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Type I Interferon Modulates Monocyte Recruitment and Maturation in Chronic Inflammation

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Chronic inflammation is characterized by continuous recruitment and activation of immune cells such as monocytes in response to a persistent stimulus. Production of proinflammatory mediators by monocytes leads to tissue damage and perpetuates the inflammatory response. However, the mechanism(s) responsible for the sustained influx of monocytes in chronic inflammation are not well defined. In chronic peritonitis induced by pristane, the persistent recruitment of Ly6C^{hi} inflammatory monocytes into the peritoneum was abolished in type I interferon (IFN-I) receptor-deficient mice but was unaffected by the absence of IFN-γ, tumor necrosis factor-α, interleukin-6, or interleukin-1. IFN-I signaling stimulated the production of chemokines (CCL2, CCL7, and CCL12) that recruited Ly6C^{hi} monocytes via interactions with the chemokine receptor CCR2. Interestingly, after 2,6,10,14-tetramethylpentadecane treatment, the rapid turnover of inflammatory monocytes in the inflamed peritoneum was associated with a lack of differentiation into Ly6C^{lo} monocytes/macrophages, a more mature subset with enhanced phagocytic capacity. In contrast, Ly6C^{hi} monocytes differentiated normally into Ly6C^{lo} cells in IFN-I receptor-deficient mice. The effects of IFN-I were specific for monocytes as granulocyte migration was unaffected in the absence of IFN-I signaling. Taken together, our findings reveal a novel role of IFN-I in promoting the recruitment of inflammatory monocytes via the chemokine receptor CCR2. Continuous monocyte recruitment and the lack of terminal differentiation induced by IFN-I may help sustain the chronic inflammatory response. ***(Am J Pathol 2009, 175:2023–2033; DOI: 10.2353/ajpath.2009.090328)***

Chronic inflammation is a pathological condition characterized by unremitting production of cytokines and other mediators in response to persistent microbial infection or chemical agents.[1] In autoimmune disorders such as systemic lupus erythematosus (SLE), endogenous antigens also may play an important role in perpetuating a chronic inflammatory state.[2] Regardless of the cause, a common feature of chronic inflammation is the persistent homing of circulating monocytes to the site of injury.[1]

Two major subsets of blood monocytes have been identified in mice. Inflammatory monocytes (also called Ly6C^{hi} monocytes), characterized by surface expression of Ly6C and the chemokine receptor CCR2, are released from the bone marrow and actively recruited to inflamed tissue.[3] Under steady-state conditions, Ly6C^{hi} monocytes mature in the circulation into residential (Ly6C^{lo}) monocytes, which express CX3CR1 and infiltrate noninflamed tissues where they differentiate into residential tissue macrophages.[3,4] On their recruitment to sites of injury, inflammatory monocytes also can differentiate into macrophages with enhanced phagocytic activity or into dendritic cells, which capture antigens for presentation to lymphocytes.[5–8] Tissue monocytes/macrophages also can amplify the immune response by secreting inflammatory cytokines and chemokines to recruit additional immune cells. In chronic inflammation, however, persistent production of these inflammatory mediators can lead to tissue damage, resulting in cycles of tissue destruction

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and repair. The mechanism(s) responsible for the continuous recruitment of monocytes in chronic inflammation are not well defined.

Intraperitoneal administration of 2,6,10,14 tetramethylpentadecane (TMPD; also known as pristane) potently induces chronic inflammation in mice. The influx of mononuclear and polymorphonuclear leukocytes to the peritoneal cavity persists for months after injection of this hydrocarbon oil.[9] The chronic inflammatory state also promotes tumorigenesis, as TMPD was found to induce plasmacytomas more than three decades ago.[10] TMPD is used to stimulate monoclonal antibody production by hybridoma cells injected i.p., an effect of chronic interleukin (IL)-6 production in response to the oil.[11] TMPD treatment stimulates the development of ectopic lymphoid tissue, a feature often associated with chronic inflammatory states.[9] Ectopic lymphoid tissue may play a key role in the induction of lupus-like autoimmune disease (autoantibodies, glomerulonephritis, arthritis, and pulmonary vasculitis) in TMPD-treated mice.[12,13]

How TMPD causes chronic inflammation is not well understood, but a pathological role of several cytokines has been suggested. IL-6 is essential for generating plasmacytomas,[14] whereas IL-12 is required for the development of TMPD-induced glomerulonephritis.[15] Recently, it was found that type I interferons (IFN-I, including IFN-α and IFN-β) and type II interferon (IFN-γ) both contribute to autoantibody production in TMPD-treated mice.[12,16] Using TMPD-induced chronic peritonitis as a model, we examined the mechanism(s) underlying the chronic recruitment of monocytes and granulocytes to the inflamed peritoneum.

Materials and Methods

Mice

These studies were approved by the institutional animal care and use committee. Wild-type C57BL/6, IL-6$^{-/-}$, TNF-$\alpha^{-/-}$, CCR2$^{-/-}$, and IL-1R$^{-/-}$ mice (all on a C57BL/6 background), C3H/HeJ, C3H/HeOuJ, BALB/c, and BALB/c.IFN$\gamma^{-/-}$ mice were from The Jackson Laboratory (Bar Harbor, ME). 129Sv/Ev type I interferon receptor α-chain-deficient (IFNAR$^{-/-}$) mice and wild-type controls (129Sv/Ev), originally from B&K Universal Limited (Grimston, Aldbrough, UK), were bred in our facility. MyD88$^{-/-}$ and IFNAR$^{-/-}$ mice (both on a C57BL/6 background) and littermate controls were bred and maintained in a specific pathogen-free facility at Osaka University. All other mice were maintained in a specific pathogen-free facility at the University of Florida. Mice (8 to 10 weeks old) received TMPD (0.5 ml i.p., Sigma-Aldrich, St. Louis, MO), 4% thioglycollate (BD Biosciences, San Jose, CA), recombinant mouse IFN-α5 (3×10^4 units in 200 μl of PBS, PBL InterferonSource, Piscataway, NJ), or PBS. Peripheral blood and peritoneal exudate cells (PECs) were isolated as described previously.[12,17] For morphological analysis, 5×10^4 sorted cells were cytocentrifuged onto glass slides (Fisher Scientific, Pittsburgh, PA) and stained using a Hema3 kit (Fisher).

Quantitative Real-Time PCR (Q-PCR)

Q-PCR was performed as described.[12] In brief, total RNA was extracted from 10^6 peritoneal cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using a SuperScript II First-Strand Synthesis Kit (Invitrogen). Q-PCR was performed using the SYBR Green Core Reagent Kit (Applied Biosystems, Foster City, CA) with an Opticon II thermocycler (MJ Research, Waltham, MA). Amplification conditions were 95°C for 10 minutes, followed by 45 cycles of 94°C for 15 seconds, 60°C for 25 seconds, and 72°C for 25 seconds. After the final extension (72°C for 10 minutes), a melting curve analysis was performed to ensure specificity of the products. Primers used in this study have been described,[18,19] except for the following: *CCL7* (forward 5′-GATCTCTGCCACGCTTCTGT-3′ and reverse 5′-ATAGCCTCCTCGACCCACTT-3′); *CCL12* (forward 5′-GTCCTCAGGTATTGGCTGGA-3′ and reverse 5′-CACTGGCTGCTTGTGATTCT-3′); *CX3CL1* (forward 5′-CGCGTTCTTCCATTTGTGTA-3′ and reverse 5′-AGCTGATAGCGGATGAGCAA-3′); *CXCL1* (forward 5′-GCTGGGATTCACCTCAAGAA-3′ and reverse 5′-TCTCCGTTACTTGGGGACAC-3′); *arginase-1* (forward 5′-GCACTGAGGAAAGCTGGTCT-3′ and reverse 5′-GACCGTGGGTTCTTCACAAT-3′); *TGF-β* (forward 5′-AGCCCGAAGCGGACTACTAT-3′ and reverse 5′-TTCCACATGTTGCTCCACAC-3′) *cathepsin S* (forward 5′-ACCTACCAAGTGGGCATGAA-3′ and reverse 5′-TGTCAGGCAATGTCCGATTA); *cathepsin L* (forward 5′-CCATCCGTCTCTCCAGTTCT-3′ and reverse 5′-ATAGCCATAGCCCACCAACA-3′); *MMP-2* (forward 5′-CAGAACACCATCGAGACCAT-3′ and reverse 5′-CCAGGTCAGGTGTGTAACCA-3′); and *MMP-9* (forward 5′-CATGCACTGGGCTTAGATCA-3′ and reverse 5′-GGCTTAGAGCCACGACCATA-3′).

Flow Cytometry

All antibodies were purchased from BD Biosciences with the exception of anti-F4/80-FITC, anti-Sca-I-PE, avidin-APC (eBioscience, San Diego, CA), and anti-CD11b-Pacific blue (Caltag Laboratories, Burlingame, CA). Cell staining was performed as described.[12] CCR2 expression on monocytes was assessed using rat anti-mouse CCR2 antibody (clone MC-21, a kind gift from Dr. Matthias Mack, Regensburg University Medical Center, Regensburg, Germany) and rat IgG2b isotype control (BD Biosciences) as described.[20] 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, Los Angeles, CA).

Monocyte Labeling

Clodronate-liposomes and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD)-liposomes were produced as described previously.[4,21] Clodronate was a gift from Roche Diagnostics. To label

Ly6C^{lo} monocytes, 150 μl of DiD-liposomes were injected i.v. into TMPD-treated mice. To label Ly6C^{hi} monocytes, clodronate-liposomes (200 μl) were injected i.v. 24 hours before the injection of DiD-liposomes.[4] Cells were analyzed 24 or 72 hours after labeling.

Cell Culture and Stimulation

PECs resuspended in complete Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum, 10 mmol/L HEPES, glutamine, and penicillin/streptomycin) were seeded on 96-well cell culture plates (10^5 cells/well). Cells were stimulated with the indicated doses of peptidoglycan, R848 (Invivogen, San Diego, CA), or lipopolysaccharide (from *Salmonella typhimurium*, Sigma-Aldrich) and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours before the supernatant was collected. IL-12, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and IL-6 enzyme-linked immunosorbent assays (BD Biosciences) were performed following the manufacturer's instructions. Optical density was converted to concentration using standard curves based on recombinant cytokines analyzed by a four-parameter logistic equation (Softmax Pro 4.3, Molecular Devices Corporation, Sunnyvale, CA).

Phagocytosis Assays

For *in vivo* phagocytosis, DiD-liposomes (50 μl) or apoptotic BW5147 lymphocytes (2×10^5 cells in 200 μl of PBS) were injected i.p. in mice treated with TMPD 2 weeks earlier, and PECs were isolated and analyzed by flow cytometry 1 hour later. DiD-labeled apoptotic BW5147 cells (>70% annexin V$^+$) were generated by heat shock as described previously.[19] For *in vitro* phagocytosis assays, bone marrow-derived macrophages (10^6 cells/well) were generated as described[19] and incubated with or without IFN-β (0.5 or 5 ng/ml) for the final 24 hours of culture. DiD-liposomes (10 μl/well) or fluorescein isothiocyanate-labeled microbeads (10:1 beads/cell ratio, Invitrogen) were added for 30 minutes at 37°C (in PBS with 0.5% bovine serum albumin). Cells were washed three times and analyzed by flow cytometry.

Statistical Analysis

For continuous variables, differences between groups were analyzed by an unpaired Student's *t*-test. Data are presented as means ± SE All tests were two-sided. $P < 0.05$ was considered significant. Statistical analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA).

Results

Persistent Recruitment of Ly6C^{hi} Monocytes after TMPD Treatment

To study the chronic inflammatory response to i.p. TMPD, we first analyzed the peritoneal cell infiltrate at various

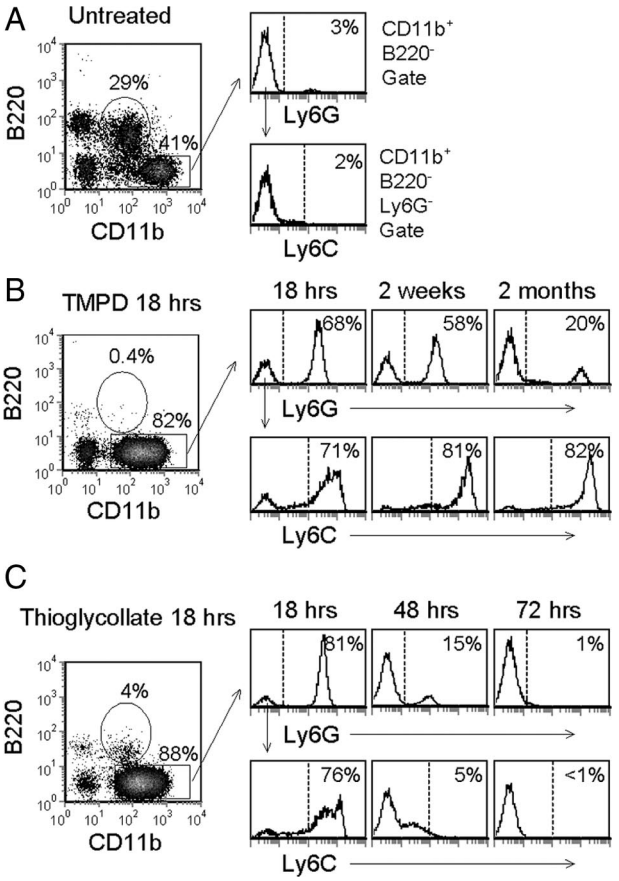

Figure 1. Chronic recruitment of Ly6C^{hi} monocytes in TMPD-induced peritonitis. **A:** Cell subset analysis of peritoneal cells from untreated C57BL/6 mice by flow cytometry. **Left:** Two-dimensional dot plot shows the presence of CD11b$^+$B220$^+$ B1 lymphocytes (circle) and CD11b$^+$B220$^-$ myeloid cells (box). **Right:** Histogram of the percentage of Ly6G$^+$ granulocytes and Ly6G$^-$ monocytes/macrophages in the myeloid cell gate (CD11b$^+$B220$^-$) (**upper panel**); histogram of Ly6C expression on monocytes/macrophages (CD11b$^+$B220$^-$Ly6G$^-$ gate) (**lower panel**). **B:** Flow cytometry analysis of PECs from TMPD-treated mice. **Left:** Two-dimensional dot plot of PECs 18 hours after treatment. **Right:** Histogram of Ly6G and Ly6C expression (as described above) at the indicated time points after treatment. **C:** Flow cytometry analysis of PECs from thioglycollate-treated mice. Panels and gating are as described in **B**. For all panels, flow cytometry plots are representative of three or more experiments.

time points. In untreated mice, peritoneal lavage yielded mainly B220$^+$CD11b$^+$ B1 lymphocytes (Figure 1A, circle; also CD19$^+$, not shown) and B220$^-$CD11b$^+$ myeloid cells (Figure 1A, box) that included granulocytes and monocytes/macrophages. Ly6G$^+$ granulocytes comprised only a small portion of the myeloid population (CD11b$^+$B220$^-$ gate; Figure 1A, right). Thus, the B220$^-$CD11b$^+$ cells were predominantly monocytes/macrophages. Consistent with other studies,[4,22] residential peritoneal macrophages did not exhibit surface expression of Ly6C (CD11b$^+$B220$^-$Ly6G$^-$ gate; Figure 1A, right), a marker highly expressed on inflammatory monocytes.

A strikingly different pattern was observed 18 hours after TMPD treatment (Figure 1B). A dramatic expansion of CD11b$^+$B220$^-$ PECs and a reduction in both B1 lymphocytes and conventional B cells were observed (Figure 1B, left). Granulocytes were the predominant population at this time point, outnumbering monocytes/macro-

phages by ~2 to 1. Unlike residential peritoneal macrophages, the majority of monocytes elicited by TMPD expressed high levels of Ly6C, suggestive of an inflammatory phenotype. The chronic nature of the response to TMPD was confirmed by the presence of granulocytes and Ly6C^{hi} monocytes in the peritoneal cavity 2 weeks and even 2 months after the initial injection (Figure 1B, right). All subsequent experiments were performed 2 weeks after TMPD injection as the absolute number of Ly6C^{hi} monocytes in the peritoneal cavity was similar at 2 weeks and 2 months (data not shown).

An early influx of granulocytes and Ly6C^{hi} monocytes was also detected in the sterile peritonitis induced by thioglycollate (Figure 1C). However, the response was transient in comparison with TMPD as Ly6C^{hi} monocytes elicited by thioglycollate were rapidly replaced by monocytes/macrophages with a mature Ly6C^{lo} phenotype (Figure 1C). By 72 hours, few granulocytes were present and almost all monocytes/macrophages in the peritoneal cavity were Ly6C^{lo}. Depletion of B lymphocytes was also not a feature of thioglycollate-induced inflammation (the reduced percentage of these cells was due to expansion of the myeloid populations).

IFN-I Signaling Modulates the Phenotype of Recruited Monocytes/Macrophages

Proinflammatory cytokines including IL-6, TNF-α, IL-1β, IFN-γ, and IFN-I have been implicated in the immune response induced by TMPD.[12,18,23,24] We asked whether these cytokines play a role in the persistent inflammatory cell influx in response to TMPD. Compared with wild-type C57BL/6 controls, mice deficient in IL-6, TNF-α, or IL-1 receptor showed a similar influx of Ly6C^{hi} monocytes to the peritoneal cavity 2 weeks after TMPD treatment (Figure 2A). The persistent inflammatory response also was not explained by the presence of endotoxin as a similar pattern was seen in C3H/HeJ mice (Figure 2A), which carry a mutation in Toll-like receptor (TLR)-4.[25]

IFN-γ has been recently shown to mediate Ly6C^{hi} monocyte recruitment in response to i.v. infection by *Listeria monocytogenes*.[26] However, the absence of IFNγ did not affect recruitment of inflammatory cells (Figure 2B) induced by TMPD. Although granulocyte influx was higher in BALB/c than in B6 mice, there was no difference in the accumulation of granulocytes, Ly6C^{hi} monocytes, or Ly6C^{lo} monocytes/macrophages in IFN$\gamma^{-/-}$ mice versus wild-type controls (Figure 2B).

Interestingly, although a sustained accumulation of granulocytes was found in TMPD-treated IFNAR$^{-/-}$, the majority of monocytes/macrophages in the peritoneal cavity exhibited a mature, Ly6C^{lo} phenotype (Figure 2C, left). A significant reduction in Ly6C^{hi} monocytes paralleled the increase of Ly6C^{lo} monocytes/macrophages in the absence of IFN-I signaling (Figure 2C, right). This was not due to genetic background differences because wild-type 129Sv and B6 mice demonstrated comparable monocyte responses to TMPD. The effects of IFNAR deficiency were also similar in both backgrounds (data not shown). Consistent with flow cytometry analysis, visual

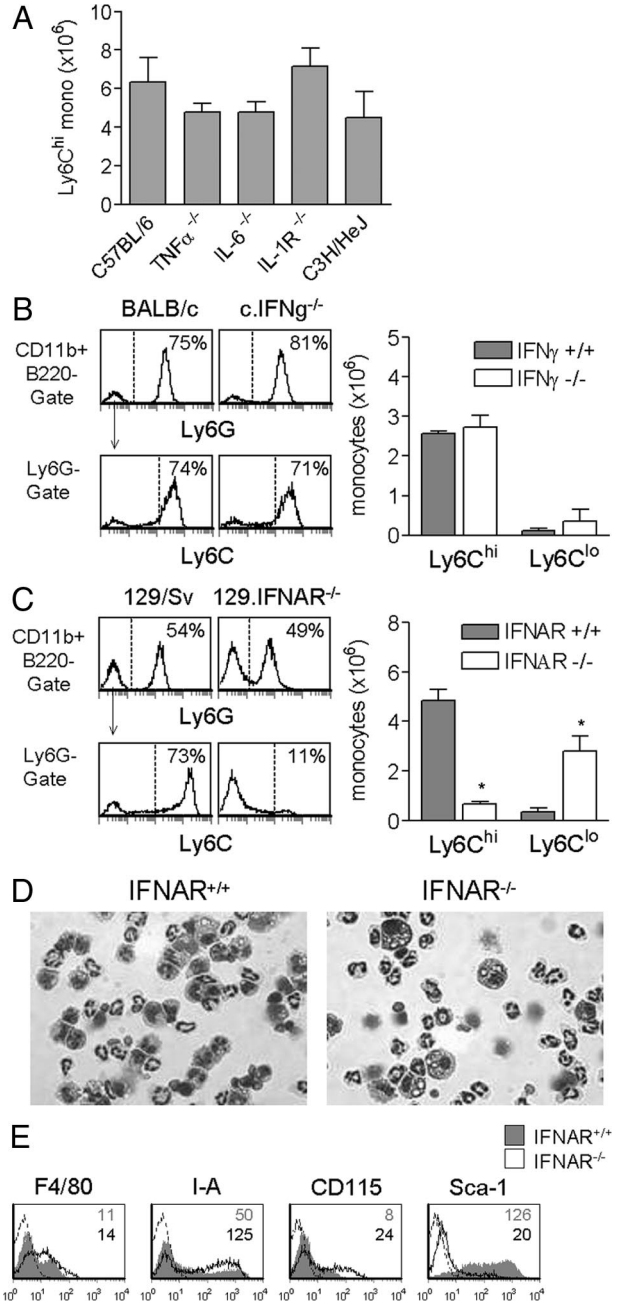

Figure 2. IFN-I signaling modulates the profile of monocytes/macrophages. **A:** Quantification of peritoneal Ly6C^{hi} monocytes in wild-type C57BL6 (n = 5), B6 IL-6$^{-/-}$ (n = 3), B6 TNF-$\alpha^{-/-}$ (n = 4), B6 IL-1R$^{-/-}$ (n = 6), and C3H/HeJ (n = 4) mice 2 weeks after TMPD-treatment. **B:** Flow cytometry analysis of PECs in TMPD-treated wild-type BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice (n = 4 per group). **Upper panels:** Histograms of the percentage of Ly6G$^+$ granulocytes and Ly6G^{lo} monocytes/macrophages in the myeloid cell gate (CD11b$^+$B220$^-$). **Lower panels:** Histogram of Ly6C expression on monocytes/macrophages (CD11b$^+$B220$^-$Ly6G$^-$ gate). Bar graph provides absolute monocyte counts in the different subsets for each group. **C:** Flow cytometry analysis of PECs in TMPD-treated wild-type 129/Sv and 129. IFNAR$^{-/-}$ mice (n = 6 per group). Plots and gating are as described in **B. D:** Morphological analysis of PECs from wild-type and IFNAR$^{-/-}$ mice 2 weeks after TMPD treatment (modified Giemsa-Wright stain). **E:** Flow cytometry analysis of surface markers on peritoneal monocytes/macrophages (CD11b$^+$B220$^-$Ly6G$^-$ gate) from wild-type and IFNAR$^{-/-}$ mice. Plotted values represent the mean fluorescent intensity. Dotted curves represent staining with isotype control antibodies. For all panels, flow cytometry plots are representative of three or more experiments. Each bar represents the mean and error bars indicate SE $^*P < 0.05$ compared with wild-type controls (Student's unpaired t-test).

examination of PECs showed numerous polymorphonuclear cells in both wild-type and IFNAR$^{-/-}$ mice (Figure 2D). Whereas cells with monocytic morphology were found in the peritoneal cavity of wild-type mice, PECs from IFNAR$^{-/-}$ mice revealed the presence of larger, vacuolated cells consistent with a mature, macrophage-like morphology (Figure 2D). Supporting this view, monocytes/macrophages from IFNAR$^{-/-}$ mice displayed greater surface expression of several macrophages markers including F4/80, I-A, and CD115 (Figure 2E). Not surprisingly, expression of the interferon-stimulated gene Sca-1 on monocytes/macrophages was greatly reduced in the absence of IFN-I signaling.

IFN-I Promotes the Recruitment of Ly6C^{hi} Monocytes

Under steady-state conditions, both residential (Ly6C^{lo}) and inflammatory monocytes (Ly6C^{hi}) are present in the circulation.[3,4] Accumulation of Ly6C^{lo} monocytes/macrophages in the peritoneum of TMPD-treated IFNAR$^{-/-}$ mice suggests that either this subset is preferentially recruited to the peritoneal cavity or that the infiltrating Ly6C^{hi} monocytes rapidly acquire a mature phenotype. To distinguish between these possibilities, we monitored monocyte influx into the peritoneal cavity (2 weeks after TMPD injection) *in vivo* using fluorescently labeled liposomes. Neither Ly6C^{lo} nor Ly6C^{hi} circulating monocytes displayed fluorescence before labeling (CD11b^{+}Ly6G^{-} gate) (Figure 3A). As reported previously,[4] i.v. injection of liposomes containing the fluorescent dye DiD (DiD-liposomes) selectively labeled Ly6C^{lo} monocytes in the peripheral blood of both wild-type and IFNAR$^{-/-}$ mice (Figure 3, B and C). Peripheral blood Ly6C^{hi} monocytes were not labeled with the procedure, probably because of their lower phagocytic capacity compared with the Ly6C^{lo} subset.[27] After 24 hours, >40% of circulating Ly6C^{lo} monocytes were DiD^{+} in TMPD-treated wild-type and IFNAR$^{-/-}$ mice, but few DiD^{+} cells had migrated into the peritoneal cavity in either strain. Thus, the absence of IFN-I signaling does not cause preferential recruitment of Ly6C^{lo} monocytes from the peripheral blood.

To track the migration of circulating Ly6C^{hi} monocytes, clodronate-liposomes were first given to deplete the Ly6C^{lo} subset. In the absence of Ly6C^{lo} counterparts, liposome uptake was evident in the Ly6C^{hi} subset as ~50% of peripheral blood Ly6C^{hi} monocytes were fluorescently labeled in TMPD-treated wild-type and IFNAR$^{-/-}$ mice 24 hours after administration of DiD-liposomes (Figure 3, D and E). Importantly, a similar percentage of Ly6C^{hi} monocytes in the peritoneal cavity of wild-type mice were DiD^{+}, indicating that most cells in this subset entered the peritoneal cavity within the past 24 hours. Although the absolute number of peritoneal Ly6C^{hi} monocytes was profoundly reduced in the absence of IFN-I signaling (Figure 2C), the influx of DiD^{+} Ly6C^{hi} monocytes was evident in IFNAR$^{-/-}$ mice (Figure 3, D and E).

We found that few DiD^{+} monocytes remained in the circulation 72 hours after injection of DiD-liposomes and

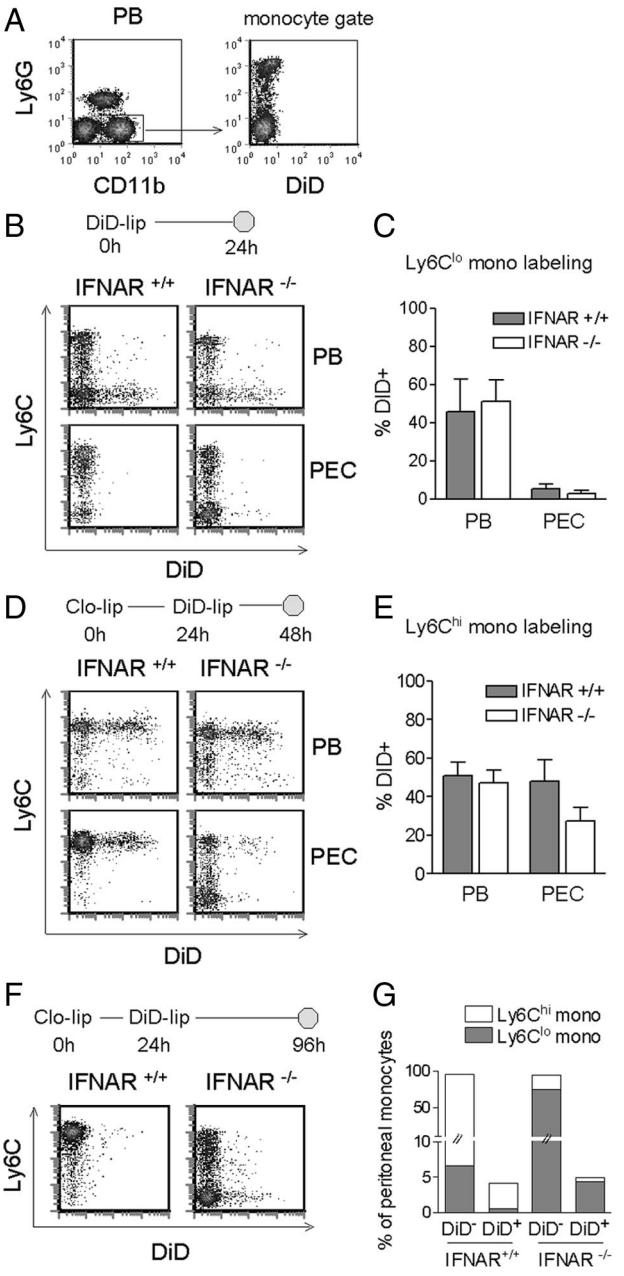

Figure 3. IFN-I signaling promotes the recruitment of Ly6C^{hi} monocytes. **A:** Gating strategy for the analysis of peripheral blood (PB) monocytes (CD11b^{+}Ly6G^{-}) used for subsequent labeling experiments. Flow cytometry analysis (**B**) and quantification (**C**) of DiD^{+} Ly6C^{lo} monocytes in the peripheral blood and peritoneum (PEC) in TMPD-treated wild-type or IFNAR$^{-/-}$ mice 24 hours after administration of DiD-liposomes ($n = 3$ per group). Flow cytometry analysis (**D**) and quantification (**E**) of DiD^{+} Ly6C^{hi} monocytes 48 hours or 96 hours (**F** and **G**) after initial pretreatment with clodronate-liposomes followed by subsequent labeling with DiD-liposomes ($n = 4$ per group). All mice were treated with TMPD 2 weeks before liposome injection. For all panels, flow cytometry plots are representative of three or more experiments. Time points for the administration of clodronate-liposomes and/or DiD-liposomes are indicated on the timeline within each figure and the circle denotes the time of analysis. Each bar represents the mean and error bars indicate SE $^{*}P < 0.05$ compared with wild-type controls (Student's unpaired *t*-test).

further influx of labeled cells to the peritoneal cavity was negligible (data not shown). This allowed us to trace the fate of DiD^{+}Ly6C^{hi} monocytes that had migrated into the peritoneal cavity. In contrast to the 48-hour time point,

few DiD$^+$ peritoneal monocytes were present in wild-type mice at 96 hours after the initial injection of clodronate-liposomes (and 72 hours after DiD-liposome treatment), suggestive of a high turnover rate of the infiltrating monocytes (Figure 3, F and G). The remaining DiD$^+$ population in wild-type mice consisted predominantly of Ly6C^{hi} monocytes. Curiously, evidence of maturation was seen in IFNAR$^{-/-}$ mice, as the majority of DiD$^+$ monocytes at this time point showed reduced or no expression of Ly6C (Figure 3, F and G). Hence, maturation of Ly6C^{hi} monocytes, rather than recruitment of the Ly6C^{lo} subset, is likely to be responsible for the accumulation of Ly6C^{lo} monocytes/macrophages in IFNAR$^{-/-}$ mice. Taken together, these data show an essential role of IFN-I in promoting the chronic recruitment of Ly6C^{hi} monocytes to the peritoneal cavity after TMPD treatment.

TMPD-Induced Monocyte Recruitment Requires CCR2

Next we asked whether administration of IFN-I alone was sufficient to initiate the recruitment of inflammatory monocytes. Indeed, i.p. injection of recombinant mouse IFN-α5 induced the migration of Ly6C^{hi} monocytes to the peritoneal cavity within 24 hours (Figure 4A). Because IFN-I is not known to possess direct chemotactic properties, we reasoned that chemokine production stimulated by IFN-I signaling may be responsible for the influx of Ly6C^{hi} monocytes. Previous studies have shown that several monocyte chemoattractants including CCL2/MCP-1, CCL7/MCP-3, and CCL12/MCP-5 are IFN-inducible genes.[28,29] Indeed, these chemokines were highly expressed in PECs from TMPD-treated wild-type mice but not from IFNAR$^{-/-}$ mice (Figure 4B). Conversely, the expression of CX3CL1/fractalkine, which mediates the migration of Ly6C^{lo} monocytes,[3] and the neutrophil attractant CXCL1/KC were not significantly affected by the absence of IFN signaling (Figure 4B). These data supported the observation that IFN-I is essential for the influx of Ly6C^{hi} inflammatory monocytes but not Ly6C^{lo} monocytes or neutrophils.

We recently reported that TMPD elicits IFN-I production via a TLR-7 and myeloid differentiation factor-88 (MyD88)-dependent pathway.[19] Supporting these findings, the expression of the IFN-I-inducible chemokines CCL2, CCL7, and CCL12 was reduced dramatically in MyD88$^{-/-}$ mice (Figure 4C). Although many chemokines are dually regulated by type I and type II interferons, this pattern was specifically dependent on IFN-I because the absence of IFN-γ had no impact on the expression of these monocyte chemoattractants (Figure 4D).

To further examine the role of these chemokines, we examined the expression on CCR2, the major receptor for CCL2, CCL7 and CCL12. Flow cytometry analysis confirmed the presence of surface CCR2 expression on Ly6C^{hi} monocytes, although the levels of expression were not altered by TMPD treatment (Figure 4E). Next we administered TMPD to mice deficient in CCR2. As shown previously,[5] CCR2 deficiency was associated with a reduction of steady-state Ly6C^{hi} monocytes in the peripheral blood (Figure 4F). However, a significant increase of peripheral blood Ly6C^{hi} monocytes was observed in both wild-type and CCR2$^{-/-}$ mice 2 weeks after TMPD treatment.

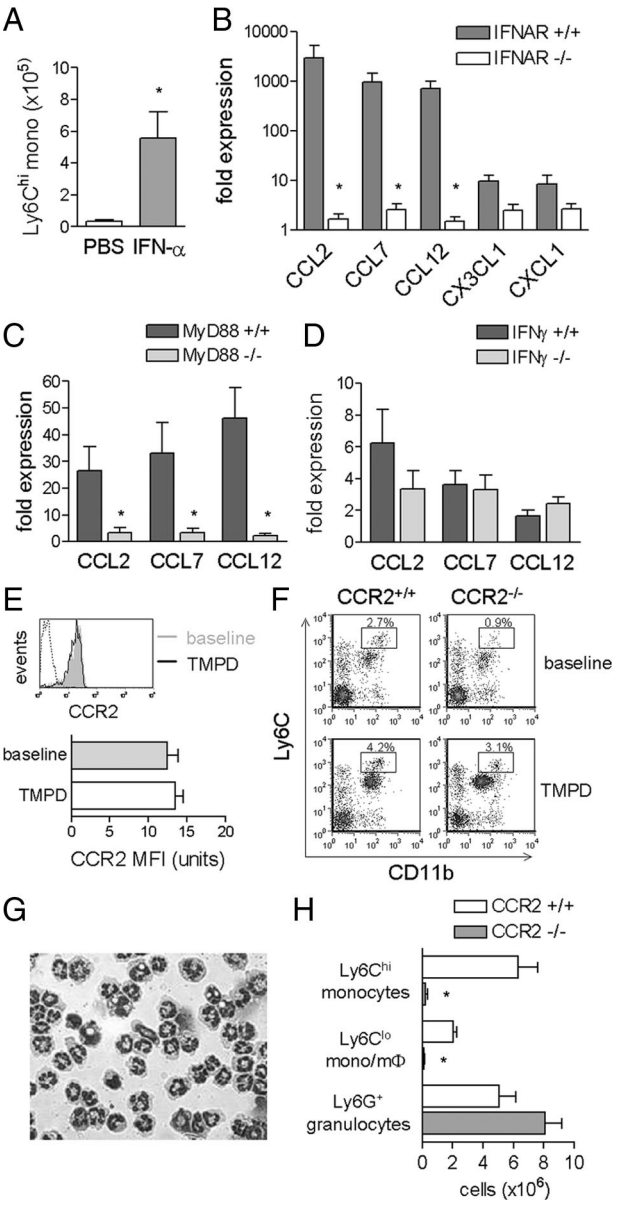

Figure 4. TMPD-induced monocyte recruitment is dependent on CCR2. **A:** Quantification of peritoneal Ly6C^{hi} monocytes in wild-type C57BL/6 mice ($n = 4$) 24 hours after treatment with PBS or recombinant murine IFN-α5 (3×10^4 units i.p.). Q-PCR of chemokine expression in PECs from wild-type 129/Sv and 129/Sv IFNAR$^{-/-}$ mice (**B**, $n = 6$ per group), wild-type C57BL/6 and B6 MyD88$^{-/-}$ mice (**C**, $n = 3$ per group), and wild-type BALB/c and BALB/c IFN$\gamma^{-/-}$ mice (**D**, $n = 4$ per group) 2 weeks after TMPD treatment. **E:** Flow cytometry analysis of CCR2 expression on Ly6C^{hi} monocytes at baseline and 2 weeks after TMPD treatment ($n = 4$ per group). Dotted curve on the histogram represents the isotype control. MFI, mean fluorescence intensity. **F:** Flow cytometry analysis of peripheral blood monocytes in wild-type C57BL/6 and CCR2$^{-/-}$ mice before and 2 weeks after TMPD treatment (live cell gate; $n = 5$ per group). Box indicates Ly6C^{hi} monocytes. Morphological analysis (**G**) and quantification (**H**) of PECs from C57BL/6 and B6 CCR2$^{-/-}$ mice 2 weeks after TMPD treatment ($n = 5$ per group). Flow cytometry plots are representative of three or more experiments. Each bar represents the mean and error bars indicate SE $^*P < 0.05$ compared with wild-type controls (Student's unpaired *t*-test).

Supporting the role of the monocyte chemoattractants, PECs from CCR2$^{-/-}$ mice were composed primarily of granulocytes 2 weeks after TMPD treatment (Figure 4, G and H). Both Ly6C^{hi} and Ly6C^{lo} monocyte subsets were largely absent in the peritoneal cavity of CCR2$^{-/-}$ animals, despite the presence of these cells in the peripheral blood (Figure 4F). The number of granulocytes, on the other hand, increased slightly compared with that in controls (Figure 4H). Taken together, these results show that the chronic influx of Ly6C^{hi} monocytes induced by TMPD requires the interaction of IFN-inducible chemokines with CCR2.

Altered Monocyte Function in TMPD-Treated Mice

To evaluate the functional significance of the preferential recruitment of Ly6C^{hi} monocytes because of IFN-I signaling, we stimulated peritoneal monocytes/macrophages from wild-type and IFNAR$^{-/-}$ mice with the TLR ligands peptidoglycan, lipopolysaccharide, and R848. Strikingly, the production of IL-6, TNF-α, and MCP-1 in response to TLR ligands was reduced dramatically in the absence of IFN-I signaling (Figure 5A). Monocytes/macrophages from TMPD-treated IFNAR$^{-/-}$ mice showed minimal production of TNF-α and MCP-1, even at the highest TLR ligand doses tested.

The lack of inflammatory cytokine production in monocytes/macrophages from TMPD-treated IFNAR$^{-/-}$ mice raised the question whether these cells differentiated toward the alternatively activated M2 monocyte/macrophages phenotype. Down-regulation of inducible nitric oxide synthase and up-regulation of arginase-1 and TGF-β expression are characteristic features of M2 macrophages.[30,31] Q-PCR analysis in peritoneal monocytes/macrophages did not reveal any differences in expression of these genes between wild-type and IFNAR$^{-/-}$ mice (Figure 5B), suggesting that alternative activation of monocytes/macrophages in the absence of IFN-I signaling is unlikely to explain the discrepancy in cytokine production. Furthermore, because monocytes also play an important role in extracellular matrix degradation and remodeling,[32–34] we examined the proteolytic function of peritoneal monocytes/macrophages from wild-type and IFNAR$^{-/-}$ mice treated with TMPD. Metalloproteinase (MMP)-9, in particular, is differentially expressed in M2 macrophages.[35] Consistent with the lack of differences in inducible nitric oxide synthase and Arg-1 expression, Q-PCR analysis revealed comparable expression levels of MMP-9 (Figure 5B). The expression of several other pro-atherosclerotic proteases including cathepsins L, cathepsin S,[36] and MMP-2[37] were also similar in peritoneal monocytes/macrophages from wild-type and IFNAR$^{-/-}$ mice.

We next assessed phagocytic function of peritoneal monocytes/macrophages *in vivo* by direct i.p. injection of DiD-labeled liposomes. In contrast with cytokine production, phagocytosis was greatly enhanced in monocytes/macrophages from IFNAR$^{-/-}$ mice compared with wild-type controls (Figure 5C). Both the percentage of cells

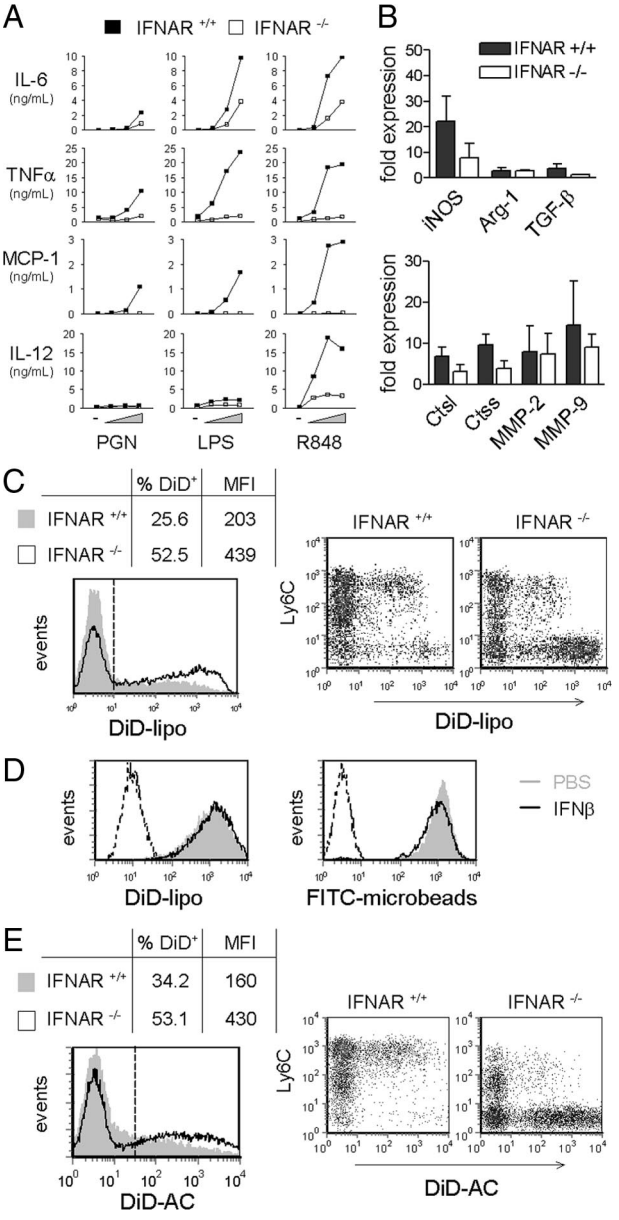

Figure 5. Functional differences in monocyte subsets from TMPD-treated mice. **A:** Enzyme-linked immunosorbent assay quantification of cytokine and chemokine production by adherent PECs from TMPD-treated wild-type or IFNAR$^{-/-}$ mice cocultured with peptidoglycan (2 μg/ml), lipopolysaccharide (1 μg/ml), or R848 (1 μg/ml) for 20 hours. **B:** Q-PCR analysis of inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1), and TGF-β expression (**top**) and cathepsin L (Ctsl), cathepsin S (Ctss), matrix metalloproteinase (MMP)-2, and MMP-9 (**bottom**) in PECs from TMPD-treated wild-type or IFNAR$^{-/-}$ mice (n = 3 to 4 per group). The mean and SE are indicated. **C:** Flow cytometry analysis of *in vivo* DiD-liposome phagocytosis by peritoneal monocytes/macrophages (CD11b^{+}B220^{-}Ly6G^{-} gate) in wild-type or IFNAR$^{-/-}$ mice 2 weeks after TMPD treatment (**left**) and two-dimensional dot plot analysis of DiD-liposome uptake by monocyte/macrophages subsets (**right**). MFI, mean fluorescence intensity of DiD^{+} cells. **D:** Flow cytometry analysis of DiD-liposome or fluorescein isothiocyanate-labeled microbead uptake in bone marrow-derived macrophages with or without IFN-β treatment (5 ng/ml) for 24 hours. Dotted curves represent staining with isotype control antibodies. **E:** Flow cytometry analysis of *in vivo* apoptotic cell uptake by peritoneal monocytes/macrophages from wild-type or IFNAR$^{-/-}$ mice 2 weeks after TMPD treatment. Plots and gating are as described above in **C**. Flow cytometry plots are representative of three or more experiments.

engulfing liposomes and the amount of uptake per cell (measured by mean fluorescence intensity) were increased in the absence of IFN-I signaling (Figure 5C, left). Further analysis revealed that the discrepancy was largely because of the predominance of Ly6C^{lo} monocytes/macrophages, which were more phagocytic than the Ly6C^{hi} monocytes, in IFNAR$^{-/-}$ mice (Figure 5C, right). It is noteworthy that although it was present in smaller numbers, the Ly6C^{lo} subset in wild-type mice was also highly phagocytic. IFN-β treatment of bone-marrow derived macrophages or isolated Ly6C^{hi} monocytes *in vitro* did not inhibit phagocytosis of liposomes or microbeads (Figure 5D and data not shown), indicating that the difference seen in wild-type and IFNAR$^{-/-}$ mice was probably due to a difference in monocyte/macrophage differentiation rather than to a direct effect of IFN-I.

We also examined the phagocytosis of apoptotic cells. Similar to the results with labeled liposomes, enhanced uptake (as measured by the percentage of cells displaying phagocytosis and mean fluorescence intensity) was found in peritoneal monocytes/macrophages from TMPD-treated IFNAR$^{-/-}$ mice compared with wild-type mice after i.p. injection of DiD-labeled apoptotic lymphocytes (Figure 5E, left). The difference was again attributable to the predominance of peritoneal Ly6C^{lo} monocytes/macrophages in IFNAR$^{-/-}$ mice (Figure 5E, right). Ly6C^{hi} monocytes displayed lower phagocytic capacities compared with Ly6C^{lo} monocytes/macrophages in both wild-type and IFNAR$^{-/-}$ mice. Therefore, IFN-I promotes the recruitment of Ly6C^{hi} monocytes that are highly responsive to pathological stimuli (eg, TLR ligands) but weakly phagocytic.

Discussion

Chronic inflammation can be caused by persistent immune responses to microbes, chemicals, or autoantigens.[1] Despite the heterogeneity of etiological factors, continuous infiltration of monocytes is a common feature of many types of chronic inflammation. Using TMPD to induce chronic peritonitis, we show in this study that the persistent recruitment of inflammatory Ly6C^{hi} monocytes was mediated by IFN-I. These antiviral cytokines activate the production of monocyte chemoattractants that recruit Ly6C^{hi} monocytes to the site of inflammation in a CCR2-dependent manner. Curiously, the rapid turnover of recruited monocytes in the peritoneum of TMPD-treated mice was associated with a lack of differentiation into phagocytic Ly6C^{lo} monocytes/macrophages, consistent with previous observations that the uptake of carbon particles is reduced substantially after TMPD injection.[38] These findings define an important role of IFN-I in maintaining certain chronic inflammatory states.

IFN-I are known primarily for their antiviral and antiproliferative properties, and a prominent role in chronic inflammation has not been established previously.[39] Studies on acute murine cytomegalovirus infection showed that early antiviral defense is orchestrated by IFN-I and the downstream production of IFN-inducible chemokines. Recruitment of natural killer cells and macrophages to the site of infection is mediated by the production of CXCL3 and CCL2, respectively.[40] Our results suggest that a similar mechanism is involved in the chronic inflammation induced by TMPD, as IFN-I (but not other inflammatory mediators such as TNF-α, IL-6, IL-1, or IFN-γ) is required for chronically recruiting inflammatory monocytes to the peritoneum. These data are consistent with the observation that the influx of Ly6C^{hi} monocytes is reduced in mice deficient in TLR7 or MyD88, two upstream mediators of TMPD-induced IFN-I production.[19] PECs from wild-type mice treated with TMPD expressed high levels of the IFN-inducible monocyte chemoattractants CCL2, CCL7, and CCL12, which promote migration of monocytes to the site of inflammation through interactions with CCR2. Because of shared receptor usage, the relative contribution of each of these chemokines is unclear, although recent studies suggest a synergy of CCL2 and CCL7 in recruiting monocytes.[41,42]

Interestingly, the chronic influx and rapid turnover of Ly6C^{hi} monocytes were accompanied by a concomitant reduction of Ly6C^{lo} monocytes/macrophages in the peritoneal cavity of TMPD-treated mice. In contrast, although Ly6C^{hi} monocytes initially were recruited after thioglycollate treatment, few were found in the peritoneal cavity after 72 hours as the majority of PECs were Ly6C^{lo} residential monocytes/macrophages. Unexpectedly, the infiltrating monocyte profile induced by TMPD depended almost completely on IFN-I signaling. Labeling studies suggested that the accumulation of Ly6C^{lo} monocytes/macrophages in IFNAR$^{-/-}$ mice was not due to direct recruitment of circulating Ly6C^{lo} monocytes. Instead, the small number of Ly6C^{hi} monocytes recruited to the peritoneal cavity later differentiated into the Ly6C^{lo} subset. This view is supported by the deficiency of both Ly6C^{hi} and Ly6C^{lo} subsets in the absence of CCR2, which mediates the migration of Ly6C^{hi} but not Ly6C^{lo} monocytes.[3,43] How IFN-I interfere with differentiation or maturation of Ly6C^{hi} monocytes into the Ly6C^{lo} subset remains unclear.

The predominance of Ly6C^{hi} monocytes is likely to amplify the inflammatory cascade as these cells produced large amounts of inflammatory mediators in response to stimulation with TLR ligands. Ly6C^{hi} monocytes may establish a vicious cycle of monocyte recruitment and cytokine/chemokine production as they also constitute a major source of IFN-I and CCL2 in TMPD-treated mice.[18] In contrast, Ly6C^{lo} monocytes/macrophages exhibited much greater phagocytic capacity for both liposomes and apoptotic lymphocytes. Enhanced phagocytic function of Ly6C^{lo} monocytes *in vivo* also is illustrated by the preferential labeling of this subset on administration of DiD-liposomes alone, whereas depletion of this subset by clodronate-liposomes was required to effectively label Ly6C^{hi} monocytes. Supporting our observations, Ly6c^{lo} monocytes are also more effective in the uptake of apoptotic cells *in vivo*.[27] These findings suggest that the relative deficiency of Ly6C^{lo} monocytes/macrophages in the presence of IFN-I impairs phagocytic capacity at the site of inflammation, leading to defective clearance of the triggering stimuli and perpetuation of the inflammatory response. This is

further suggested by the defective clearance of carbon particles after i.p. TMPD injection.[38] The importance of monocyte maturation to the resolution of inflammation also is emphasized by recent studies of a skeletal injury model in which inflammatory Ly6C^{hi} monocytes are actively recruited to the site of injury and subsequently develop into Ly6C^{lo} monocytes/macrophages, which contribute to phagocytosis and tissue repair.[44]

The link between IFN-I production and Ly6C^{hi} monocyte influx is corroborated by findings from experimental peritonitis induced by other hydrocarbons. Although less potent than TMPD, *n*-hexadecane is a strong inducer of MCP-1 expression and Ly6C^{hi} monocyte recruitment.[18,45] In contrast, squalene and mineral oil treatment elicited little IFN-I production and the monocytes found in the peritoneal cavity were primarily of the Ly6C^{lo} subset, recapitulating the pattern in TMPD-treated IFNAR$^{-/-}$ mice.[18,23] The ability to recruit granulocytes, on the other hand, was similar among all of these hydrocarbons, supporting the specific role of IFN-I on monocyte migration.

A potential limitation of the current study is the possibility that monocyte homeostasis and maturation might be altered by clodronate-liposomes. It is noteworthy that this method was originally used to demonstrate the maturation of monocytes from Ly6C^{hi} into the Ly6C^{lo} phenotype.[4] Whereas adoptive transfer of monocyte subsets may circumvent the use of clodronate,[5] the lengthy process of *ex vivo* cell sorting may also affect the monocyte maturation and/or function. Indeed, *in vitro* experiments revealed that Ly6C^{hi} monocytes are converted to the Ly6C^{lo} phenotype with enhanced phagocytic function within 2 to 3 days (our unpublished observation) compared with 5 to 7 days *in vivo*.[4]

Our findings suggest a potential role of IFN-I in clinical conditions associated with chronic inflammation. In SLE, a chronic autoimmune condition characterized by the production of antibodies to numerous self-antigens, serum levels of IFN-I correlate with disease severity, kidney inflammation, and the presence of autoantibodies.[46,47] Numerous IFN-inducible chemokines, including CCL2 and CCL7, also are overexpressed in patients with lupus.[28] Interestingly, consistent with the present observations, defective phagocyte function is well established in SLE.[2] Aberrant clearance of apoptotic debris is thought to provide autoantigens that initiate the autoimmune response.[2] The production of lupus autoantibodies is also abolished in several mouse models in the absence of IFN-I signaling.[48–50] Whether dysregulated IFN-I production in SLE disrupts the balance of inflammatory versus residential monocyte subsets and/or contributes to the phagocytosis defect warrants detailed investigation. It is noteworthy that excess expression of IFN-I and interferon-stimulated genes is evident at sites of inflammation including the skin and kidneys of patients with lupus.[51,52] Migration of inflammatory monocytes to these sites in response to local secretion of IFN-inducible chemokines may perpetuate the vicious cycle of chronic inflammatory responses associated with the skin rashes and glomerulonephritis seen in SLE.

In addition, IFN-I may contribute to chronic inflammation in atherosclerosis. Circulating Ly6C^{hi} monocytes are chronically elevated in mice with atherosclerotic heart disease, and these inflammatory monocytes infiltrate the atheromatous lesions in apolipoprotein E-deficient mice.[8,43] Recombinant IFN-I administration accelerates plaque development in mice and human plaque tissue exhibits elevated expression of IFN-α as well as CCL2.[53,54] In view of our findings, it will be interesting to determine whether a similar IFN-I-chemokine feedback loop contributes to the chronic monocytosis in atherogenesis or the accelerated atherosclerosis of patients with SLE.[55]

In conclusion, this study defines an essential role of IFN-I in the recruitment of monocytes in the TMPD-induced model of chronic inflammation. These findings suggest that therapies aimed to neutralize IFN-I and/or downstream chemokines may be effective in treating lupus and other chronic inflammatory conditions.

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