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Monocytes from *Irf5*$^{-/-}$ mice have an intrinsic defect in their response to pristane-induced lupus‡

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Abstract

The transcription factor interferon regulatory factor 5 (IRF5) has been identified as a human systemic lupus erythematosus (SLE) susceptibility gene by numerous joint linkage and genome-wide association studies. Although IRF5 expression is significantly elevated in primary blood cells of SLE patients, it is not yet known how IRF5 contributes to SLE pathogenesis. Recent data from mouse models of lupus indicate a critical role for IRF5 in the production of pathogenic autoantibodies and the expression of Th2 cytokines and type I IFN. In the current study, we examined the mechanism(s) by which loss of *Irf5* protects mice from pristane-induced lupus at early time points of disease development. We demonstrate that *Irf5* is required for Ly6C(hi) monocyte trafficking to the peritoneal cavity (PC), which is believed to be one of the initial key events leading to lupus pathogenesis in this model. Chemotaxis assays using peritoneal lavage from pristane-injected *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates support an intrinsic defect in *Irf5*$^{-/-}$ monocytes. We found the expression of chemokine receptors CXCR4 and CCR2 to be dysregulated on *Irf5*$^{-/-}$ monocytes and less responsive to their respective ligands, CXCL12 and CCL2. Bone marrow reconstitution experiments further supported an intrinsic defect in *Irf5*$^{-/-}$ monocytes since *Irf5*$^{+/+}$ monocytes were preferentially recruited to the PC in response to pristane. Together, these findings demonstrate an intrinsic role for IRF5 in the response of monocytes to pristane, and their recruitment to the primary site of inflammation that is thought to trigger lupus onset in this experimental model of SLE.

Introduction

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder characterized by multiple immunologic abnormalities that lead to a break in self-tolerance and the production of autoantibodies (1, 2). Patients display elevated type I IFN in their serum and IFN gene signature in their blood cells that correlates with disease activity and severity (3). At this point, nearly all key elements of the immune system have been implicated in the pathogenesis of SLE (1). Monocytes/macrophages have been increasingly recognized to play a dynamic role in the initiation and perpetuation of SLE given their hallmark functions in phagocytosis, antigen presentation and cytokine production (4). Increased influx of monocytes to sites of inflammation and aberrant expression of cytokines and surface activation markers on monocytes have been documented in SLE patients (4, 5). Recent

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studies in the pristane-induced model of murine lupus revealed that monocytes play a key pathogenic role by acting as a major producer of type I IFNs (6, 7).

The transcription factor interferon (IFN) regulatory factor 5 (IRF5) has been identified as an SLE susceptibility gene in numerous large-scale genetic association studies (8–13). IRF5 controls multiple inflammatory and immune responses through its regulation of type I IFN expression and IFN stimulated genes (ISGs) (14–17). IRF5 is also a key mediator of MyD88-dependent Toll-like receptor (TLR) signaling, thus a critical factor controlling the expression of proinflammatory cytokines (18). We have recently demonstrated that mice lacking *Irf5* are protected from pristane-induced lupus in part due to significant alterations in cytokine expression, including elevated Th2 cytokines and significant weakening of the type I IFN gene signature (19). In support of this, overexpression of IRF5 was reported to induce M1 polarization (proinflammatory) in human macrophages and promote the Th1/Th17 response, while knockdown of IRF5 induced the M2 anti-inflammatory phenotype (20). In human SLE, we have shown that IRF5 expression is significantly elevated in primary peripheral blood mononuclear cells (PBMC) of SLE patients, as compared to healthy donors, and upregulation specifically associated with the *IRF5* SLE risk haplotype in SLE monocytes (21). Subsequently, we demonstrated that IRF5 was constitutively activated (nuclear-localized) in SLE monocytes, and not other immune cell populations examined from SLE patients, further supporting a pathogenic role for IRF5 in SLE monocytes (22).

Given that our current findings in human SLE point towards a pathogenic role for IRF5 in SLE monocytes (21, 22), combined with the fact that monocytes are one of the key pathogenic triggers in pristane-induced lupus (6, 7, 23), we sought to use this model to examine further the functional role(s) of IRF5 in the early stages of disease development that require monocyte trafficking to the PC. We show that mice lacking *Irf5* are significantly impaired in their chronic recruitment of Ly6C(hi) monocytes to the PC due to intrinsic defects in their chemokine receptor expression.

Materials and Methods

Mice

Irf5$^{-/-}$ mice backcrossed eight generations to C57BL6 were obtained from Dr. Ian Rifkin (Boston University School of Medicine) by approval from T. Taniguchi (University of Tokyo, Tokyo, Japan) and T. Mak (University of Toronto, Toronto, Ontario, Canada) (18). Wild-type C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). After obtaining the *Irf5*$^{-/-}$ mice, they were back-crossed two additional times to parental C57Bl/6 in order to obtain heterozygotes for intercrossing to obtain a new cohort of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates, by standard breeding techniques. Littermate *Irf5*$^{+/+}$ mice were used as controls. The *Dock2* mutation was analyzed by PCR genotyping of purified RNA from PBMC of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates as described (24). All *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates used in this study lacked the *Dock2* mutation. CD45.1 congenic mice B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ were purchased from The Jackson Laboratory (Bar Harbor, ME). Six to eight-week-old mice received a single intraperitoneal (*i.p.*) injection of 0.5 ml of pristane (Sigma-Aldrich), 1.5 ml 4% thioglycollate medium (Sigma-Aldrich) or PBS. Mice were sacrificed at indicated time-points post-injection. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey, New Jersey Medical School.

Cell counting and flow cytometry

Cell counting was performed on a VI-Cell® (Beckman Coulter, Inc) or FACSCalibur flow cytometer (BD Biosciences) using CountBright™ Absolute Counting Beads (Invitrogen)

according to manufacturer's instruction. All antibodies were purchased from Biolegend except for anti-CXCR4-PE (clone 2B11; eBioscience) and anti-CCR2-PE (clone 475301; R&D Systems). The monocyte/macrophage population was gated on using anti-CD11b-FITC (clone M1/70) and anti-Ly6G-APC (clone 1A8) antibodies; monocyte subsets were distinguished from this population using anti-Ly6C-PE/CY7 (clone HK1.4) antibodies. To examine bone marrow chimeric mice, anti-CD45.1-PE (clone A20) and anti-CD45.2-PerCP/Cy5.5 (clone 104) antibodies were used. To examine monocyte maturation, anti-I-A-PE (clone M5/114.15.2) and anti-CD86-AF700 (clone GL-1) antibodies were used, and anti-IgG2b-PE Isotype Control (clone RTK4530) antibodies were used throughout the study. Surface staining was performed at 4° C for 20 min with an optimized amount of primary antibody or the appropriate isotype control. Intracellular staining was carried out in 0.5% saponin at 4° C for 30 min following surface staining. Samples were acquired on a FACSCalibur or LSR II (BD Biosciences) and data analyzed with FlowJo (Tree Star, Inc.) software.

Isolation of monocytes

Bone marrow cells collected from femurs and tibias were incubated with Biotin anti-mouse Ly6G Ab (clone 1A8; Biolegend) followed by incubation with Streptavidin microbeads (Miltenyi Biotec). $Ly6G^+$ cells were depleted by magnetic separation with MS columns (Miltenyi Biotec). The negative fraction was incubated with CD11b microbeads (Miltenyi Biotec) and $CD11b^+$ cells were isolated by magnetic separation. Alternatively, monocytes were isolated using EasySep® mouse monocyte enrichment kit (Stemcell Technologies). Briefly, bone marrow cells were incubated with antibody cocktail against non-monocytes followed by incubation with biotin selection cocktail and magnetic particles. Labeled cells were removed using EasySep magnet and monocytes were enriched. The two methods yielded a similar percentage of enriched monocytes per sample.

Chemotaxis Assay

Bone marrow cells were pooled from *Irf5*$^{+/+}$ or *Irf5*$^{-/-}$ littermates and monocytes isolated using the EasySep® monocyte enrichment kit described above. 0.05 to 0.1 million cells were seeded in each of 6.6 mm transwells with 5 μm pores (Corning). Peritoneal lavage fluid or chemokine was added to the bottom well as the chemoattractant and RPMI-1640 plus 1% BSA medium was used as control. All chemokines were purchased from R&D Systems. Cultures were incubated under 5% CO_2, 37° C for 3 hrs. Cells that migrated across the insert were trypsinized, collected and counted on a FACSCalibur (BD Biosciences).

Chemokine and chemokine receptor measurement

MILLIPLEX MAP multiplex mouse cytokine/chemokine kit (Millipore, Billerica, MA) was used to determine chemokine expression level according to the manufacturer's instruction. Samples were analyzed with a Luminex 100 Multi-Analyte Profiling System (Luminex Corp, Austin, TX). Cytokine/chemokine concentrations were determined by standard curve, which were generated using the mixed standard provided with the kit. For the analysis of chemokine receptor transcript expression, bone marrow monocytes were enriched, as described above, and RNA extracted using the RNeasy plus mini kit (Qiagen). Total RNA was converted to cDNA and the Mouse Chemokines and Receptors qPCR array (SABiosciences) was performed on the ABI 7300 Real-Time PCR system (Applied Biosystems) using RT^2 qPCR master mixes (SABiosciences). Raw data were analyzed with SDS software (Applied Biosystems) and the web-based PCR array data analysis tool (SABiosciences).

Dual luciferase assay

HEK 293T cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) and 1 IU penicillin/1 μl/ml streptomycin (Mediatech, Hemdon, VA) at 37°C in a humidified incubator with 5% CO_2/95% air. Cells were co-transfected with pGL3b-*CCR2* or -*CXCR4* promoter reporter plasmids and pCAGEN-*mIRF5* or empty vector control plasmid and *pRL* using Lipofectamine® 2000 Reagent (Invitrogen) as previously described (14). Luciferase activity was measured 24 hr post-transfection and normalized to Renilla activity.

Generation of bone marrow chimeric mice

Bone marrow cells from CD45.1 $Irf5^{+/+}$ and CD45.2 $Irf5^{-/-}$ C57BL/6 mice were harvested, treated with red blood cell lysis buffer (eBioscience), mixed at a 1:1 ratio and used as donor cells. Recipient $Irf5^{+/+}$ CD45.2 C57BL/6 mice received 10 Gy of irradiation (split dose, 3 hrs apart) and were *i.v.* injected with 10 million donor cells 4 hrs after second irradiation. 6 weeks post-transplantation, each mouse was given 0.5 ml of pristane intraperitoneally and sacrificed 4 weeks later for PECs, spleen, blood and bone marrow. The monocyte/macrophage population was gated on using anti-Cd11b-FITC and anti-Ly6G-APC antibodies.

Phagocytosis assay

Splenic macrophages were isolated by positive selection with CD11b magnetic beads. Cells were seeded in a 96-well plate format and pre-incubated with LPS (100 ng/mL) or medium alone for 35 min (25). Cells were then incubated with 1 mg/mL FITC dextran for 40 min followed by 1 min incubation with 0.25 mg/mL trypan blue to quench extracellular signal. Phagocytosis was assessed by measuring fluorescein counts at absorbance 485/535.

Statistical analysis

Statistical analysis was done with the two-tailed Students *t* test or Mann-Whitney *U* test when variables were not normally distributed. Data are presented as mean ± SD (normal distribution) or mean ± SEM (non-normal distribution). p value <0.05 was considered significant. Statistical analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA).

Results

Pristane-induced Ly6C(hi) monocyte recruitment to the PC is impaired in $Irf5^{-/-}$ mice

Pristane (or 2,6,10,14-tetramethylpendadecane; TMPD) is a type of hydrocarbon oil that induces SLE in a variety of mouse strains (23). Diseased mice produce autoantibodies against small nuclear ribonucleoproteins (snRNPs) and dsDNA and develop immune complex-mediated glomerulonephritis (23). $Irf5^{-/-}$ mice are protected from pristane-induced lupus (19, 26). An early key event in this model is the recruitment of IFN-producing monocytes to the PC following *i.p.* injection of pristane (6, 7, 23). Type I IFN then stimulates the expression of inflammatory cytokines and chemokines leading to the sustained infiltration of leukocytes and formation of ectopic lymphoid tissues in the PC (6, 7, 27). There are two subsets of monocytes in murine blood; one subset expresses high levels of CX3CR1 and low levels of Ly6C and CCR2; the other subset expresses low levels of CX3CR1 and high levels of Ly6C and CCR2 (28, 29). The Ly6C(hi) subset is preferentially recruited by pristane and is the major producer of Type I IFN in this model (6, 7). In naïve C57BL/6 mice, the major components of peritoneal exudate cells (PECs) are $CD11b^+B220$(dim) B1 cells and CD11b(hi)$Ly6G^-Ly6C^-$ resident macrophages (7). Upon

i.p. injection of pristane, however, $CD11b^+Ly6G^+$ neutrophils and $CD11b^+Ly6G^-$ monocytes are recruited to the PC within 8 hrs (data not shown) and influx persists for months after (30) while B1 cells are dramatically reduced in the PC (7). Consistent with previous findings (6, 7), we found that the majority of monocytes elicited by pristane express high levels of Ly6C (Fig. 1A); we also found that there were less than 2% of B1 cells in the PC after pristane injection (Supplemental Fig. 1). We compared the numbers of peritoneal monocytes in $Irf5^{+/+}$ (*wt*) and $Irf5^{-/-}$ littermates before pristane injection and 24, 48 hrs, 1, 2, 4 and 6 weeks after injection. No significant difference was observed at 24 and 48 hrs post-injection (data not shown). The earliest time point that showed a significant difference in the recruitment of monocytes/macrophages to the PC between littermates was at 2 weeks post-injection (Fig. 1B); this difference was even more striking at 4 and 6 weeks (Fig. 1C). Similar to total cell numbers in the PC, the frequency of $CD11b^+Ly6G^-$ monocytes was also reduced in pristane-injected $Irf5^{-/-}$ mice (Supplemental Fig. 2A); the reduction in the Ly6C(hi) subset is not due to alterations in Ly6C expression between littermates (Supplemental Fig. 2B). A significant decrease in the number of $Irf5^{-/-}$ Ly6C(lo) cells in the PC was only observed at the later timepoints. Examination of *IRF5* expression in sorted blood cells revealed dramatically higher levels in Ly6C(hi) monocytes as compared to Ly6C(lo) (Supplemental Fig. 3). In contrast to the pristane-induced chronic inflammation model, *i.p.* injection of thioglycollate (TG) induces acute inflammation with recruitment of monocytes/macrophages to the PC; recruitment generally peaks around 72 hrs post-injection and then declines (31). Upon examination of Ly6C(hi) monocyte recruitment to the PC in response to TG, we found no significant difference between littermates; notably, the recruitment of Ly6C(lo) cells was significantly decreased in $Irf5^{-/-}$ mice (Fig. 1D). In either model, the observed defect in monocyte migration to the PC was not due to differences in PC cellular apoptosis (data not shown). These data support a critical role for IRF5 in the recruitment of monocytes to the PC after induction of chronic or acute inflammation.

$Irf5^{-/-}$ monocytes are defective in their response to peritoneal lavage of pristane-injected mice

Impaired Ly6C(hi) monocyte recruitment to the PC in pristane-injected $Irf5^{-/-}$ mice could be due to an intrinsic defect in $Irf5^{-/-}$ monocytes or an extrinsic defect in the signaling environment of the PC. To test these two possibilities, we performed chemotaxis assays on naïve monocytes from $Irf5^{+/+}$ and $Irf5^{-/-}$ littermates using peritoneal lavage fluid from littermates 4 weeks post-pristane injection as the chemoattractant. Naïve monocytes were enriched from bone marrow cells by magnetic depletion of non-monocytes; a similar percent enrichment per sample and number of cells plated per experiment was achieved (Figs. 2A–B). Consistent with previous reports, enriched bone marrow monocytes are predominantly Ly6C(hi) (data not shown) (32, 33). We found that significantly fewer monocytes from $Irf5^{-/-}$ mice, as compared to $Irf5^{+/+}$ littermates, could migrate towards peritoneal lavage fluid from pristane-injected $Irf5^{+/+}$ mice (Fig. 2C), supporting an intrinsic defect in $Irf5^{-/-}$ monocytes. Little difference between the migration efficiency of $Irf5^{+/+}$ and $Irf5^{-/-}$ monocytes to $Irf5^{-/-}$ lavage was observed.

Expression of a subset of chemokine receptors is dysregulated on $Irf5^{-/-}$ monocytes

Chemokine receptors are critical for monocyte trafficking under both steady-state and inflammatory conditions (34). *CX3CR1* is required for Ly6C(lo) monocytes to migrate to the periphery to replenish tissue macrophages during homeostasis (35). *CCR1*, *CCR2*, *CCR5*, *CCR8*, *CXCR2* and *CXCR4* have been implicated in monocyte recruitment during infection and inflammation; they regulate monocyte egress from bone marrow, migration from the circulation to inflamed tissue, and/or homing to bone marrow (34). The observed decrease in the response of $Irf5^{-/-}$ monocytes to pristane could be due to defects in chemokine receptor expression, resulting in the inability of $Irf5^{-/-}$ monocytes to “sense”

inflammatory signals. To test this, we isolated naïve monocytes from the bone marrow of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates, that are predominantly Ly6C(hi), and examined the expression of chemokine receptors using a Mouse Chemokines and Receptors qPCR array (SABiosciences). Receptors that were upregulated or downregulated more than two-fold in *Irf5*$^{-/-}$ monocytes as compared to *Irf5*$^{+/+}$ are listed, along with their respective ligands, in Table 1. We found a striking decrease in *CXCR4* receptor expression on monocytes from *Irf5*$^{-/-}$ mice, as well as decreases in *CXCR3*, *CCRL*1, *CCR5*, and *CCR2*, and increases in *CCR1* and *CX3CR1* expression.

Impaired CCR2 and CXCR4 protein expression and function on Irf5$^{-/-}$monocytes

To determine whether there is a correlation between chemokine receptor transcript expression and protein expression on *Irf5*$^{-/-}$ monocytes, we collected blood and bone marrow from naïve and pristane-injected *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates and examined surface or intracellular staining of the chemokine receptors CXCR4, CXCR3, CCR2 and CCR5 on monocyte subsets. No significant difference in the expression of CCR5 or CXCR3 was observed on monocytes from the blood or bone marrow of naïve and pristane-injected littermates (data not shown). Instead, a significant decrease in CCR2 expression was found on naïve blood monocytes of *Irf5*$^{-/-}$ mice, as compared to *Irf5*$^{+/+}$, and the decrease was observed in both Ly6C(hi) and Ly6C(lo) subsets (Fig. 3A&B). CCR2 expression is not altered by pristane treatment (7). Since nothing is known of CXCR4 expression in the pristane-induced model of lupus, we examined expression on monocytes from both naïve and pristane-injected mice. No defect in CXCR4 expression was observed on naïve blood or bone marrow monocytes of *Irf5*$^{-/-}$ mice (Fig. 3C and data not shown); however, a decrease on bone marrow monocytes of pristane-injected *Irf5*$^{-/-}$ mice was observed (Fig. 3D–F). Although the mean fluorescence intensity (MFI) of CXCR4 staining was not significantly reduced, the percentage of CXCR4 expressing cells was significantly decreased (Fig. 3D–F). Similar reductions were found on blood monocytes from pristane-injected *Irf5*$^{-/-}$ mice (data not shown).

In order to understand the functional consequence of impaired chemokine receptor expression on *Irf5*$^{-/-}$ monocytes, we examined the ability of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ monocytes to migrate towards specific receptor ligands using the chemotaxis assay. Naïve monocytes from either *Irf5*$^{+/+}$ or *Irf5*$^{-/-}$ littermates showed little response to RANTES and IP10 (Fig. 3G); however, a significant defect in the ability of *Irf5*$^{-/-}$ monocytes to migrate towards the *CXCR4* ligand, CXCL12, was found. In addition, a consistent, yet non-significant, reduction in the migration ability of *Irf5*$^{-/-}$ monocytes towards the *CCR2* ligand, CCL2, was observed (Fig. 3G). To begin to address how IRF5 might be regulating the expression of these two genes, we performed promoter reporter assays in HEK 293T cells and found a small yet significant transactivation of both reporters by IRF5 (Fig. 3H). Together, these data suggest that the observed defect in both *in vivo* and *in vitro Irf5*$^{-/-}$ monocyte recruitment is due to the impaired expression and function of *CCR2* and *CXCR4* receptors.

Expression of monocyte-related chemokines is not compromised in the PC of Irf5$^{-/-}$ mice

It has been shown that IRF5 expression promotes M1 polarization and Th1/Th17 cytokine expression while inhibiting M2-associated cytokines (19, 20, 36). Among the cytokines currently known to be regulated by IRF5, such as IFN-α, IL-6, IL-12, and TNF-α, some have been reported to be elevated in the serum of SLE patients (2, 18, 37–39). To this effect, we recently demonstrated that serum levels of the Th1 cytokine IL-6 was decreased, while Th2 cytokines IL-4, IL-5 and IL-10 were increased in pristane-injected *Irf5*$^{-/-}$ mice (19). In addition, we showed that *CCL2* (*MCP-1*) expression was significantly reduced in *Irf5*$^{-/-}$ bone marrow cells (19). Although data in Figs. 1–3 support an intrinsic defect in *Irf5*$^{-/-}$ monocytes that results in impaired recruitment to the PC, we cannot exclude an extrinsic

defect in serum or PC chemokine expression in *Irf5*$^{-/-}$ mice that may also contribute to impaired recruitment. To further examine this, we measured sera and PC chemokine levels using the MILLIPLEX MAP multiplex mouse cytokine/chemokine kit (Millipore) at 2 weeks post-pristane injection. Interestingly, we found no significant difference in the levels of monocyte-related chemokines (CCL2, IP10, RANTES, MIP1a and MIP1b) in the peritoneal lavage fluid between *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates; however, decreases were observed for some of these chemokines in the sera of *Irf5*$^{-/-}$ mice (Fig. 4). Although a significant defect in IP10 expression was observed in sera from *Irf5*$^{-/-}$ mice, it is currently not known whether IP10 mediates monocyte recruitment in the pristane model of murine lupus and neither *Irf5*$^{+/+}$ or *Irf5*$^{-/-}$ monocytes responded to IP10 *in vitro* (Fig. 3G). Nonetheless, these data provide further support that impaired recruitment of *Irf5*$^{-/-}$ monocytes to the PC in response to pristane is primarily due to intrinsic defects in chemokine receptor expression and not the chemokines themselves.

Irf5$^{+/+}$ monocytes/macrophages are preferentially recruited to the PC of pristane-injected chimeras

To unambiguously confirm the intrinsic defect in *Irf5*$^{-/-}$ monocytes, we generated bone marrow chimeras by injecting 1:1 mixed CD45.1 *Irf5*$^{+/+}$ and CD45.2 *Irf5*$^{-/-}$ bone marrow cells to lethally irradiated CD45.2 *Irf5*$^{+/+}$ mice (Fig. 5A). Six weeks after reconstitution, pristane was administered to the reconstituted mice, and 4 weeks later, the percentages of CD45.1 and CD45.2 monocytes/macrophages in the bone marrow, blood, PC and spleen were determined (Fig. 5B). Before injection of pristane, a slight enrichment of *Irf5*$^{+/+}$ monocytes was observed in blood and this held true after pristane injection; however, a significant defect in the recruitment of *Irf5*$^{-/-}$ CD11b^{+}Ly6G^{-} bone marrow cells to the blood and PC of chimeras was observed as the ratio of *Irf5*$^{+/+}$ (CD45.1) to *Irf5*$^{-/-}$ (CD45.2) monocytes went up from ~1 in bone marrow to ~1.8 in the blood and ~2.2 in the PC (Fig. 5C). Less than 5% of CD11b^{+}Ly6G^{-} cells from chimeras were CD11b^{+}B220(dim) B1 cells (data not shown). These data support the impaired recruitment of *Irf5*$^{-/-}$ monocytes to the PC in response to pristane due to an intrinsic defect in *Irf5*$^{-/-}$ monocytes.

Enhanced phagocytic activity but not maturation of Irf5$^{-/-}$ monocytes/macrophages

In human SLE, impaired cell clearance and accumulation of apoptotic debris is thought to contribute to a break in self-tolerance leading to the production of pathogenic autoantibodies (40). Indeed, cells from SLE patients appear more susceptible to apoptosis than healthy donors, yet their macrophages are impaired in their ability to engulf apoptotic material (41, 42). In the pristane-induced model of murine lupus, it was found that the rapid turnover of recruited monocytes in the PC of pristane-injected wild-type mice was associated with a lack of differentiation into more phagocytic Ly6C(lo) monocytes/macrophages (7), which was consistent with previous observations that the uptake of carbon particles are substantially reduced after pristane injection (43). Furthermore, pristane induces apoptosis both *in vivo* and *in vitro* and is thought to be one of the critical first events in the pathogenesis of pristane-induced lupus that is similar to human SLE (44). First, we examined whether loss of *Irf5* alters monocyte/macrophage differentiation. At 4 weeks post-pristane injection, we examined activation/maturation markers I-A (MHC II) and CD86 on PC monocytes from *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates and found no significant difference in expression (Fig. 6A). We next examined *in vitro* phagocytosis and found that macrophages from *Irf5*$^{-/-}$ mice had enhanced phagocytic ability in their response to LPS as compared to macrophages from *Irf5*$^{+/+}$ littermates (Fig. 6B). These data support a potentially new role for IRF5 in phagocytosis that is not due to alterations in the activation/maturation of monocytes.

Discussion

The recruitment of monocytes to inflamed tissues is critical for host defense against a variety of pathogens; however, this response can be a double-edged sword creating a state of chronic inflammation if not properly regulated (34). Monocyte trafficking is directed by adhesion proteins, chemokines and their receptors (34), yet the mechanisms and factors controlling these molecules have yet to be fully elucidated. Others and we have recently demonstrated in the pristane-induced model of chronic inflammation that results in the development of lupus-like disease, that mice lacking the transcription factor *Irf5* are protected from disease development (19, 26, 36). We found that protection of *Irf5*$^{-/-}$ mice from lupus onset and pathogenic autoantibody production was due in part to a weakened type I IFN signature and significant skewing of the cytokine milieu towards a more protective Th2-like environment (19). In the current study, we examined the role of IRF5 in the early stages of lupus development that require monocyte trafficking to the PC in response to *i.p.* injection of pristane. We found a significant reduction in the migration of Ly6C(hi) monocytes to the PC as early as 2 wks post-injection (Fig. 1). These data are reminiscent of findings from pristane-injected *IFNAR*$^{-/-}$ mice except that we did not observe a concomitant increase in Ly6C(lo) monocytes/macrophages in the PC (7); *TLR7*$^{-/-}$ and *MyD88*$^{-/-}$ mice also gave similar decreases in Ly6C(hi) monocyte recruitment to the PC but the Ly6C(lo) subset was not analyzed (45). While preparing this manuscript, a paper by Xu *et al.* was published that reported the similar finding of reduced Ly6C(hi) monocyte recruitment to the PC of pristane-injected *Irf5*$^{-/-}$ mice yet, identical to the pristane-injected *IFNAR*$^{-/-}$ mice, they observed a significant increase in the percentage of Ly6C(lo) cells in the PC at 2 wks post-injection (36). The difference between our findings may in part be explained by the complete abolishment of the type I IFN gene signature in their pristane-injected *Irf5*$^{-/-}$ mice (36), while we only observed a significant weakening of the IFN signal (19). It was found that accumulation of the Ly6C(lo) subset in the PC of *IFNAR*$^{-/-}$ mice was due to enhanced maturation of Ly6C(hi) monocytes to Ly6C(lo) rather than the preferential recruitment of the Ly6C(lo) subset (7). We did not observe a significant difference in the expression of activation/maturation markers I-A and CD86 on peritoneal monocytes between *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates supporting the observed lack of increase in the Ly6C(lo) subset (Fig. 6) (46).

It was recently reported that several independent colonies of *Irf5*$^{-/-}$ mice in the United States are contaminated with a spontaneous genomic duplication and frameshift mutation in the guanine exchange factor dedicator of cytokinesis 2 (*Dock2*) gene (24). Dock2, an atypical Rac activator, is essential for TLR7/9-mediated IFN-α induction in PDCs, T and B lymphocyte migration, atrophy of lymphoid follicles and loss of marginal zone B cells (46–48). Previously observed phenotypes of the *Irf5*$^{-/-}$ mice, such as altered marginal zone B cells, changes in PDC number and function, and type I IFN responses, are now being put into question by these findings (24). It was not reported by Xu and colleagues whether their *Irf5*$^{-/-}$ mice were screened for the *Dock2* mutation that could potentially contribute to the complete abolishment of the type I IFN signature (36). We have reported the genotype of our *Irf5*$^{-/-}$ mice and they contain homozygous wild-type *Dock2* alleles (19). For the most part, results from three independent studies on pristane-injected *Irf5*$^{-/-}$ mice have been quite similar (19, 26, 36). Nonetheless, it will be critical to our understanding of IRF5 function to replicate and confirm previous findings in *Irf5*$^{-/-}$ mice that have been genotyped for the *Dock2* mutation.

The mechanism(s) of impaired Ly6C(hi) monocyte recruitment to the PC of pristane-injected *Irf5*$^{-/-}$ mice was not examined in the recent study by Xu and colleagues (36). Here, we found the defect in Ly6C(hi) monocyte recruitment to the PC of *Irf5*$^{-/-}$ mice to be due, at least in part, to decreased expression of chemokine receptors *CCR2* and *CXCR4* on

Ly6C(hi) monocytes of *Irf5*$^{-/-}$ mice (Fig. 3). Previous studies have shown that *CCR2* is required for monocyte egress from the bone marrow to the circulation during homeostasis and inflammation (49–51). More importantly, it has been shown that the chronic influx of monocytes to the PC by pristane is dependent on *CCR2*; both Ly6C(hi) and Ly6C(lo) monocyte subsets were largely absent in the PC of *ccr2*$^{-/-}$ mice (7). We observed normal egress of Ly6C(hi) monocytes from the bone marrow of *Irf5*$^{-/-}$ mice to the circulation and CCR2 protein expression on *Irf5*$^{-/-}$ bone marrow monocytes was not changed between *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ mice (data not shown); however, CCR2 expression was significantly reduced on circulating *Irf5*$^{-/-}$ blood monocytes (Fig. 3). While little is known of CXCR4 expression and function in the pristane-induced model of murine lupus, expression of CXCR4 has been shown to be upregulated on Ly6C(hi) monocytes of lupus-prone murine strains and accumulation of CXCR4 expressing monocytes/macrophages has been observed in the kidneys of diseased mice (52). CXCR4 antagonism has also been shown to restore monocyte numbers in the circulation following monocyte depletion by CCL2 blocking molecules and administration of CXCR4 antagonists protects B6.*Sle1.Yaa* mice from lupus (53). Similar to findings of CCR2 expression (7), CXCR4 expression on monocytes was not increased after pristane injection (data not shown); however, CXCR4 protein levels were significantly reduced on bone marrow (Fig. 3F) and blood (data not shown) monocytes from pristane-injected *Irf5*$^{-/-}$ as compared to *Irf5*$^{+/+}$ mice. To put these findings into perspective with the *Dock2* mutation, while homing of lymphocytes to secondary lymphoid tissues has been shown to be defective in *Dock2*$^{-/-}$ mice, the numbers of monocytes in the periphery of *Dock2*$^{-/-}$ mice were maintained and *Dock2*$^{-/-}$ monocytes responded normally *in vitro* to CCL2 and CXCL12 ligands (47). Data in Fig. 3G support that the observed intrinsic defects in CCR2 and CXCR4 expression on *Irf5*$^{-/-}$ Ly6C(hi) monocytes and their inability to respond to CCL2 and CXCL12 are not due to confounding genotype results as current data support that *Dock2* is dispensable for monocyte trafficking (47). This data is further enhanced by the fact that IRF5 can regulate the *CCR2* and *CXCR4* promoters (Fig. 3H). Thus, these data instead point towards a critical role for IRF5 in regulating the molecules, *i.e.* chemokine receptors, which are important for pristane-induced monocyte trafficking to the PC.

In vivo data from *Irf5*$^{-/-}$ mice (Fig. 1) and bone marrow chimerical mice (Fig. 5C) provide clear support for the intrinsic defect in *Irf5*$^{-/-}$ monocytes. In response to pristane, data from both of these models reveal a defect in the recruitment of *Irf5*$^{-/-}$ monocytes to the PC; however, data from bone marrow chimerical mice suggests that the primary defect may be in the initial response of *Irf5*$^{-/-}$ bone marrow monocytes to signals in the blood environment since a significant decrease in the recruitment of *Irf5*$^{-/-}$ monocytes from the bone marrow to the blood was observed (Fig. 5C). Results from the *in vitro* chemotaxis assay also support a defect in the response of *Irf5*$^{-/-}$ bone marrow monocytes to peritoneal lavage fluid from pristane-injected *Irf5*$^{+/+}$. Somewhat surprising, however, was the finding of no significant difference between the recruitment of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ bone marrow monocytes to peritoneal lavage from *Irf5*$^{-/-}$ mice (Fig. 2C). This small discrepancy is likely a result of *in vivo* versus *in vitro* experimentation and the different signals monocytes are exposed to in each compartment (bone marrow, blood or PC). The *in vitro* chemotaxis assay simply measured the response of bone marrow monocytes to signals from peritoneal lavage fluid; unlike the *in vivo* systems, it did not take into account signals from the blood that the bone marrow monocytes would first be exposed to before recruitment to the PC. Another possible explanation is that bone marrow monocytes and blood monocytes lacking *Irf5* respond differently to signals from the PC. Further studies will be required to clearly define the role of IRF5 in these two cell populations.

In conclusion, this study defines an essential role for IRF5 in the recruitment of monocytes to the PC in the pristane-induced model of chronic inflammation and murine lupus. Given

the recent finding that IRF5 is already activated (nuclear-localized) in human SLE monocytes (22), supporting a pathological function for IRF5 in human disease, it will be interesting to expand these types of studies to examine the functional role of IRF5 in monocytes/macrophages from SLE patients. Together, these findings clearly demonstrate an intrinsic role for IRF5 in the regulation of inflammatory monocyte migration through its ability to control chemotactic responses to CCL2 and CXCL12 and provide significant new insight into how the dysregulation of IRF5 expression and activation may contribute to human SLE pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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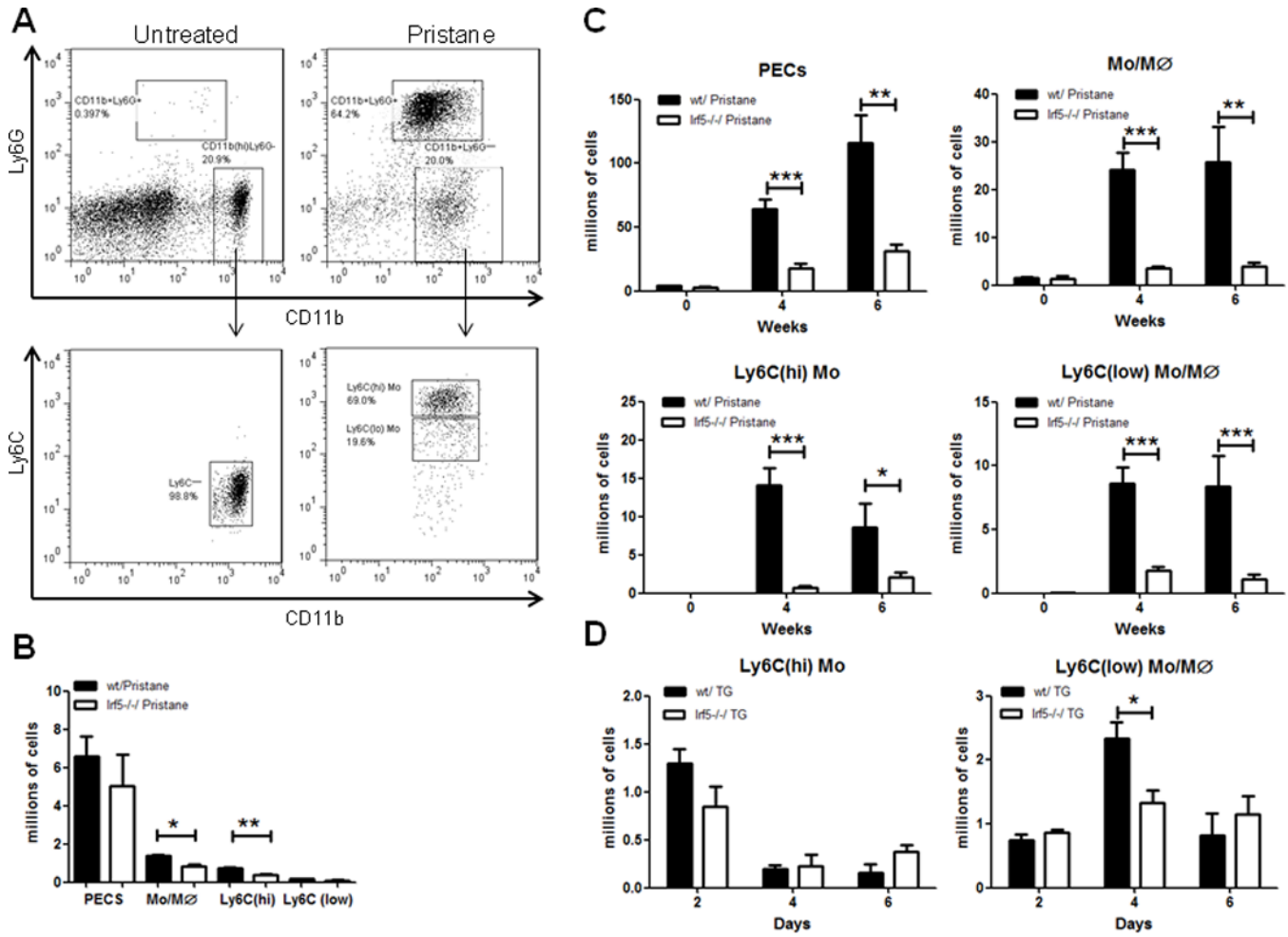

Figure 1. Ly6C(hi) monocyte recruitment to the PC after pristane-injection is impaired in *Irf5*$^{-/-}$ mice

Irf5$^{+/+}$ (wt) and *Irf5*$^{-/-}$ littermates were *i.p.* injected with 0.5 ml of PBS or pristane and PECs collected by peritoneal lavage with 3 ml PBS. (**A**) Gating strategy for the analysis of resident macrophages (CD11b(hi)Ly6G−), pristane-elicited monocytes/macrophages (CD11b^{+}Ly6G^{-}) in PECs and monocyte subsets (Ly6C(hi) and Ly6C(lo)) used for subsequent labeling experiments. Representative flow cytometry analysis of resident and pristane-elicited PECs in *Irf5*$^{+/+}$ C57BL/6 mice. (**B**) Flow cytometry quantification of PECs, monocyte/macrophage (Mo/Mϕ), Ly6C(hi) and Ly6C(lo) monocyte subsets at 2 weeks post-pristane injection. (**C**) Same as in (**B**) except quantification was performed at 4 and 6 weeks post-injection and cells counted by Vi-Cell. (**D**) Flow cytometry quantification of Ly6C(hi) and Ly6C(lo) PECs after 1.5 ml *i.p.* injection of thioglycollate (TG). n = 4–7 mice per group, representative of two independent experiments. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$ by unpaired Student *t* test.

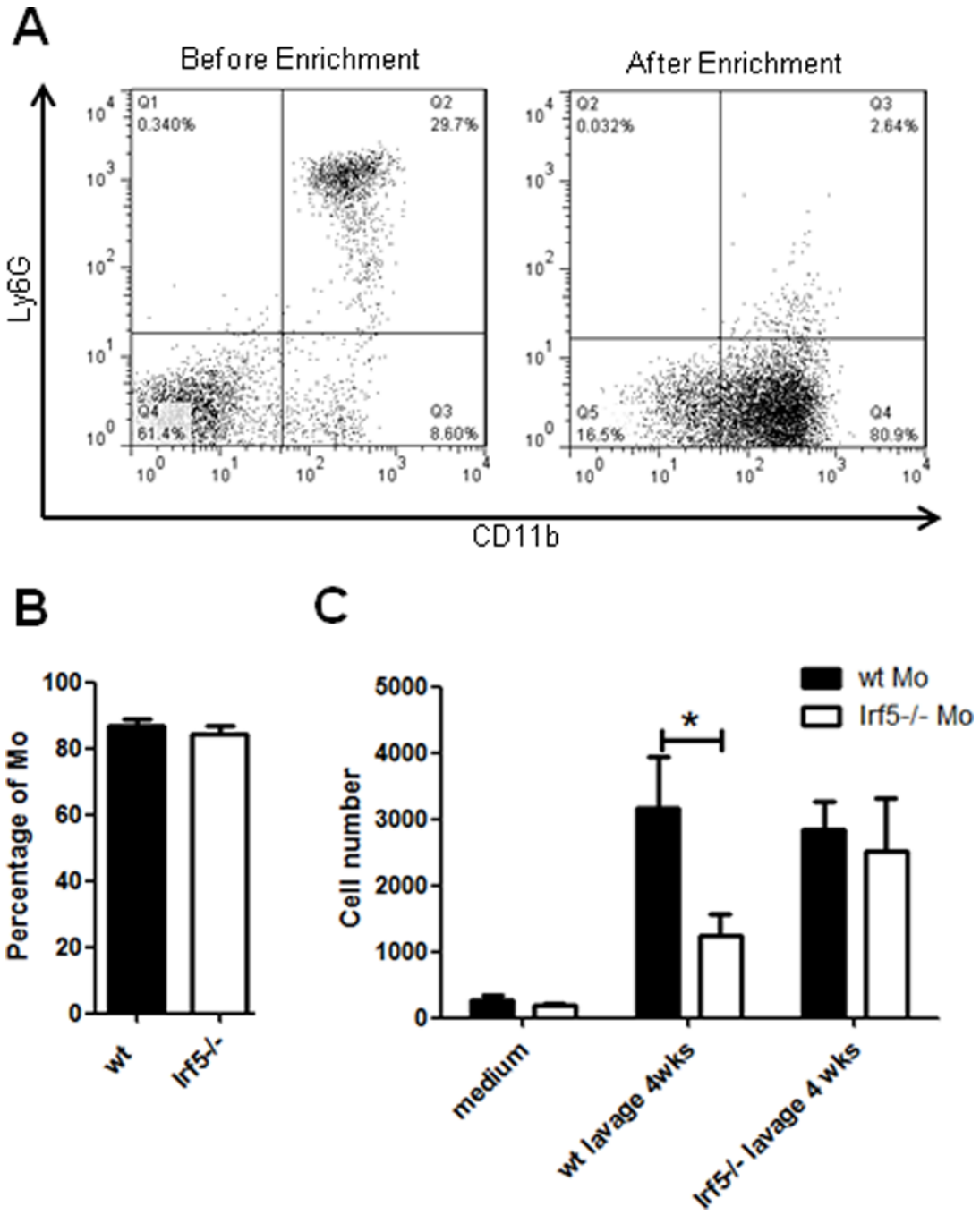

Figure 2. *Irf5*$^{-/-}$ monocytes are defective in their migration towards peritoneal lavage fluid from pristane-injected *Irf5*$^{+/+}$ mice

Monocytes were enriched from bone marrow cells of naïve *Irf5*$^{+/+}$ (wt) and *Irf5*$^{-/-}$ littermates. (**A**) Representative flow cytometry analysis of CD11b^{+}Ly6G^{-} monocytes from *Irf5*$^{+/+}$ C57BL/6 mice before and after enrichment. (**B**) Percentages of CD11b^{+}Ly6G^{-} monocytes were assessed by flow cytometry; similar percentages were obtained for each genotype after enrichment. (**C**) Peritoneal lavage fluid was collected from six littermates per genotype at 4 weeks post-pristane injection and used as chemoattractant in the chemotaxis assay. The number of *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ monocytes that migrated to bottom wells in response to peritoneal lavage fluid were quantified on flow cytometry using counting beads.

n = 10 mice per group, representative of three independent experiments. * $p < 0.05$ by Mann-Whitney U test.

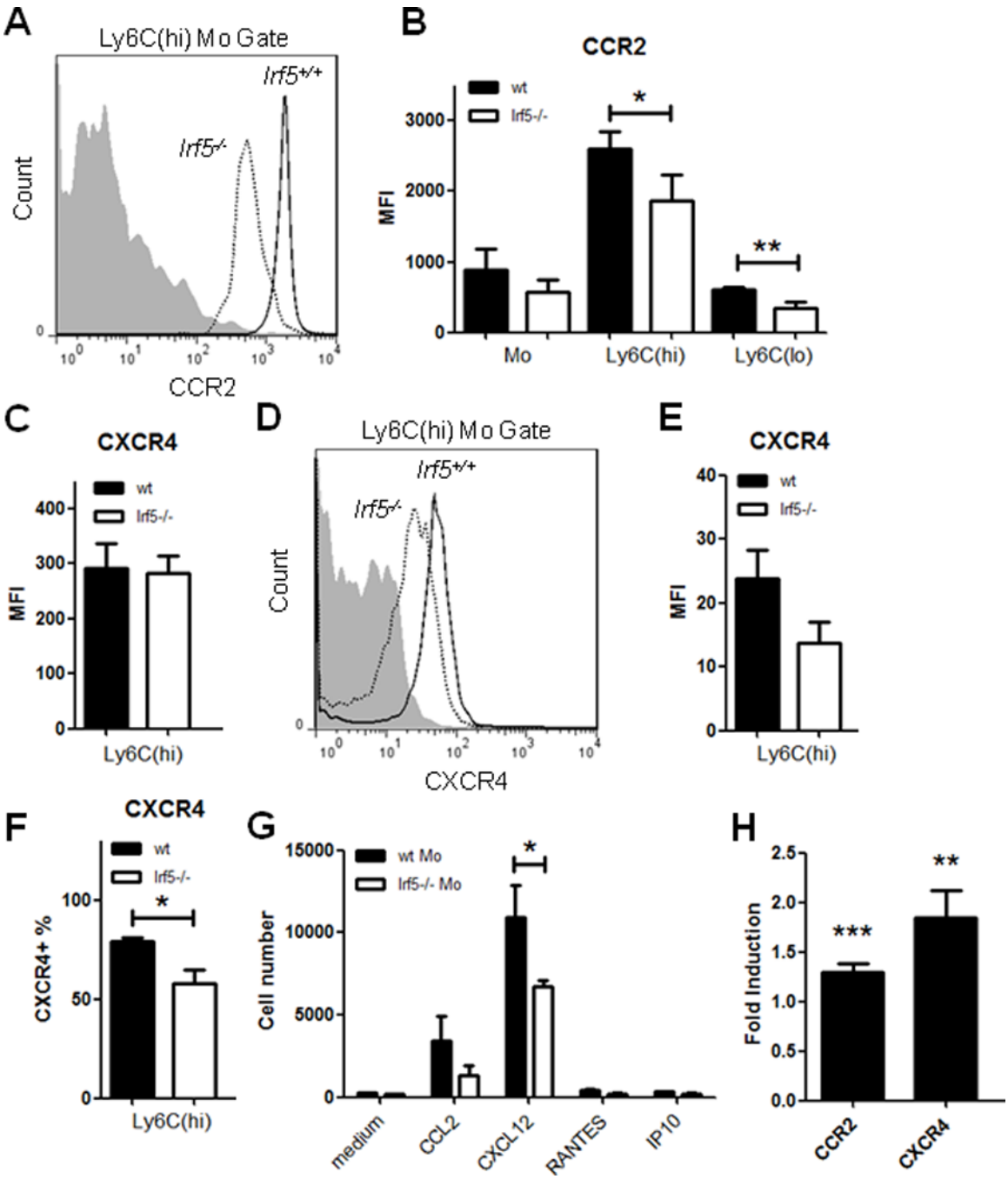

Figure 3. Decreased expression of CCR2 and CXCR4 on *Irf5*$^{-/-}$ monocytes

Blood and bone marrow cells were collected from naïve and pristane-injected *Irf5*$^{+/+}$ (wt) and *Irf5*$^{-/-}$ littermates. Surface staining was performed with anti-CD11b, -Ly6G, -Ly6C and -CCR2 antibodies, and intracellular staining with anti-CXCR4 antibodies; receptor expression was analyzed by flow cytometry. (**A**) Representative histogram of CCR2 expression on naïve blood Ly6C(hi) monocytes from littermates and (**B**) quantification of the mean fluorescence intensity (MFI) on Ly6C(hi) and Ly6C(lo) monocytes; n = 10–11 mice per genotype. Gray area represents isotype control staining in (**A** and **D**). (**C**) Quantification of the MFI of CXCR4 on bone marrow monocytes from naïve mice. (**D**)

Same as in (**A**) except CXCR4 expression was analyzed on bone marrow monocytes 4 weeks post-pristane injection. (**E**) Quantification of the MFI of CXCR4 and (**F**) percentage of CXCR4 expressing cells in Ly6C(hi) bone marrow monocytes from pristane-injected mice. n = 3 mice per genotype. (**G**) *Irf5*$^{-/-}$ monocytes are defective in their response to CCR2 and CXCR4 ligands. Enriched naïve bone marrow monocytes from *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates were seeded inside transwells and medium alone or supplemented with 50 ng/ml CCL2, CXCL12, RANTES or 300 ng/ml IP10 added to the other side as chemoattractants. Cells that migrated to bottom wells were quantified. n = 6 mice per genotype. Data are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$ by Mann-Whitney *U* test. (**H**) IRF5 regulates chemokine receptor promoter reporter activity. HEK 293T cells were transiently co-transfected with pGL3b-*CCR2* or *CXCR4* promoter reporters with pCAGEN-mIRF5 or empty vector and pRL. Luciferase activity was measured and normalized to Renilla activity. Data is presented as relative induction of luciferase activity by IRF5 over control empty vector. Data are representative of three independent experiments performed in duplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by unpaired Student *t* test.

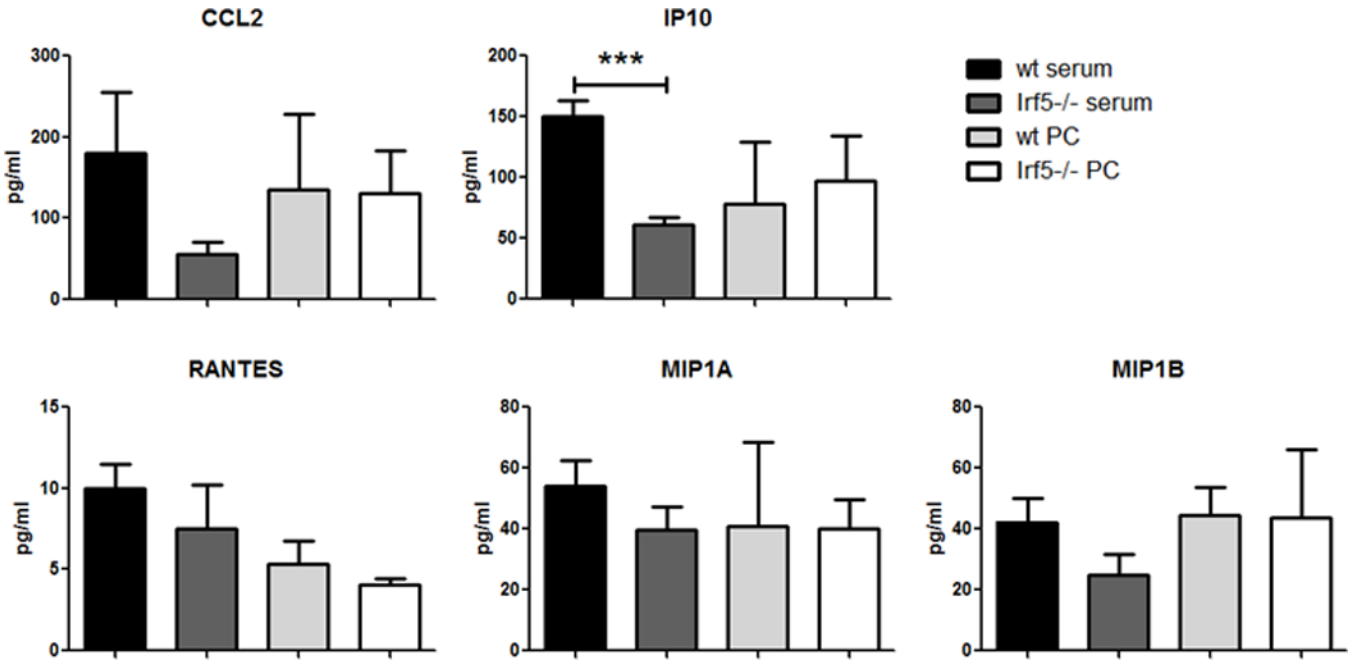

Figure 4. Expression of monocyte-related chemokines is not impaired in the PC of *Irf5*$^{-/-}$ mice

Serum and peritoneal lavage fluid (PC) from *Irf5*$^{+/+}$ (wt) (n = 2–5) and *Irf5*$^{-/-}$ (n = 4–6) littermates were harvested at 2 weeks post-pristane injection. Chemokine levels were determined by beads-based immunoassay. *** $p < 0.001$ by unpaired Student *t* test.

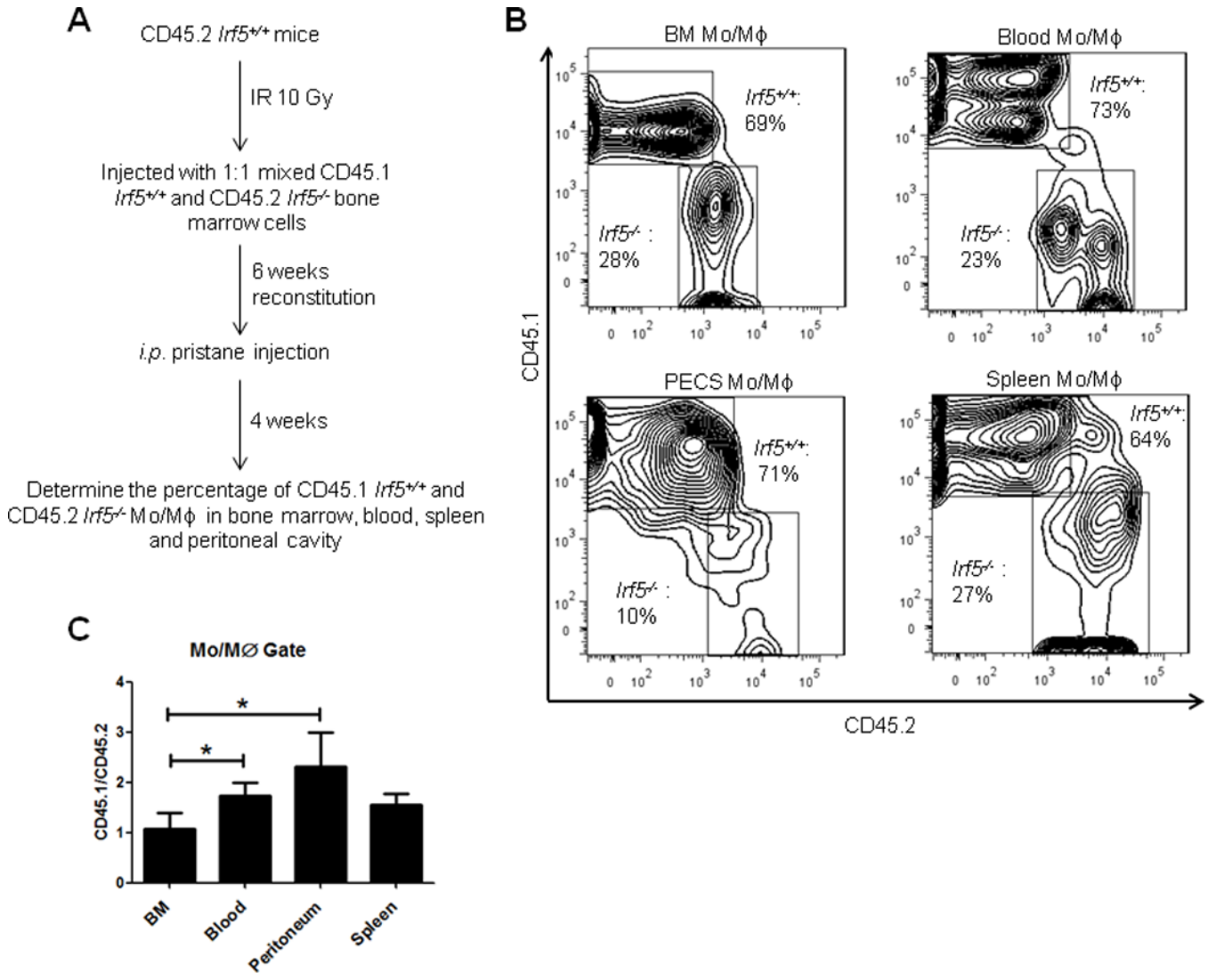

Figure 5. Impaired recruitment of *Irf5*$^{-/-}$ monocytes to the PC of pristane-injected bone marrow chimeras

(**A**) Schematic of bone marrow reconstitution model. (**B**) Representative contour plots showing percentages of CD45.1 *Irf5*$^{+/+}$ and CD45.2 *Irf5*$^{-/-}$ cells in the monocytes/macrophages (Mo/Mϕ) gate in different compartments. (**C**) Ratio of CD45.1 and CD45.2 Mo/Mϕ in the bone marrow (BM), blood, PC (PECs) and spleen of chimerical mice. n = 10 mice; representative of two independent experiments. * $p < 0.05$ by paired Student *t* test.

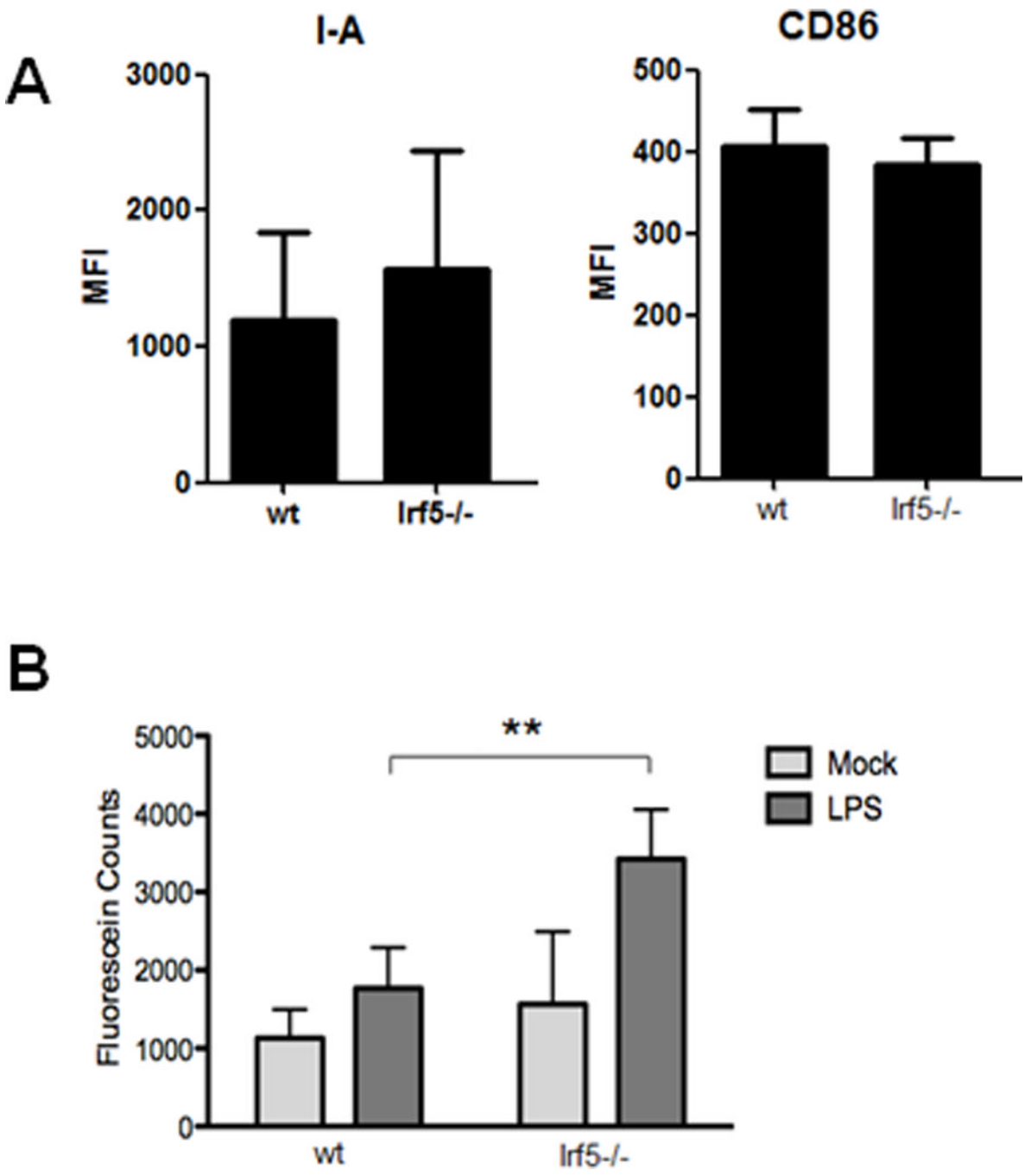

Figure 6. CD11b$^+$ splenic macrophages from *Irf5*$^{-/-}$ mice display enhanced phagocytosis

(**A**) PECs were collected from *Irf5*$^{+/+}$ (*wt*) and *Irf5*$^{-/-}$ littermates 4 wks post-pristane injection. Expression of activation/maturation surface markers I-A (MHC II) and CD86 were examined on CD11b$^+$Ly6G$^-$ monocytes. n = 5 mice per genotype. (**B**) Splenic macrophages from *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ mice were isolated by positive selection and *in vitro* phagocytosis determined by measuring fluorescein counts. n = 4–5 per genotype; $^{**}p < 0.01$ by unpaired Students *t* test.

Table 1

Chemokine receptors differentially expressed on naïve bone marrow monocytes from *Irf5*$^{-/-}$ mice[a].

Gene	Fold Change[b]	Ligands
Ccr1	3.56	RANTES
Cx3cr1	17.31	CX3CL1
Cxcr4	−52.46	CXCL12
Cxcr3	−8.19	IP10
Ccrl1	−5.83	CCL19, CCL21
Ccr5	−3.69	RANTES, MIP1A/B
Ccr2	−2.08	CCL2, CCL7, CCL12

[a]Bone marrow cells were pooled from n=4 *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ littermates to isolate monocytes.

[b]denotes the fold change as compared to naïve *Irf5*$^{+/+}$ monocytes; (+) upregulated or (−) downregulated.