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IL-1α Modulates Neutrophil Recruitment in Chronic Inflammation Induced by Hydrocarbon Oil

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Exposure to naturally occurring hydrocarbon oils is associated with the development of chronic inflammation and a wide spectrum of pathological findings in humans and animal models. The mechanism underlying the unremitting inflammatory response to hydrocarbons remains largely unclear. The medium-length alkane 2,6,10,14 tetramethylpentadecane (also known as pristane) is a hydrocarbon that potently elicits chronic peritonitis characterized by persistent infiltration of neutrophils and monocytes. In this study, we reveal the essential role of IL-1α in sustaining the chronic recruitment of neutrophils following 2,6,10,14 tetramethylpentadecane treatment. IL-1α and IL-1R signaling promote the migration of neutrophils to the peritoneal cavity in a CXCR2-dependent manner. This mechanism is at least partially dependent on the production of the neutrophil chemoattractant CXCL5. Moreover, although chronic infiltration of inflammatory monocytes is dependent on a different pathway requiring TLR-7, type I IFN receptor, and CCR2, the adaptor molecules MyD88, IL-1R–associated kinase (IRAK)-4, IRAK-1, and IRAK-2 are shared in regulating the recruitment of both monocytes and neutrophils. Taken together, our findings uncover an IL-1α–dependent mechanism of neutrophil recruitment in hydrocarbon-induced peritonitis and illustrate the interactions of innate immune pathways in chronic inflammation. ***The Journal of Immunology*****, 2011, 186: 1747–1754.**

Chronic inflammation is characterized by unremitting immune responses to persistent microbial infection or chemical agents (1). Continued influx of leukocytes and local production of inflammatory mediators are common features at sites of chronic inflammation. Although chemokine gradients play a prominent role in leukocyte migration, the mechanisms responsible for the sustained chemokine production and subsequent influx of neutrophils and monocytes in chronic inflammation are not well defined.

Exposure to naturally occurring hydrocarbon oils is associated with the development of chronic inflammation and a variety of pathological findings in humans and animal models (2–5). Due to their ability to enhance and sustain inflammation, hydrocarbons are often used as adjuvants in vaccines (6, 7). Among the most potent hydrocarbons in eliciting chronic inflammation is the medium-length alkane 2,6,10,14 tetramethylpentadecane (TMPD; also known as pristane). A single i.p. dose of TMPD elicits infiltration of neutrophils and monocytes into the peritoneal cavity for several months (8). The chronic inflammatory response promotes the formation of plasmacytomas and lipogranulomas, a form of ectopic lymphoid tissue (5, 9). Depending on the genetic background, persistent inflammation in TMPD-treated mice also promotes the development of a plethora of autoimmune manifestations including autoantibodies, glomerulonephritis, arthritis, and pulmonary vasculitis (9–13). In addition, TMPD augments mAb production by hybridoma cells by stimulating IL-6 production (14).

Recent studies have begun to unravel the mechanisms responsible for the chronic inflammation induced by TMPD. The response to TMPD is orchestrated by major components of the innate immune system. The continued influx of Ly6C^{hi} inflammatory monocytes to the peritoneal cavity requires the presence of type I IFN (IFN-I) production downstream of TLR-7 signaling (15). IFN-I activates the production of the monocyte chemoattractants CCL2, CCL7, and CCL12, which collectively recruit monocytes to the site of inflammation in a CCR2-dependent manner (16). The persistent infiltration of neutrophils, in contrast, remains largely unexplained. In this study, we aimed to define the mechanism of neutrophil recruitment in TMPD-induced chronic inflammation.

Materials and Methods

Mice

These studies were approved by the Institutional Animal Care and Use Committee. Wild-type C57BL/6, TNF-α$^{-/-}$, CCR2$^{-/-}$, and IL-1R$^{-/-}$ mice (all on a C57BL/6 background), BALB/c, CXCR2$^{-/-}$ (BALB/c background), C3H/HeJ, C3H/HeOuJ, and CBA/CaJ mice were from The Jackson Laboratory (Bar Harbor, ME). FcRγ-chain$^{-/-}$ mice were from Taconic Farms (Hudson, NY), and 129/Sv mice were from B&K Universal Limited (Grimston, Aldbrough, England). Mice were maintained in a specific pathogen-free facility at the Malcolm Randall Veterans Affairs Medical Center (Gainesville, FL). MyD88$^{-/-}$, apoptosis-associated speck-

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Abbreviations used in this article: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; IFN-I, type I IFN; IRAK, IL-1R–associated kinase; IRF-7, IFN regulatory factor-7; PEC, peritoneal exudate cell; Q-PCR, quantitative PCR; TMPD, 2,6,10,14 tetramethylpentadecane.

like protein containing a caspase recruitment domain (ASC)$^{-/-}$, Nalp3$^{-/-}$, caspase-1$^{-/-}$, IL-1R–associated kinase (IRAK-1)$^{-/-}$, IRAK-2$^{-/-}$, IRAK-1$^{-/-}$IRAK-2$^{-/-}$, IRAK-4$^{-/-}$, and IFN regulatory factor-7 (IRF-7)$^{-/-}$ mice (on a C57BL/6 background), and littermate controls were bred and maintained in a specific pathogen-free facility at Osaka University. Mice (8–10-wk-old) received 0.5 ml i.p. injection of TMPD, pentadecane, *n*-hexadecane, squalene (Sigma-Aldrich, St. Louis, MO), or mineral oil (Harris Teeter, Matthews, NC). Peripheral blood and peritoneal exudate cells (PECs) were isolated as described (9). For morphological analysis, neutrophils were sorted using PE-conjugated anti-Ly6G and magnetic bead-conjugated anti-PE Abs (17). Fifty thousand sorted cells were cytocentrifuged onto glass slides (Fisher Scientific, Pittsburgh, PA) and stained using the Hema3 kit (Fisher Scientific). For IL-1α and CXCL5 blockade, mice treated with TMPD for 2 wk were given 200 μg anti–IL-1α neutralizing Abs, hamster IgG (Biolegend, San Diego, CA), anti-CXCL5 neutralizing Abs, or rat IgG1 isotype control Abs (R&D Systems, Minneapolis, MN) i.p., and analysis was performed after 24 h.

Real-time quantitative PCR

Quantitative PCR (Q-PCR) was performed as previously described (17). Briefly, total RNA was extracted from 10^6 peritoneal cells using TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the Superscript II First-Strand Synthesis Kit (Invitrogen). Q-PCR was performed using the SYBR Green JumpStart Kit (Sigma-Aldrich) with an Opticon II thermocycler (Bio-Rad, Hercules, CA). Amplification conditions were: 95°C for 10 min, followed by 45 cycles of 94°C for 15 s, 60°C for 25 s, and 72°C for 25 s. After the final extension (72°C for 10 min), a melting-curve analysis was performed to ensure specificity of the products. Gene expression was normalized to 18S RNA, and expression relative to the sample with the lowest expression was calculated using the $2^{-\Delta\Delta Ct}$ method (18). Primers used in this study were all described previously (15, 16) except for CXCL2 (forward: 5′-AAGTTTGCCTTGACCCTGAA-3′; reverse: 5′-CGAGGCACATCAGGTACGAT-3′) and CXCL3 (5′-forward: CCACTCTCAAGGATGGTCAA-3′; reverse: 5′-GGATGGATCGCTTTTCTCTG-3′).

Flow cytometry

The following conjugated Abs were used: anti–CD11b-PE, anti–CD8-allophycocyanin, anti–CD4-FITC, anti–CD11c-PE, anti–B220-allophycocyanin–Cy5.5, anti–Ly6G-PE, anti–Ly6C-FITC, anti–Ly6C-biotin, anti–Siglec-F–biotin (all from BD Biosciences, San Jose, CA), anti–CCR3-Alexa 647, anti–CD11b-Pacific Blue (Biolegend), and avidin-allophycocyanin (eBioscience, San Diego, CA). Cells were then stained with an optimized amount of primary Abs or the appropriate isotype control for 10 min at room temperature as previously described (15). Fifty thousand events per sample were acquired using a CYAN ADP flow cytometer (Beckman Coulter, Hialeah, FL) and analyzed with FCS Express 3 (De Novo Software, Ontario, Canada).

ELISA

CXCL1, CXCL2 (PeproTech, Rocky Hill, NJ), and CXCL5 ELISA (R&D Systems) were performed following the manufacturer's instructions. OD was converted to concentration using standard curves based on recombinant chemokines analyzed by a four-parameter logistic equation (Softmax Pro 4.3; Molecular Devices, Sunnyvale, CA).

Cell culture

NIH3T3 cells were cultured in complete DMEM (containing 10% FBS, 10 mM HEPES, glutamine, penicillin/streptomycin, and 10 U/ml heparin) and seeded on 24-well cell-culture plates (10^5 cells/well). Cells were stimulated with PBS or 500 pg/ml rIL-1α (Biolegend), IL-1β (BD Bioscience), or IFN-β (PBL Laboratories, Piscataway, NJ) and incubated at 37°C in a 5% CO_2 atmosphere for 6 h. RNA extraction and Q-PCR were performed as described above.

Statistical analysis

For quantitative variables, differences between groups were analyzed by the unpaired Student *t* test. Data are presented as mean ± SE. All tests were two-sided. A p value <0.05 was considered significant. Statistical analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA).

Results

Chronic recruitment of neutrophils induced by TMPD and other hydrocarbons

The inflammatory response to TPMD is characterized by chronic accumulation of monocytes and neutrophils in the peritoneal cavity (8). To better understand the mechanism of neutrophil recruitment in this model, we first studied the time course of neutrophil accumulation. In untreated or PBS-treated wild-type C57BL/6 mice, very few neutrophils (characterized by surface expression of the myeloid marker CD11b and the neutrophil-specific marker Ly6G) were present in the peritoneal cavity (Fig. 1*A*). Neutrophils begin to accumulate within 24 h of TMPD treatment, comprising about one third of the PECs (Fig. 1*A*). The absolute number of neutrophils peaked after 2 wk and remained stable for 4–6 wk before a gradual decline was observed (Fig. 1*B* and data not shown). Magnetic bead sorting of PECs using Abs to Ly6G yielded polymorphonuclear cells morphologically consistent with neutrophils (Fig. 1*C*). Based on these findings, all subsequent analyses were performed 2 wk after TMPD treatment. Nevertheless, neutrophils were detectable up to 6 mo post TMPD treatment (Fig. 1*A*, 1*B*), illustrating the chronicity of the inflammatory response. The infiltration of eosinophils, which are distinguished from neutrophils by morphology and surface expression of CCR3 and Siglec-F (19), was minimal in TMPD-treated mice (Supplemental Fig. 1).

In addition, an expansion of the neutrophil compartment in the peripheral blood also was evident following TMPD treatment. Whereas small numbers of inflammatory monocytes displaying high surface expression of Ly6C and no expression of Ly6G were seen in the peripheral blood of untreated mice (Fig. 1*D*, ovals), larger numbers of neutrophils expressing intermediate levels of Ly6C (boxes) in addition to Ly6G were present. Two weeks after TMPD injection, the proportions of both Ly6C^{hi} monocytes and neutrophils in the peripheral blood were increased by 2–3-fold compared with baseline levels (Fig. 1*D*).

These changes were not strain specific, as neutrophil influx was also detected in other wild-type strains injected with TMPD, although the greatest effect was seen in BALB/c mice (Table I). Furthermore, we extended our analysis to other naturally occurring hydrocarbons including mineral oil, squalene, pentadecane, and *n*-hexadecane. Similar to TMPD, all of these hydrocarbons elicited chronic recruitment of neutrophils into the peritoneal cavity (Fig. 1*E*). This number of neutrophils in PECs 2 wk after mineral oil or squalene treatment was similar to TMPD, whereas hexadecane or pentadecane treatment elicited slightly greater levels of neutrophils.

TMPD-induced neutrophil recruitment is mediated by IL-1R signaling

The mechanism of hydrocarbon-induced chronic neutrophil recruitment remains unclear and appears independent of the pathways required for monocyte migration. Recent studies demonstrated that although TLR-7, IFN-I receptor, and CCR2 are essential for the persistent recruitment of Ly6C^{hi} monocytes into the peritoneal cavity following TMPD treatment, these components are all dispensable for the neutrophil response (15, 16). To identify the mechanism(s) responsible for the chronic neutrophil influx, we first examined the role of several proinflammatory mediators previously implicated in the immune response to TMPD (9, 10, 17, 20). Compared to wild-type controls, mice deficient in TNF-α or FcR γ-chain all showed comparable levels of neutrophil recruitment 2 wk after TMPD injection. The response was also similar in C3H/HeJ mice, suggesting that endotoxin is not responsible for our observations (Fig. 2*A*). Moreover, the infiltration of neutrophils in this model was not dependent on Nalp3/cryopyrin, ASC, or caspase-1 (Fig. 2*B*). These components of the inflammasome complex are key mediators of neutrophil migration in several models of sterile inflammation (21–23).

Interestingly, the accumulation of neutrophils in the peritoneal cavity was largely abolished in the absence of IL-1R type I (Fig.

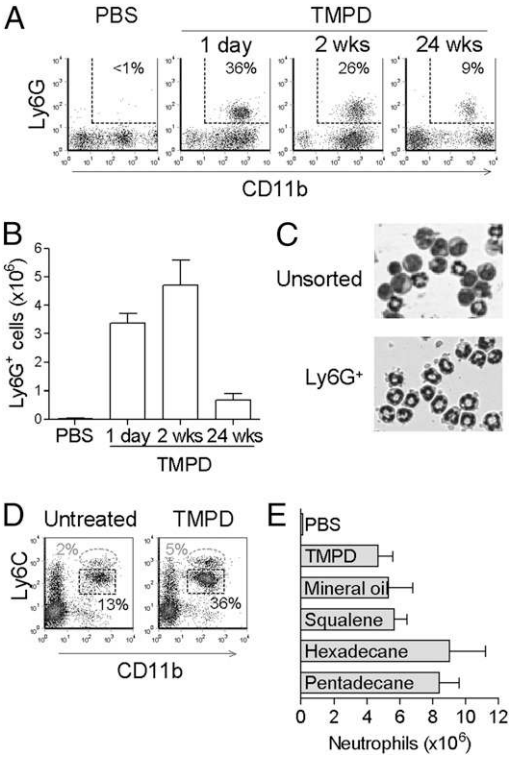

FIGURE 1. TMPD induces chronic recruitment of neutrophils in mice. Flow cytometry (*A*) and quantification of PECs (*B*) in wild-type C57BL/6 mice treated with PBS or TMPD at the indicated time points after treatment ($n = 4$ per group). Neutrophils are characterized by coexpression of CD11b and Ly6G. The percentage of neutrophils is indicated within each plot. *C*, Morphologic analysis of PECs from TMPD-treated mice before and after magnetic bead sorting using anti-Ly6G Abs (original magnification ×200). *D*, Flow cytometry of peripheral blood cells in C57BL/6 mice before and 2 wk after TMPD treatment. Dotted ovals indicate $Ly6C^{hi}$ monocytes ($CD11b^+Ly6C^{hi}$) and boxes indicate neutrophils ($CD11b^+Ly6C^{mid}$). The percentage of both populations is indicated within each plot. *E*, Quantification of PECs in C57BL/6 mice treated with PBS or various hydrocarbon oils for 2 wk ($n = 4$ per group). For bar graphs, each bar represents the mean and error bars indicate SE.

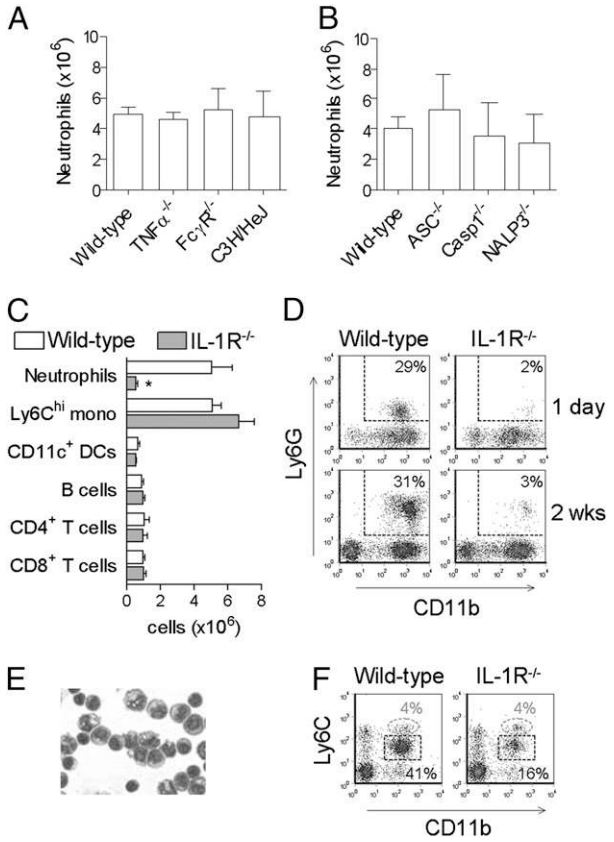

FIGURE 2. TMPD-induced neutrophil recruitment is mediated by IL-1R signaling. *A*, Quantification of $Ly6G^+$ neutrophils in PECs from TMPD-treated wild-type C57BL/6, TNF-$\alpha^{-/-}$, FcRγ-chain$^{-/-}$, and C3H/HeJ mice ($n = 6$ to 7 per group). *B*, Quantification of $Ly6G^+$ neutrophils in PECs from TMPD-treated C57BL/6, $ASC^{-/-}$, caspase-$1^{-/-}$, and $NALP3^{-/-}$ mice ($n = 8$ per group). *C*, Comparison of PEC populations in TMPD-treated C57BL/6 and IL-$1R^{-/-}$ mice ($n = 6$ per group). *D*, Flow cytometry of PECs from C57BL/6 and IL-$1R^{-/-}$ mice 1 d or 2 wk after TMPD treatment. The percentage of neutrophils is indicated within each plot. *E*, Morphologic analysis of PECs from TMPD-treated IL-$1R^{-/-}$ mice (original magnification ×200). *F*, Flow cytometry of peripheral blood cells in TMPD-treated C57BL/6 and IL-$1R^{-/-}$ mice. Dotted ovals indicate $Ly6C^{hi}$ monocytes ($CD11b^+Ly6C^{hi}$), and boxes indicate neutrophils ($CD11b^+Ly6C^{mid}$). The percentages of cells in these populations are indicated within each plot. For all panels, unless otherwise noted, all mice were treated with TMPD 2 wk prior to analysis. Each bar in bar graphs represents the mean and error bars indicate SE. $^*p < 0.05$ compared with wild-type controls (Student's unpaired *t* test).

2*C*). This effect was specific as numbers of $Ly6C^{hi}$ monocytes, B lymphocytes, T lymphocytes, and dendritic cells in the peritoneal exudate were comparable to wild-type mice. The depletion of neutrophils was evident at 1 d or 2 wk post TMPD treatment, indicating that IL-1R signaling mediates both acute and chronic neutrophil recruitment in this model (Fig. 2*D*). Consistent with these findings, morphological analysis of PECs from TMPD-treated IL-$1R^{-/-}$ mice showed a predominance of monocytes and lymphocytes, whereas few neutrophils were present (Fig. 2*E*). Moreover, expansion of the neutrophil compartment in the peripheral blood also was abrogated in the absence of IL-1R (Fig. 2*F*). These data suggest that neutrophil recruitment in the TMPD model of chronic inflammation is specifically mediated by IL-1R signaling.

Table I. Quantification of peritoneal exudate cells in wild-type mouse strains 2 wk after TMPD treatment

Strain	*n*	Neutrophils	$Ly6C^{hi}$ Monocytes	Dendritic Cells	B Cells	$CD4^+$ T Cells	$CD8^+$ T Cells
C57BL/6	6	4.32 ± 0.60	4.80 ± 0.87	0.50 ± 0.07	0.85 ± 0.23	0.90 ± 0.27	0.90 ± 0.23
BALB/cJ	6	9.49 ± 0.43	2.36 ± 0.15	0.23 ± 0.02	0.68 ± 0.05	0.64 ± 0.11	1.13 ± 0.18
129/Sv	6	3.23 ± 0.54	4.85 ± 0.48	0.31 ± 0.08	0.84 ± 0.20	1.03 ± 0.17	0.79 ± 0.14
C3H/OuJ	6	4.80 ± 1.60	4.50 ± 1.58	0.35 ± 0.07	0.90 ± 0.26	0.35 ± 0.07	0.23 ± 0.06
CBA/CaJ	4	3.37 ± 0.38	3.65 ± 1.04	0.27 ± 0.07	0.83 ± 0.24	1.20 ± 0.15	0.37 ± 0.03

All values represent cell number × 10^6. Cell populations were quantified by flow cytometry using the following surface markers: neutrophils ($CD11b^+$ $Ly6G^+$), $Ly6C^{hi}$ monocytes ($CD11b^+Ly6C^{hi}$), dendritic cells ($CD11C^+I^A/I^{E+}$), B lymphocytes ($B220^+CD11C^-$), $CD4^+$ T cells ($CD4^+CD11C^-CD11b^-$) and $CD8^+$ T cells ($CD8^+CD11C^-CD11b^-$).

MyD88 and IRAKs modulate both monocyte and neutrophil recruitment

To elucidate the pathway downstream of IL-1R, we tested the effects of TMPD in mice deficient of the adaptor molecules involved in IL-1R signaling including MyD88 and members of the IRAK family (24–26). Consistent with the essential role of MyD88 and IRAK-4 in IL-1R signaling, the number of neutrophils in the peritoneal cavity after TMPD treatment was significantly reduced in the absence of these molecules (Fig. 3*A*). Unlike IL-1R$^{-/-}$ mice, which demonstrated normal recruitment of Ly6C^{hi} monocytes (Fig. 2*C*), TMPD-treated IRAK-4$^{-/-}$ mice exhibited a drastic reduction of these inflammatory monocytes in the peritoneal cavity (Fig. 3*B*). As described previously (15), this also was seen in MyD88-deficient mice. In the absence of significant monocyte and neutrophil influx, the total number of PECs was reduced by >90% in MyD88$^{-/-}$ and IRAK-4$^{-/-}$ mice compared with wild-type mice (not shown).

IRAK-1 and IRAK-2 differentially regulate the signaling cascade downstream of IRAK-4 (27). Curiously, the recruitment of neutrophils was partially intact in the absence of either of these kinases (Fig. 3*B*). Combined deficiency of IRAK-1 and IRAK-2 was required to recapitulate the observations in IRAK-4$^{-/-}$ mice, suggesting that IRAK-1 and IRAK-2 can partially compensate for one another in the inflammatory response to TMPD.

Although the mechanisms of monocyte and neutrophil recruitment both require MyD88 and IRAKs, these pathways can be distinguished by downstream utilization of IRF-7. IRF-7 interacts with MyD88 and IRAKs to promote TLR-induced IFN-I production but does not participate in IL-1R signaling (28, 29). Consistent with requirement of TLR-7 activation and IFN-I production for monocyte recruitment (15, 16), IRF-7 deficiency resulted in defective accumulation of Ly6C^{hi} monocytes, but not neutrophils, following TMPD treatment (Fig. 3*A*, 3*B*). Taken together, these data suggest that IL-1R signaling specifically mediates neutrophil influx, whereas the adaptor molecule MyD88 and IRAKs 1/2/4 are shared with the pathway used for monocyte recruitment.

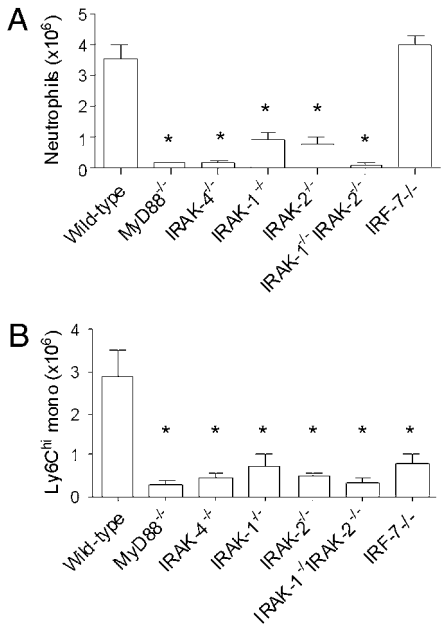

FIGURE 3. MyD88 and IRAK-4 mediate the recruitment of both neutrophils and inflammatory monocytes induced by TMPD. Quantification of neutrophils (*A*) and Ly6C^{hi} monocytes (*B*) in PECs from wild-type C57BL/6 (n = 7), MyD88$^{-/-}$ (n = 6), IRAK-4$^{-/-}$ (n = 8), IRAK-1$^{-/-}$ (n = 8), IRAK-2$^{-/-}$ (n = 8), IRAK-1$^{-/-}$ IRAK-2$^{-/-}$ (n = 6), and IRF-7$^{-/-}$ (n = 9) mice 2 wk after TMPD treatment. Each bar represents the mean, and error bars indicate SE. *$p < 0.05$ compared with wild-type controls (Student's unpaired t test).

IL-1α, but not IL-1β, is responsible for the influx of neutrophils

Because the IL-1R mediates responses to both IL-1α and IL-1β, we next evaluated the role of these cytokines in TMPD-treated mice. Compared to wild-type controls, IL-1β–deficient mice exhibited comparable levels of neutrophil recruitment 2 wk after TMPD injection (Fig. 4*A*, 4*B*). In line with these findings, the absence of caspase-1, a protease that generates the active form of IL-1β, did not impact the neutrophil response (Fig. 2*B*). To address the role of IL-1α, we tested the effects of monoclonal neutralizing Abs against IL-1α in mice treated with TMPD. A single dose of anti–IL-1α Abs reduced the infiltration of neutrophils by ~40–50% (Fig. 4*C*, 4*D*). This effect was specific to neutrophils as the numbers of monocytes and lymphocytes in the peritoneal cavity were not affected (data not shown). Therefore, IL-1α is the primary mediator of neutrophil influx in this model.

IL-1α promotes neutrophil recruitment by inducing CXCL5 expression

To further understand the mechanism of neutrophil migration driven by IL-1α, we studied the involvement of chemokines downstream of IL-1R signaling. Several members of the CXC chemokine family are potent neutrophil chemoattractants produced in response to IL-1R stimulation (30–32). In vitro studies using cultured fibroblasts showed that IL-1α and IL-1β are equally

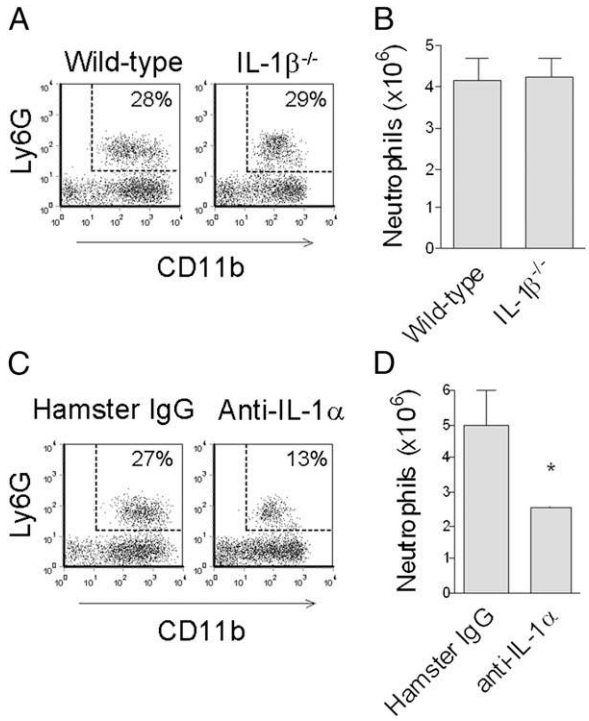

FIGURE 4. TMPD-induced neutrophil recruitment requires IL-1α, but not IL-1β. Flow cytometry (*A*) and quantification of neutrophils (*B*) in wild-type C57BL/6 and IL-1β$^{-/-}$ mice 2 wk after TMPD treatment (n = 7 per group). Flow cytometry (*C*) and quantification of neutrophils (*D*) in C57BL/6 mice injected with 200 μg hamster IgG or anti–IL-1α Abs 2 wk after TMPD treatment (n = 5 per group). Analysis was performed 24 h after Ab treatment. For all flow cytometry plots, the percentage of neutrophils is indicated within each plot. Each bar in bar graphs represents the mean, and error bars indicate SE. *$p < 0.05$ compared with wild-type or isotype controls (Student's unpaired t test).

effective in upregulating the transcription of the neutrophil chemoattractants CXCL1 and CXCL5 (Supplemental Fig. 2). In PECs from TMPD-treated mice, expression of the neutrophil chemoattractants CXCL1, CXCL2, CXCL3, and CXCL5 as well as the monocyte chemoattractants CCL2, CCL12, and CX_3CL1 all were detectable using Q-PCR (Fig. 5*A*). However, the transcript levels of CXCL5 were reduced significantly in IL-1R–deficient mice compared with wild-type controls, whereas the expression of other CXC chemokines and monocyte chemoattractants was largely unaffected. Supporting these findings, CXCL5 protein was readily detectable in the peritoneal lavage fluid at 1 d, 2 wk, and 1 mo post TMPD treatment (Fig. 5*B*). CXCL5 levels were drastically reduced in the peritoneal lavage fluid from TMPD-treated IL-$1R^{-/-}$ mice (Fig. 5*C*), whereas deficiency of IL-1β did not impact the production of this chemokine. The expression of CXCL5, but not CXCL1, CXCL2, or CXCL3, was similarly reduced by the administration of anti–IL-1α Abs (Fig. 5*D*). IL-1α blockade also reduced CXCL5 protein levels in the peritoneal lavage fluid (Fig. 5*E*).

In contrast to CXCL5, the amounts of CXCL1 in the peritoneal lavage fluid from TMPD-treated mice were small (Fig. 5*F*). Interestingly, although significant amounts of CXCL2 were detected in the lavage fluid, the production of this chemokine is intact in the absence of IL-1R (Fig. 5*G*). Because IL-1 signaling is responsible for ~90% of neutrophil influx both acutely and chronically after TMPD treatment (Fig. 2*D*), CXCL2 is unlikely to be the major neutrophil chemoattractant in this model.

To further evaluate whether CXCL5 is critical to neutrophil migration in this model, we tested the effect of TMPD in mice deficient of CXCR2, the primary receptor for CXCL5. Compared to wild-type BALB/c controls, $CXCR2^{-/-}$ mice exhibited ~90% reduction of neutrophil influx to the peritoneal cavity in response to TMPD treatment, recapitulating the observations in IL-$1R^{-/-}$ mice (Fig. 5*H*, 5*I*). Finally, we investigated the effect of CXCL5 blockade on granulocyte recruitment. Administration of neutralizing Abs to CXCL5 reduced the infiltration of granulocytes to the peritoneal cavity by ~30% compared with treatment with isotype control Abs (Fig. 5*J*). Taken together, these data suggest that IL-1α and IL-1R signaling promotes the chronic infiltration of granulocytes at least in part by inducing CXCL5 expression (Fig. 6).

Discussion

The proinflammatory effects of naturally occurring hydrocarbon oils were described more than a half century ago (7). These properties have been applied to the development of vaccines, in which hydrocarbons are commonly incorporated as adjuvants to augment the response to immunization (6, 7). In contrast, exposure to hydrocarbons is associated with the development of chronic inflammation and a variety of pathological findings including plasmacytoma formation, lymphoid neogenesis, and autoimmune manifestations (2–5). The mechanism(s) of hydrocarbon-induced inflammation remains largely unexplained.

TMPD is a medium-length hydrocarbon that elicits a potent chronic inflammatory response in mice characterized by persistent infiltration of neutrophils and monocytes (8). In this study, we illustrate the essential role of IL-1α in TMPD-induced neutrophil recruitment. IL-1α activates a pathway that requires IL-1R, MyD88, IRAK1/2, and IRAK-4, leading to a signaling cascade that culminates in the production of the neutrophil chemoattractant CXCL5 (Fig. 6). Blockade of IL-1α reduced the production of CXCL5 and infiltration of neutrophils to the peritoneal cavity in TMPD-treated mice. In contrast, neutrophil influx was

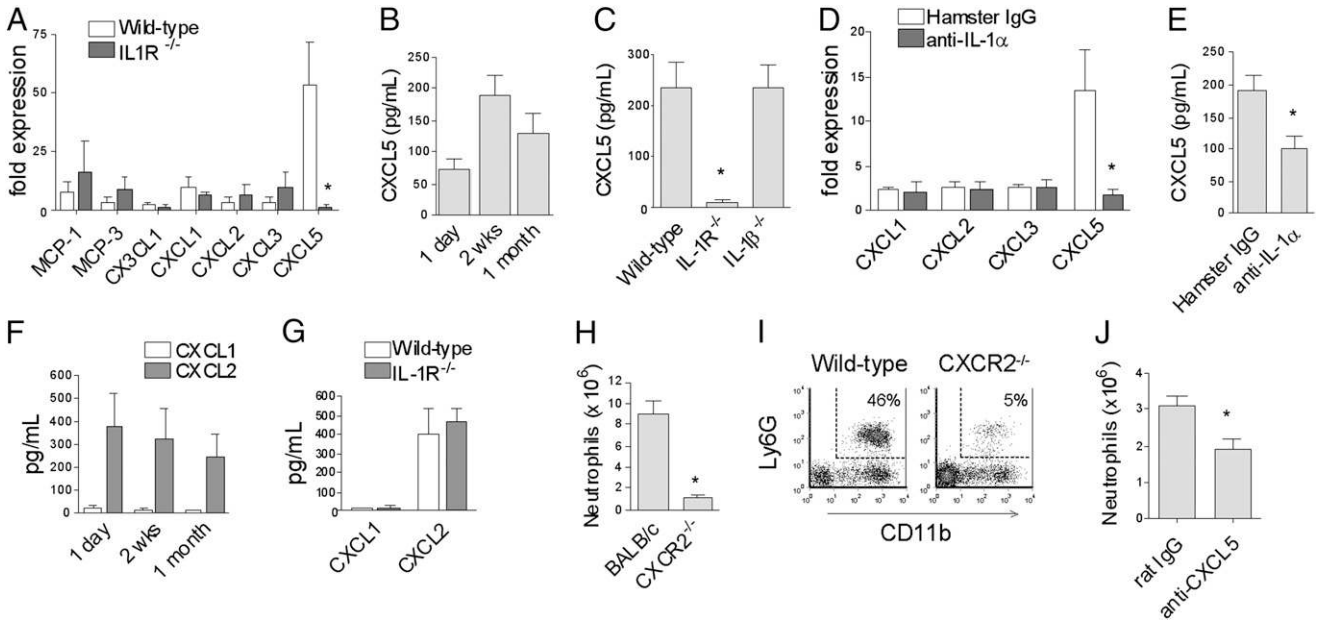

FIGURE 5. IL-1α regulates neutrophil migration by inducing expression of CXCL5 in TMPD-treated mice. *A*, Q-PCR analysis of chemokine expression in PECs from TMPD-treated C57BL/6 ($n = 5$) and IL-$1R^{-/-}$ mice ($n = 6$). ELISA quantification of CXCL5 in peritoneal lavage fluid from C57BL/6 mice treated with TMPD (*B*) for the indicated duration ($n = 4$ to 5 per group) and C57BL/6 ($n = 5$), IL-$1R^{-/-}$ ($n = 6$), and IL-$1\beta^{-/-}$ mice ($n = 4$) (*C*) 2 wk after TMPD treatment. *D*, Q-PCR analysis of chemokine expression in PECs from TMPD-treated wild-type C57BL/6 mice injected with 200 μg hamster IgG or anti–IL-1α Abs ($n = 5$ per group). *E*, ELISA quantification of CXCL5 in peritoneal lavage fluid from TMPD-treated C57BL/6 mice injected with hamster IgG or anti–IL-1α Abs ($n = 5$ per group). ELISA quantification of CXCL1 and CXCL2 in peritoneal lavage fluid from C57BL/6 mice treated with TMPD (*F*) for the indicated duration ($n = 4$ to 5 per group) and C57BL/6 ($n = 5$) and IL-$1R^{-/-}$ ($n = 6$) (*G*) 2 wk after TMPD treatment. Quantification (*H*) and flow cytometry analysis (*I*) of neutrophils in wild-type BALB/c and $CXCR2^{-/-}$ mice after TMPD treatment ($n = 7$ per group). *J*, Quantification of neutrophils in C57BL/6 mice injected with 200 μg anti-CXCL5 Abs or isotype control IgG1 2 wk after TMPD treatment ($n = 5$ per group). Unless otherwise indicated, all mice were treated with TMPD 2 wk prior to analysis. Each bar represents the mean and error bars indicate SE. $^*p < 0.05$ compared with wild-type or isotype controls (Student's unpaired *t* test).

not impacted by the absence of other proinflammatory mediators including IL-1β, TNF-α, IL-6, and TLR-4. Both IFN-γ and IFN-I are also dispensable (16). This mechanism is also different from the pathway used in chronic monocyte recruitment in the TMPD model (15, 16). The persistent influx of monocytes is mediated by the production of several CC-chemokines downstream of TLR-7 activation and IFN-I production (Fig. 6). Interestingly, the IL-1R and TLR signaling cascades share key signaling molecules including MyD88 and IRAK-4 (24–26). As a result, the recruitment of both neutrophils and monocytes is abolished in the absence of these molecules. These pathways diverge downstream of IRAK signaling. IFN-I production and monocyte recruitment initiated by TLR-7 depend on the transcription factor IRF-7, whereas IL-1R signaling and neutrophil recruitment is IRF-7 independent and likely mediated by NF-κB activation (33, 34).

How TMPD triggers the release of IL-1α remains to be solved. Recent evidence suggests that dying cells and cellular debris from the chronic inflammatory response may contribute to the release of this cytokine. Introduction of necrotic cells to the peritoneal cavity triggers neutrophil migration in an IL-1α–dependent manner (35). Interestingly, the release of IL-1α during necrosis, but not apoptosis, distinguishes the inflammatory response to the two types of cell death (36). Furthermore, necrotic cell debris is also a source of RNA-associated autoantigens (such as components of small ribonucleoproteins) (37–39), which may be responsible for the activation of TLR-7 and subsequent recruitment of Ly6C^{hi} monocytes in this model (Fig. 6). The target(s) of IL-1α is also not completely understood. Although gene expression data in PECs suggest a role of these inflammatory cells in the production of neutrophil chemoattractants, mesothelial cells also could be a major source of these chemokines. A recent study demonstrates that IL-1α released from dying cells stimulates the migration of neutrophils by inducing CXCL1 expression by mesothelial cells (40). Although a similar mechanism may be involved in TMPD-induced chronic inflammation, CXCL5 rather than CXCL1 seems to play a more prominent role in TMPD-induced neutrophil recruitment. Additional studies will be needed to distinguish the roles of mesothelial cells and PECs in this model.

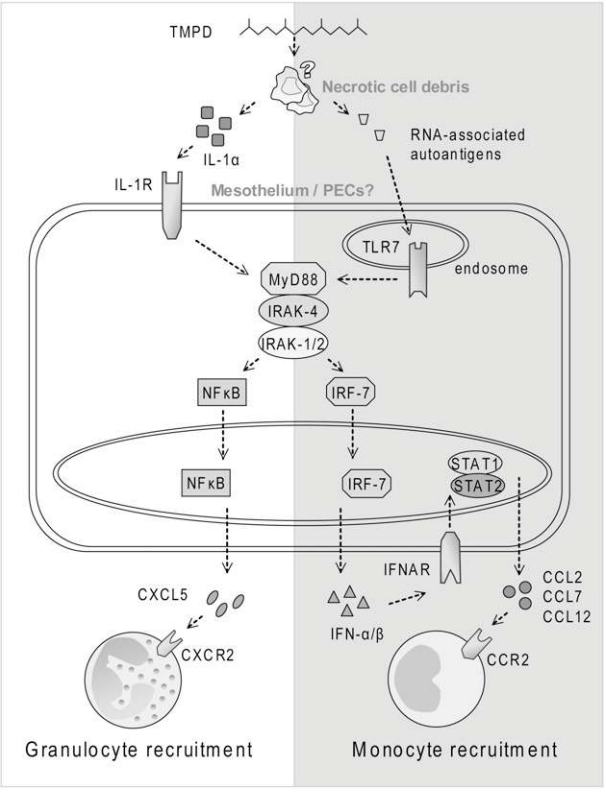

FIGURE 6. Proposed pathways of neutrophil and monocyte recruitment in TMPD-induced chronic inflammation. Neutrophil pathway (*left side*): TMPD treatment results in the release of IL-1α, likely from necrotic cells. In target cells such as mesothelial cells or peritoneal exudate cells, IL-1α stimulates the IL-1R complex, which initiates a signaling cascade that requires MyD88 and IRAKs, culminating in the activation of NF-κB and production of the neutrophil chemoattractant CXCL5. Neutrophils migrate to CXCL5 via a CXCR2-dependent mechanism. Monocyte pathway (*right side*): TMPD treatment results in the activation of TLR-7, which elicits the production of IFN-I through a MyD88- and IRAK-4–dependent pathway. IFN-I binds to IFNAR and subsequent signaling events result in the production of IFN-stimulated chemokines including CCL2, CCL7, and CCL2. These chemokines mediate the migration of Ly6C^{hi} monocytes through their interaction with CCR2.

In addition to modulating chemokine production, IL-1α and IL-1R signaling possess other functions that may fuel the chronic inflammatory response. Transgenic overexpression of IL-1α in mice is sufficient to trigger a form of arthritis characterized by a predominance of macrophages and neutrophils in the synovium (41). As TMPD-treated BALB/c mice develop an inflammatory arthritis (42), it will be of interest to examine the effect of IL-1R deficiency on the pathogenesis of arthritis in this model. IL-1α also is thought to be responsible for the maintenance of granulopoiesis through the induction of neutrophil–M-CSF expression (41, 43). Indeed, the proliferation of not only neutrophil/macrophage progenitors, but also multipotent progenitors and hematopoietic stem cells, is supported by IL-1R signaling (44). This chemokine-independent mechanism may be responsible for the peripheral granulocytosis in TMPD-treated mice, although the effects of TMPD on the different progenitor populations have not been assessed.

CXCL5, also known as epithelial cell-derived neutrophil attractant 78, directs the migration of neutrophils primarily via the receptor CXCR2 (45, 46). The interaction between CXCL5 and CXCR2 is critical for neutrophil recruitment in several models of inflammatory disease (47–50). CXCL5 expression is induced by IL-1α, IL-1β, and TNF-α through activation of NF-κB (51–54), whereas IFN-α and IFN-γ both suppress the production of this chemokine (55, 56). Production of CXCL5 downstream of kinin B1 receptor activation plays an important role in IL-1β–induced neutrophil migration (57). Interestingly, CXCL5 is also a mediator of neutrophil migration in the inflammatory response induced by IL-23 and IL-17 (58). Although our data suggest that IL-1α is responsible for CXCL5 production in the TMPD model, whether the IL-23–IL-17 axis modulates IL-1α or CXCL5 expression warrants further investigation. It is noteworthy that our observations are also distinct from the inflammatory response to turpentine oil, which relies on IL-β production (59, 60). Furthermore, the monocyte chemoattractant CCL2 has been implicated in the chronic infiltration of neutrophils in a model of adjuvant-induced vasculitis (61). The involvement of CCL2 and its receptor CCR2, however, seems limited to the regulation of monocyte migration in mice exposed to i.p. TMPD.

A potential limitation of the current study is the partial response exhibited by IL-1α and CXCL5 blockade. Whereas the influx of neutrophils was inhibited by >90% in IL-1R$^{-/-}$ and CXCR2$^{-/-}$ mice, neutralization of IL-1α or CXCL5 only achieved a reduction of 30–40%. This level of response is comparable to another study that administered these Abs i.p (35, 58). Although the partial response is likely due to the unremitting inflammatory response to TMPD and/or the presence of neutrophils in the peritoneal cavity prior to administration of Abs, it remains possible that other

cofactors are involved. For example, IL-1 signaling can directly activate the vascular endothelium to augment neutrophil chemotaxis (62).

In conclusion, our study elucidates the molecular pathway responsible for neutrophil recruitment in hydrocarbon-induced chronic inflammation. These findings highlight the role of IL-1α in maintaining persistent neutrophil recruitment to sites of inflammation at least in part by regulating CXCL5 production. In view of the induction of lupus by TMPD, the current data also may be of interest with regard to the recent evidence that proinflammatory neutrophils are involved in the pathogenesis of vascular disease in lupus patients (63). Whether therapeutic interventions targeting IL-1α, CXCL5, or CXCR2 are effective in chronic inflammatory or vascular diseases or in the inflammatory arthritis induced by TMPD warrants further evaluation.

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Disclosures

The authors have no financial conflicts of interest.

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