflammation concurrent with amplification of the Ptgs2 pathway. Our results thus provide novel insights into the dynamic regulation of *Ptgs2* expression during acute inflammation.

It is now widely appreciated that temporal regulation of lipid mediator production governs the duration of acute inflammatory responses. For instance, leukotrienes and prostaglandins regulate early phases of inflammation including vascular permeability, vascular caliber, leukocyte trafficking and phagocytosis [30]. Their levels decline rapidly as inflammation begins to resolve; a phase that is regulated by distinct families of lipid mediators that terminate leukocyte trafficking and promote macrophage-mediated clearance of apoptotic cells [30, 31]. Thus, a comprehensive understanding of how lipid mediator signaling is triggered and terminated is important for understanding why in some cases inflammation resolves appropriately while under other conditions it persists chronically at a low levels of activation. Previous studies have shown that *Ptgs2* is dynamically regulated during the initiation and resolution of acute inflammation [5]. The increase in *Ptgs2* during the

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initiation of inflammation leads to the generation of PGE_2 and is associated with increased edema and leukocyte trafficking, while a later wave of *Ptgs2* expression gives rise to PGD_2 and its degradation products (i.e., 15-deoxy PGJ_2). Inhibition of Ptgs2 during the early phase of inflammation blocks leukocyte infiltration, whereas inhibiting Ptgs2 during the later phase enhances leukocyte accumulation[5]. The findings of the present study add new mechanistic insight into this temporal regulation of *Ptgs2* by elucidating that Atf3 plays a role in blunting the first wave of *Ptgs2* transcription to control the magnitude of the inflammatory response. As *Atf3* itself is induced by the same stimuli that induce *Ptgs2* expression, these results add to a growing body of literature demonstrating that Atf3 is a central hub that controls the tone of the inflammatory response to a level appropriate to the extant pathogen load [32].

Several inflammatory stimuli have been shown to increase *Ptgs2* expression, including bacterial lipopolysaccharides (LPS), fungal pathogens, and inflammatory cytokines, such as TNF-α and IL-1β [6]. Interestingly, prostaglandins, such as PGE_2, can also stimulate *Ptgs2* expression as part of a positive feedback loop that amplifies the inflammatory response [6]. Transcription factors linked to these inflammatory signaling pathways, including AP-1, NF-κB (p65/p50), Sp1 and C/EBP, have been implicated in the induction of *Ptgs2* and several of these transcription factors bind to the transcriptional coactivator, p300, which is essential for *Ptgs2* transcription [6, 33]. Notably, the *Ptgs2* promoter contains two cyclic AMP responsive elements (CRE) that have been shown to be important in *Ptgs2* induction in response to a diverse array of stimuli, including phorbol esters, the v-*src* oncogene, and LPS[6, 34–36]. Once generated, Ptgs2 protein is subject to degradation via the endoplasmic reticulum-associated degradation pathway (ERAD), a process that is delayed by NSAIDs[36]. In addition to degradation, transcriptional repression of *Ptgs2* may represent another critical control point in regulating the levels of Ptgs2. Indeed, mutation of the E-box region of the *Ptgs2* promoter, which is adjacent to one of the ATF/CRE sites (i.e., CRE-1), increases *Ptgs2* expression in response to LPS, indicating that an endogenous repressor acts as a negative regulator of *Ptgs2* transcription [36]. Moreover, other factors, such as PARP-1, have been shown to repress *Ptgs2* transcription by binding to previously unrecognized repression elements in the *Ptgs2* promoter far upstream of the transcription start site (−655/−632) [7]. Our results strengthen the view that transcriptional repression of *Ptgs2* is an important regulatory mechanism controlling the expression of *Ptgs2* during acute inflammation by demonstrating that Atf3 is rapidly recruited to the *Ptgs2* promoter and negatively regulates *Ptgs2* expression.

It has been previously reported that Atf3 negatively regulates gene transcription by recruiting histone deacetylates (HDACs), such as HDAC1, to alter chromatin structure and thus access to transcription factors [11]. In addition to HDAC1, recent studies have also demonstrated that a sumoylated form of CEBPB, denoted LAP1, recruits HDAC4 to blunt human *PTGS2* expression [8]. Thus, it is likely that binding of Atf3 blunts *Ptgs2* transcription by regulating chromatin remodeling, given the proximity of ATF/CRE binding sites to both NF-kB and AP-1 binding sites in the *Ptgs2* promoter. Future studies will be required to fully elucidate whether this mechanism underlies the ability of Atf3 to modulate *Ptgs2* expression, although our results support the notion that loss of Atf3 increases *Ptgs2*

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expression via binding to the promoter region. Interestingly, a splice variant of Atf3 lacking the DNA binding domain (i.e., Atf3 Zip2), has been shown to regulate NF-κB-dependent transcription of anti-apoptotic genes by displacing CBP/p300 binding to activated NF-κB [37]. Given the critical role of p300 in the transcriptional regulation of *Ptgs2* induction, it is intriguing to speculate that Atf3 and its alternatively spliced forms may have multiple distinct roles in regulating *Ptgs2* transcription.

Atf3 is induced by several stress signals, including ischemia, ER stress, and oxidative stress [10]. Importantly, Atf3 has emerged as a central negative regulator of TLR signaling and Atf3 itself is induced by the inflammatory signaling pathways (e.g., NF-κB) engaged by ligation of TLR receptors [11, 12]. Although Atf3 was initially studied in the context of signaling through TLR4 [11], it is now clear that Atf3 negatively regulates gene expression induced by several TLRs, including TLR3, 5, 7 and 9 [12]. In addition, activation of the TLR2/6 heterodimer by yeast zymosan induces rapid induction of *Atf3* expression in both macrophages and dendritic cells [12]. In our study, we used fungal zymosan as a model stimulus because it robustly stimulates lipid mediator biosynthesis in leukocytes [38]. We found that stimulation of macrophages with zymosan leads to a robust induction of both *Ptgs2* and *Atf3*. The temporal relationship between *Atf3* and *Ptgs2* is similar to the established negative relationship between Atf3 and other pro-inflammatory cytokines, such as IL-6 and IL-12b [11]. This relationship allows for the rapid induction and repression of *Ptgs2*, such that its levels start declining by 6h post-stimulation. This time course is similar to the development of inflammation *in vivo*, which in this model reaches a maximum at 4–6 h and begins to resolve by 24h [15]. Indeed, our results demonstrate increased *Ptgs2* levels and increased production of downstream prostanoids in the context of *Atf3* deficiency both *in vitro* and *in vivo*. This dynamic regulation is likely to be important for enabling the resolution of inflammation, as prostanoids, such as PGE_2, are known to suppress macrophage phagocytosis of apoptotic cells [15, 39].

It is notable that loss of Atf3 increased recruitment of PMN in peritonitis, which we attribute in part to a dysregulation of prostanoid biosynthesis. This conclusion is based on the observation that both *Ptgs2* and PGE_2/D_2 levels were increased in inflammatory exudates of *Atf3*-deficient mice and that inhibition of PGE_2/D_2 receptors or COX-2, largely blunted PMN infiltration. These findings are consistent with prior work showing that inhibition of COX-2 with NSAIDs diminishes the onset of acute inflammation in several distinct models [5, 15, 40, 41]. Interestingly, a recent study by Boespflug *et al.* demonstrated that Atf3 has a dual effect on PMN recruitment [42]. They showed that, in a murine model of airway inflammation driven by LPS, levels of Cxcl1 were elevated in the *Atf3*-deficient airway. However, no changes in PMN infiltration were observed, presumably due to an inherent chemotaxis defect of the *Atf3*-deficient PMN. Thus, *Atf3*-deficiency increases Cxcl1, a chemotaxic factor for PMN, in the airway but decreases the inherent ability of PMN to migrate, resulting in no net change in PMN infiltration in the lung. In our study, we did not observe any differences in exudate levels of Cxcl1 at this time point and PMN levels were actually increased in *Atf3*-deficient mice. The discrepancy between our results and those of Beospflug et al. could be attributed potentially to different model stimuli (TLR4 vs. TLR2/6), or the timing of exudate collection. Alternatively, this difference could be due to

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the fact that PMN recruitment in our peritonitis model is driven largely by resident tissue macrophages [22], whereas the study by Boespflug *et al.* found that Atf3-driven changes in Cxcl1 production were due primarily to lung epithelial cells [42]. Nonetheless, our observations are unlikely to be model-specific because, in addition to the acute peritonitis model, we also observed that *Atf3*-deficient mice have elevated PMN infiltration into skin wounds. Moreover, a similar increase in PMN recruitment has been reported in *Atf3*-deficient mice during renal ischemia/reperfusion injury [43]. We note that one limitation of our study was the inability to selectively interfere with *Atf3* expression in tissue macrophages given that the mice used here were globally deficient in *Atf3*. In addition, the increase in PMN recruitment in *Atf3*-deficient mice is likely multifactorial and cannot be solely attributed to COX-2. Clearly, future studies are warranted to interrogate fully the role of Atf3 in leukocyte trafficking and how it relates to the suppressive effects of Atf3 on *Ptsg2* transcription in determining the overall inflammatory response.

Inhibition of COX enzymes through NSAIDs has become the mainstay of therapy for chronic inflammation [4, 44], although little is understood about transcriptional repression of *Ptgs2*. Therefore, dysregulation of the Atf3-Ptgs2 axis may be an important contributing factor underlying increased *Ptgs2* expression in chronic diseases and this axis may be a new target for novel pharmacologic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Ptgs2 and Atf3 are induced by inflammatory stimuli in macrophages.
- Atf3 binds to the promoter region of the Ptgs2 gene.
- Loss of Atf3 in macrophages increases Ptgs2 levels.
- Atf3 KO mice have increased leukocytes and Ptgs2 levels during acute inflammation.

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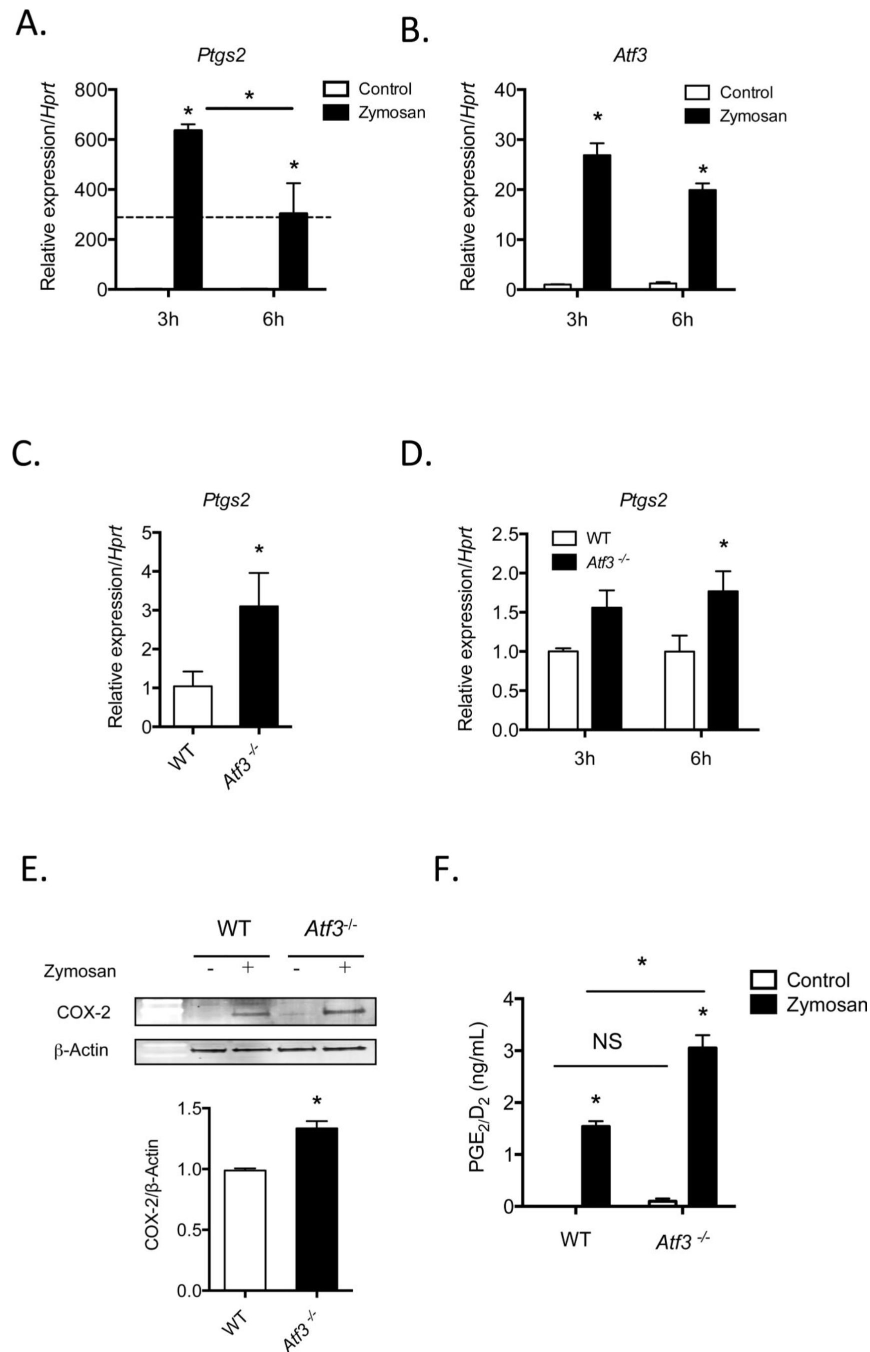

Figure 1. Atf3 negatively regulates *Ptgs2* expression in macrophages

(A, B) Levels of *Ptgs2* and *Atf3* mRNA in resident peritoneal macrophages stimulated without or with zymosan for 3 or 6h. (C) Expression of *Ptgs2* mRNA in unstimulated resident peritoneal macrophages isolated from WT or *Atf3*$^{-/-}$ mice. (D) Levels of *Ptgs2* mRNA in resident peritoneal macrophages isolated from WT or *Atf3*-deficient mice and stimulated with zymosan for 3 or 6h. (E) Western blot of COX-2 protein in WT or *Atf3*-deficient macrophages stimulated with zymosan for 6h, with quantification shown in the lower panel. (F) PGE_2/D_2 levels in supernatants of WT and *Atf3*$^{-/-}$ macrophages stimulated

without (control) or with zymosan (6h). Results are mean ± SEM, n=3–7/group. *$P<0.05$; NS=not significant

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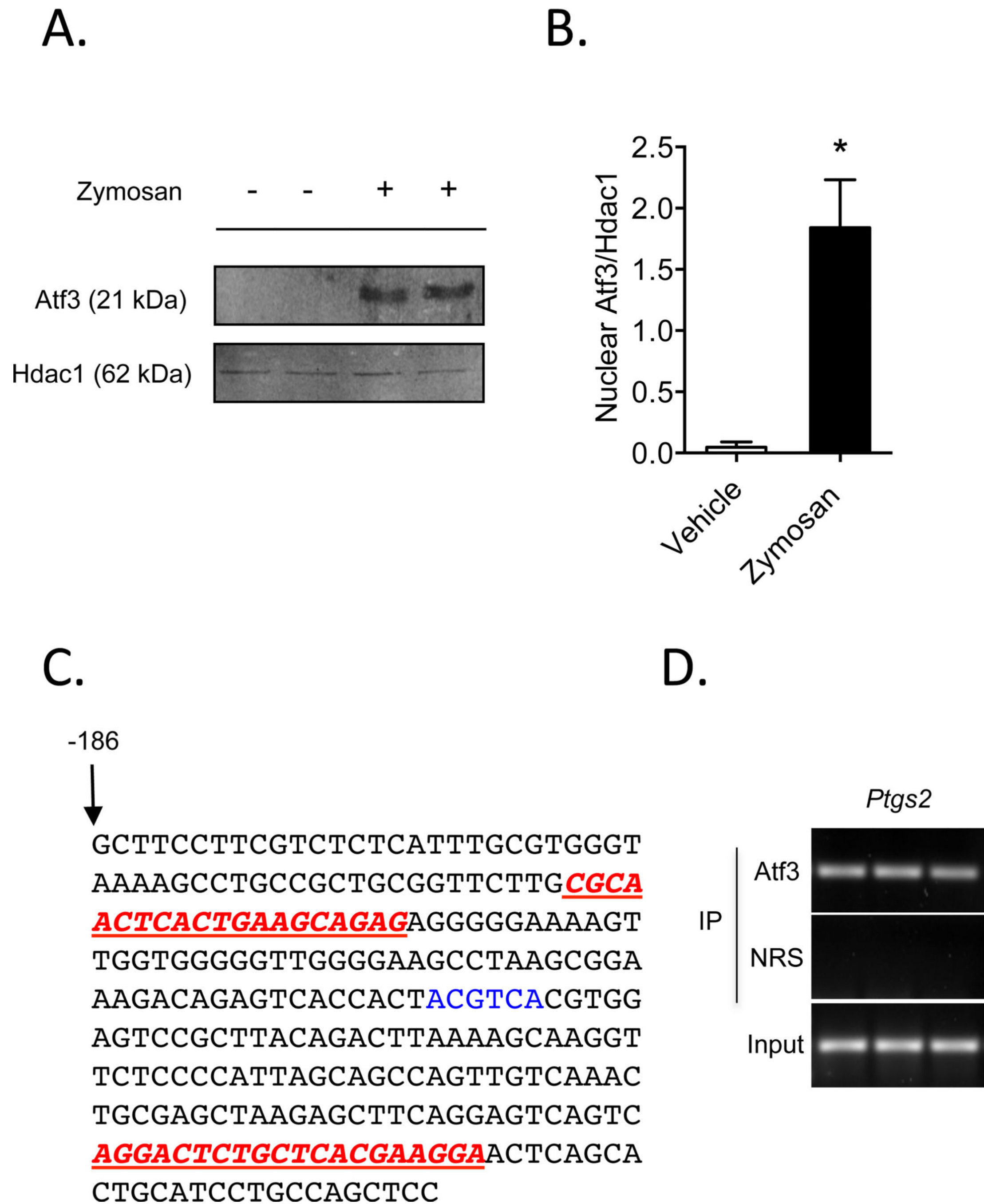

Figure 2. Atf3 is recruited to the murine *Ptgs2* promoter in activated macrophages

(A) Nuclear localization of Atf3 in zymosan-stimulated RAW 264.7 macrophages. Histone deacetylase (Hdac)-1 is shown as a control for nuclear isolation and quantification of band intensities is shown in panel B. (C) The abbreviated sequence of the *Ptgs2* promoter, with primers used for the ChIP assay (underlined in red) and an ATF/CRE binding site (highlighted in blue) shown. (D) ChIP analysis of Atf3 bound to the *Ptgs2* promoter in macrophages stimulated with zymosan, with non-immune rabbit serum (NRS) control and total DNA input (3%) shown. Results are mean ± SEM, n=3/group. *P<0.05

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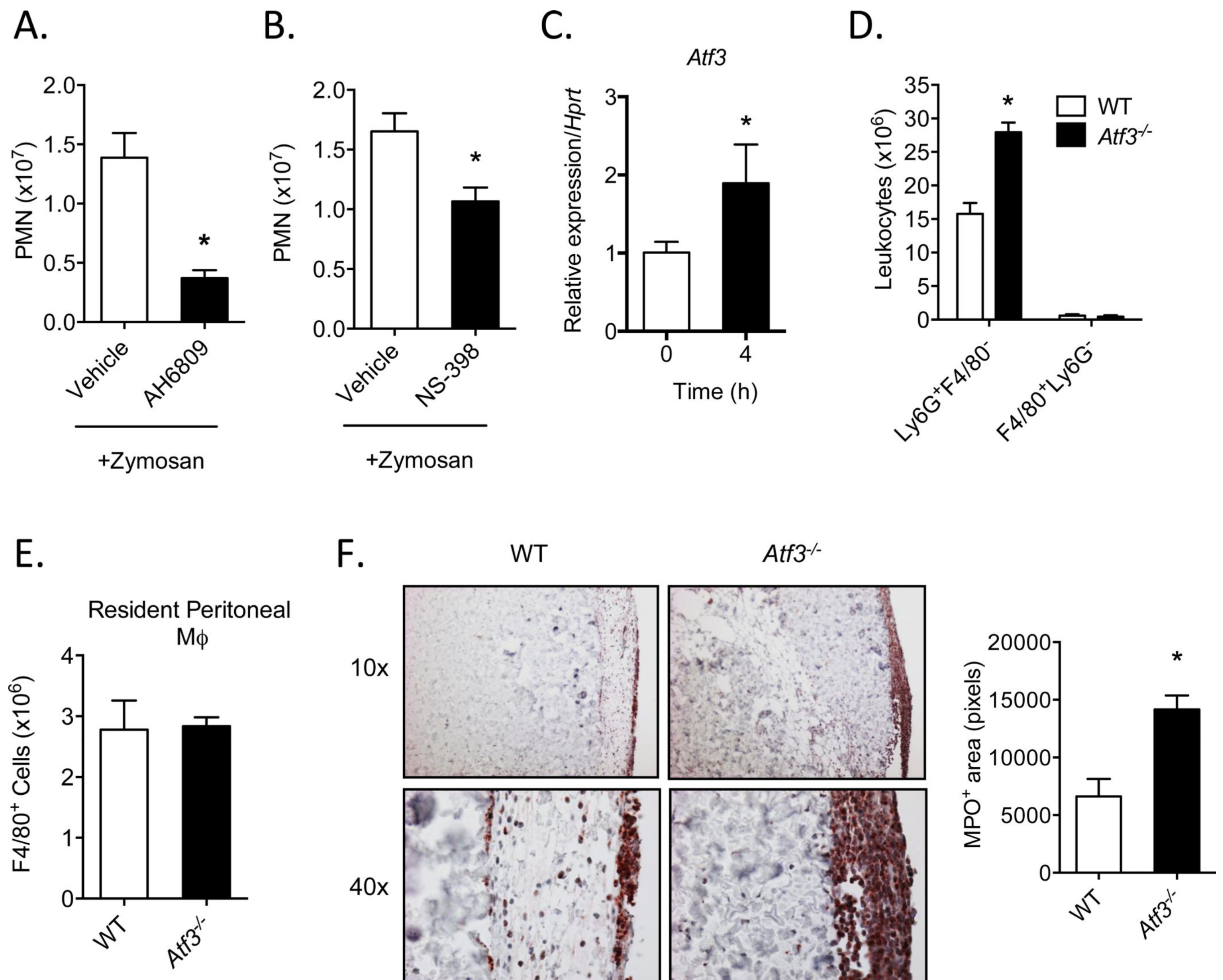

Figure 3. Atf3 negatively regulates leukocyte trafficking during acute inflammation

(A, B) Neutrophil (PMN) levels in WT mice administered zymosan (4h) and treated with vehicle (sterile saline), E-prostanoid/D-prostanoid (EP2 and DP1) receptor antagonist, AH6809 (A), or COX-2 inhibitor, NS-398 (B). (C) Expression of *Atf3* mRNA in peritoneal leukocytes isolated from naïve mice or during acute peritonitis stimulated by zymosan. (D) Neutrophil (Ly6G$^+$F4/80$^-$) and macrophage (F4/80$^+$Ly6G$^-$) levels in WT and *Atf3*-deficient mice 4h after induction of peritonitis. (E) Total resident F4/80$^+$ macrophage levels in the peritoneum of naïve WT and *Atf3*-deficient mice. (F) Histological analysis of myeloperoxidase (MPO)-positive cells in cutaneous wounds of WT and *Atf3*-deficient mice 24h after wounding, with quantitation of the total MPO$^+$ area in the right panel (4 fields per animal). Results are mean ± SEM, n=3–8/group. *P<0.05

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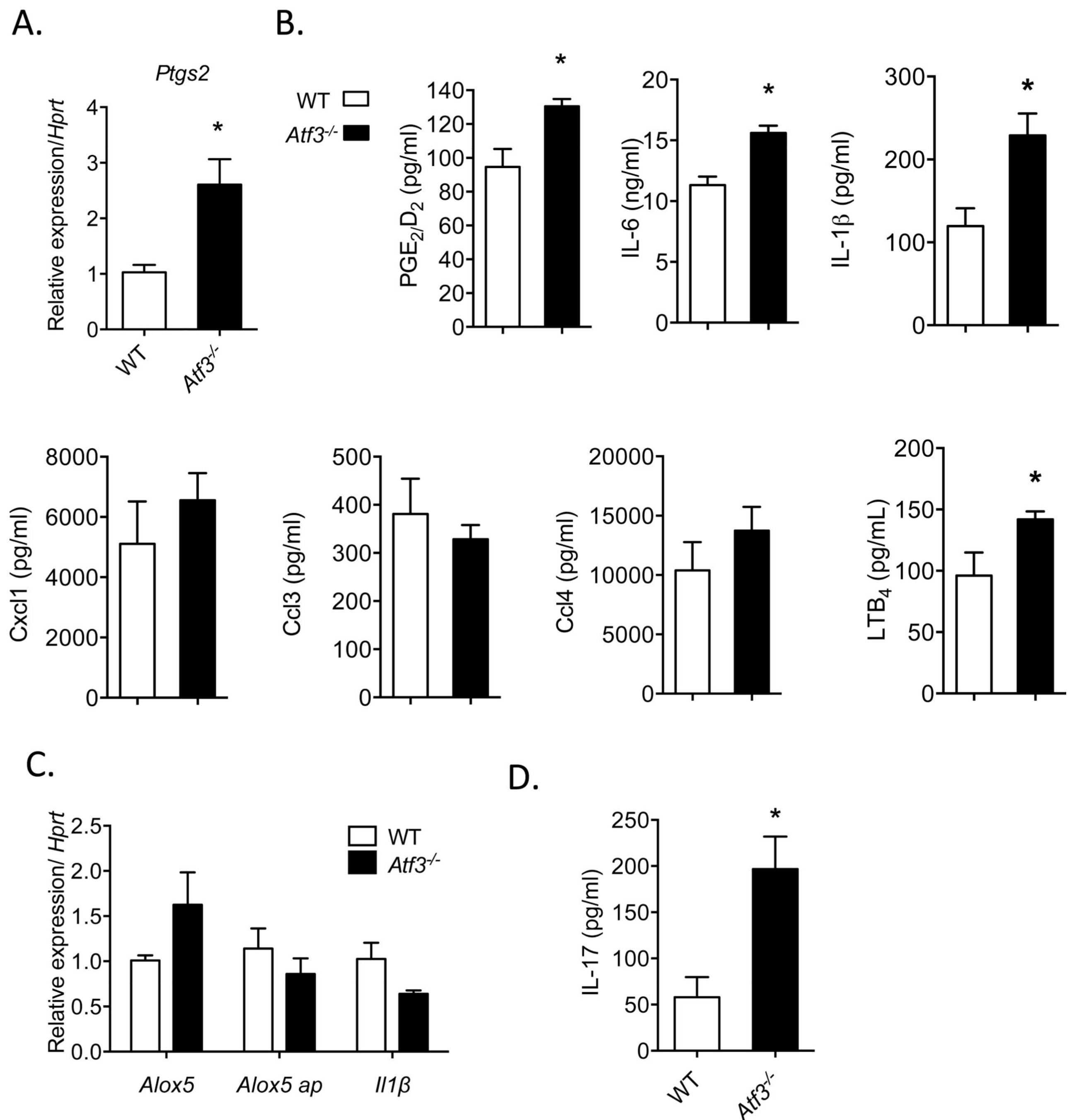

Figure 4. Increased leukocyte accumulation in *Atf3*-deficient mice is associated with the Ptgs2/ IL-17 axis

(A) Levels of *Ptgs2* mRNA in leukocytes obtained from inflammatory exudates of WT and *Atf3*-deficient mice undergoing peritonitis (4h). (B) Levels of PGE_2/D_2, inflammatory cytokines and chemokines IL-6, IL-1β, Cxcl1, Ccl3, and Ccl4, and leukotriene B_4 (LTB_4) in peritoneal exudates of WT and *Atf3*-deficient mice collected 4h after zymosan administration. (C) Expression of *Alox5*, *Alox5 activating protein (ap)* and *Il-1*β mRNA in WT or *Atf3*-deficient resident peritoneal macrophages stimulated with zymosan for 6h. (D)

Levels of IL-17 in peritoneal exudates of WT and *Atf3*-deficient mice undergoing peritonitis (4h). Results are mean ± SEM, n=3–8/group. *P<0.05

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