

PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Local apoptosis mediates clearance of macrophages from resolving inflammation in mice

Emmanuel L. Gautier,¹ Stoyan Ivanov,¹ Philippe Lesnik,² and Gwendalyn J. Randolph¹¹Department of Pathology & Immunology, Washington University in St. Louis, St. Louis, MO; and ²Institut National de la Santé et de la Recherche Médicale UMR-S 939, Hôpital de la Pitié, Pavillon Benjamin Delessert, Paris, France

Key Points

- Macrophage migration to lymph nodes during acute inflammation is quantitatively minor.
- Macrophages are cleared from acute inflammation by local death.

Chronic inflammatory diseases such as atherosclerosis are characterized by an accumulation of macrophages. To design therapies that would reduce macrophage burden during disease, understanding the cellular and molecular mechanisms that regulate macrophage removal from sites of resolving inflammation is critical. Although past studies have considered the local death of macrophages or the possibility that they emigrate out of inflammatory foci, methods to quantify death or emigration have never been employed. Here, we applied quantitative competition approaches and other methods to study resolution of thioglycollate-induced peritonitis, the model in which earlier work indicated that emigration to lymph nodes accounted for macrophage removal. We show that migration to lymph nodes occurred in a CC chemokine receptor 7-independent

manner but, overall, had a quantitatively minor role in the removal of macrophages. Blocking migration did not significantly delay resolution. However, when macrophages resistant to death were competed against control macrophages, contraction of the macrophage pool was delayed in the apoptosis-resistant cells. These data refute the concept that macrophages are dominantly cleared through emigration and indicate that local death controls macrophage removal. This finding alters the emphasis on which cellular processes merit targeting in chronic diseases associated with accumulation of macrophages. (*Blood*. 2013;122(15):2714-2722)

Introduction

Resolution of acute inflammation is a crucial step toward return to tissue homeostasis after initiation of tissue injury.¹ In the case of infection, it is self-evident that clearance of the inciting microorganism or microorganisms would be necessary before resolution could be completed, but there are a growing list of health conditions not explicitly associated with infection in which low-grade chronic inflammation is observed, including metabolic diseases (obesity, atherosclerosis), neurodegenerative diseases, and cancers.²⁻⁴ A better understanding of the mechanisms that support resolution of inflammation might facilitate new strategies aimed to combat these diseases.⁵

Initiation of inflammation is typically characterized by early neutrophil recruitment, followed by an influx of monocytes that develop into inflammatory macrophages. The neutrophils undergo death within hours and are cleared by macrophages,^{6,7} with efferocytosis of neutrophils handled by neighboring macrophages. Efferocytosis triggers the production of mediators such as transforming growth factor β 1, which in turn generate macrophages that facilitate resolution and tissue repair.^{8,9} Novel lipid mediators participate critically in control of these steps.⁷ Although there is consensus that the fate of neutrophils in acute inflammation is regulated through local death and efferocytosis,^{6,7} with a small number of neutrophils emigrating to draining lymph nodes (LNs),¹⁰ consensus is still lacking on the fate of monocyte-derived cells that become inflammatory macrophages. Two concepts are apparent in the literature: inflammatory macrophages die locally,¹¹⁻¹⁴ or

alternatively, these cells are primarily cleared by emigration to draining LNs.¹⁵ Although none of the studies is quantitative, the latter concept has received the most attention, cited as a classic in the field (www.nature.com/ni/focus/inflammation/classics/macrophages.html).

Accordingly, we earlier wondered whether the failure of resolution in atherosclerosis¹⁶ might be linked to the failure of macrophages to acquire the appropriate emigratory phenotype.¹⁷ However, although some evidence supports the link between atherosclerosis and emigration to LNs,¹⁸⁻²⁰ emigration from atherosclerotic plaques is not required to promote clearance of macrophages from plaques.²¹ Instead, a combination of macrophage death and cessation of monocyte entry is sufficient for macrophage contraction during disease regression.²¹ These observations and the overall qualitative nature of the previous studies^{11-13,15} led us to reexamine the cellular mechanisms that underlie removal of macrophages from sites of resolving inflammation. We chose to use the model of thioglycollate-induced peritonitis that previously claimed a key role for emigration to LNs and added techniques to quantitatively evaluate emigration and death. We show that emigration to LNs plays only a minor quantitative role in the removal of macrophages relative to a dominant role for local death. Considering that the peritonitis model used in our study was the same as that used in the work claiming that emigration to LNs mediates macrophage removal, our findings indicate that the concept of emigration to LNs as a quantitatively

Submitted January 11, 2013; accepted August 14, 2013. Prepublished online as *Blood* First Edition paper, August 23, 2013; DOI 10.1182/blood-2013-01-478206.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2013 by The American Society of Hematology

significant driver of macrophage clearance no longer holds experimental support.

Methods

Animals and treatments

C57BL/6J mice carrying the Ly5.1 allele (CD45.2) were purchased from The Jackson Laboratories or the National Cancer Institute. CD45.1 C57BL/6 mice were from the National Cancer Institute. CD45.2 and CD45.1 mice were bred to obtain CD45.1 × CD45.2 F1 mice. CC chemokine receptor (CCR)7^{-/-}, CCR2^{-/-}, and Bim^{-/-} mice were obtained from The Jackson Laboratories. Bone marrow cells from CD68-hBcl2 (Mφ-hBcl2) mice²² were used to transplant lethally irradiated mice, as described.²³

Resolving acute peritonitis was induced by intraperitoneal injection of 1 mL sterile thioglycollate (Sigma-Aldrich; 3% weight/volume). Pertussis toxin (PTX; Sigma-Aldrich) was injected intraperitoneally (2.5 μg/mouse). PKH26 Red Fluorescent Cell Linker Kit for Phagocytic Cell Labeling was from Sigma-Aldrich; 200 μL of a 5-μM solution was injected intraperitoneally. Mice were housed in a specific pathogen-free environment and used in accordance with protocols approved by the Animal Care and Utilization Committees at Mount Sinai School of Medicine or Washington University.

Antibodies

Anti-mouse CD115 (AFS98), F4/80 (BM8), CD45 (30-F11), MHC-II (IA-IE, M5/114.15.2), CD45.2 (104), CD45.1 (A20), and CD11c (N418) were from eBiosciences. Anti-mouse Gr-1 (Ly-6C/G, RB6-8C5) and CD36 (HM36) were from Biolegend. Anti-mouse F4/80 (CI:A3-1) was from Serotec. Apoptosis was determined using annexin V after cell surface labeling by following the manufacturer's instructions (Miltenyi Biotec).

Microarray analysis

Male C57BL/6 mice at 6 weeks of age were used for gene expression analysis in macrophages. Inflammatory peritoneal macrophages were identified as CD115⁺ F4/80^{int} B220⁻ MHC-II⁺ or MHC-II⁻ and sorted using a BD FACSAria II cell sorter. Resident peritoneal macrophages, including CD115⁺ F4/80^{hi} MHC-II⁻ B220⁻ and CD115⁺ F4/80^{lo} MHC-II⁺ B220⁻ populations, were sorted at steady state and after thioglycollate injection. Microarrays were carried out as part of the Immunological Genome Project. Flow plots of sorted cells can be found on the Immunological Genome Project website (www.immgen.org). RNA was prepared from sorted populations from C57BL/6J mice after double sorting directly into TRIzol reagent (www.immgen.org/Protocols/Total RNA Extraction with Trizol.pdf). Then it was amplified and hybridized on the Affymetrix Mouse Gene 1.0 ST array. For data analysis, Immunological Genome Project data sets were used. Raw data were normalized using the RMA algorithm. Extensive quality control documents are available on the Immunological Genome Project website (www.immgen.org/Protocols/ImmGen QC Documentation_ALL-DataGeneration_0612.pdf). All data sets have been deposited at the National Center for Biotechnology Information/Gene Expression Omnibus under accession number GSE15907 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15907). Data were analyzed with the GenePattern genomic platform (www.broadinstitute.org/cancer/software/genepattern/). A common threshold for positive expression at 95% confidence across the data set was determined to be 120 (www.immgen.org/Protocols/ImmGen QC Documentation_ALL-DataGeneration_0612.pdf). Differences in gene expression were identified and visualized with the Multiplot module of GenePattern. Clustering analysis considered the 15% most variable genes. Genes were clustered (centered on the mean) with the Hierarchical Clustering module of GenePattern, employing Pearson's correlation as a metric, and data were visualized with the Hierarchical Clustering Viewer heat-map module. Enrichment for gene ontology terms was done using List2Networks software.²⁴

Tissue sample preparation for flow cytometry

Peritoneal exudates were collected in 4 mL cold Hanks' balanced saline solution supplemented with EDTA (0.3 μM final) and bovine serum

albumin (0.06% final) into the peritoneal cavity. LN and omentum cell suspensions were obtained after digestion of the teased organ in collagenase D (Roche) for 30 minutes. Cell suspensions were then stained with appropriate antibodies for 30 minutes on ice. Data were acquired on a BD FACS Canto II or BD Fortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

Adoptive transfer and competition assay

Peritoneal macrophages were retrieved by lavage from donors injected with thioglycollate 5 days earlier. These macrophages were derived from CD45.1 wild-type (WT) mice, CD45.2 CCR7^{-/-} mice, CD68-hBcl2 mice (human Bcl2 cDNA expression driven by the mouse CD68 promoter),²² or CD45.2 Bim^{-/-} mice. Mixtures composed of 1:1 CD45.1 WT macrophage and CD45.2 macrophages from one of the respective CD45.2 strains were made. These mixtures were injected in CD45.1 × CD45.2 F1 recipients that also received thioglycollate 5 days earlier, with a total of 2 to 5 million macrophages from the 1:1 mixture injected into each recipient. Ratios at the time of injection were assessed by flow cytometry on the mixture prepared for injection. At day 8, the peritoneal lavage or LN was analyzed for the presence and ratio of the transferred cells.

Efferocytosis assay

Freshly isolated thymocytes were labeled with carboxyfluorescein diacetate succinimidyl ester (10 μM) and then treated with UV light for 10 minutes, followed by a 3-hour incubation at 37°C. Apoptotic thymocytes (2.5 × 10⁷ per mouse, 40% annexin V⁺) were then injected intraperitoneally in mice that had received thioglycollate 5 days earlier. Peritoneal lavage was performed 30 minutes after carboxyfluorescein diacetate succinimidyl ester-labeled apoptotic thymocyte injection, and uptake was determined by flow cytometry.

Statistical analysis

Data are expressed as mean ± SEM. Statistical differences were assessed using a 2-tailed *t* test or analysis of variance (with Tukey's posttest analysis), using GraphPad Prism software. A *P* value of less than .05 was considered statistically significant.

Results

Resident and inflammatory macrophages during onset and resolution of acute inflammation

On induction of peritonitis after thioglycollate administration intraperitoneally, a prominent recruitment of leukocytes into the peritoneal cavity occurred within 24 hours, as expected,¹⁵ followed by gradual clearance to reach steady-state numbers 8 days later (Figure 1A). Peak leukocyte accumulation at 24 hours was associated with a massive influx of neutrophils (Gr-1⁺ CD115^{lo} F4/80⁻) and eosinophils (Gr-1^{int} CD115^{lo} F4/80⁺) (Figure 1B). Neutrophils were cleared completely within 5 days, whereas eosinophils remained longer (Figure 1B). Macrophages prominently expressed CD115 (Csf-1 receptor). We noted, as previously described,²⁵ that 2 resident macrophage populations were present in the steady-state peritoneum: CD115⁺ F4/80^{lo} CD11c^{lo} CD36⁻ MHC-II⁺ macrophages (5%-10% of all CD115⁺ macrophages) and CD115⁺ F4/80^{hi} CD11c⁻ MHC-II⁻ (90%-95% of all CD115⁺ macrophages; Figure 1C, day 0). These populations are macrophages, as evidenced from gene expression profiling.²⁶

Inflammatory macrophages appeared in the peritoneal cavity after induction of inflammation (Figure 1C; day 1, 5, 8, and 14) and were distinguished from resident macrophage populations by intermediate cell surface levels of F4/80 (Figure 1C). In addition, these inflammatory macrophages (CD115⁺ F4/80^{int}) expressed CD11c and

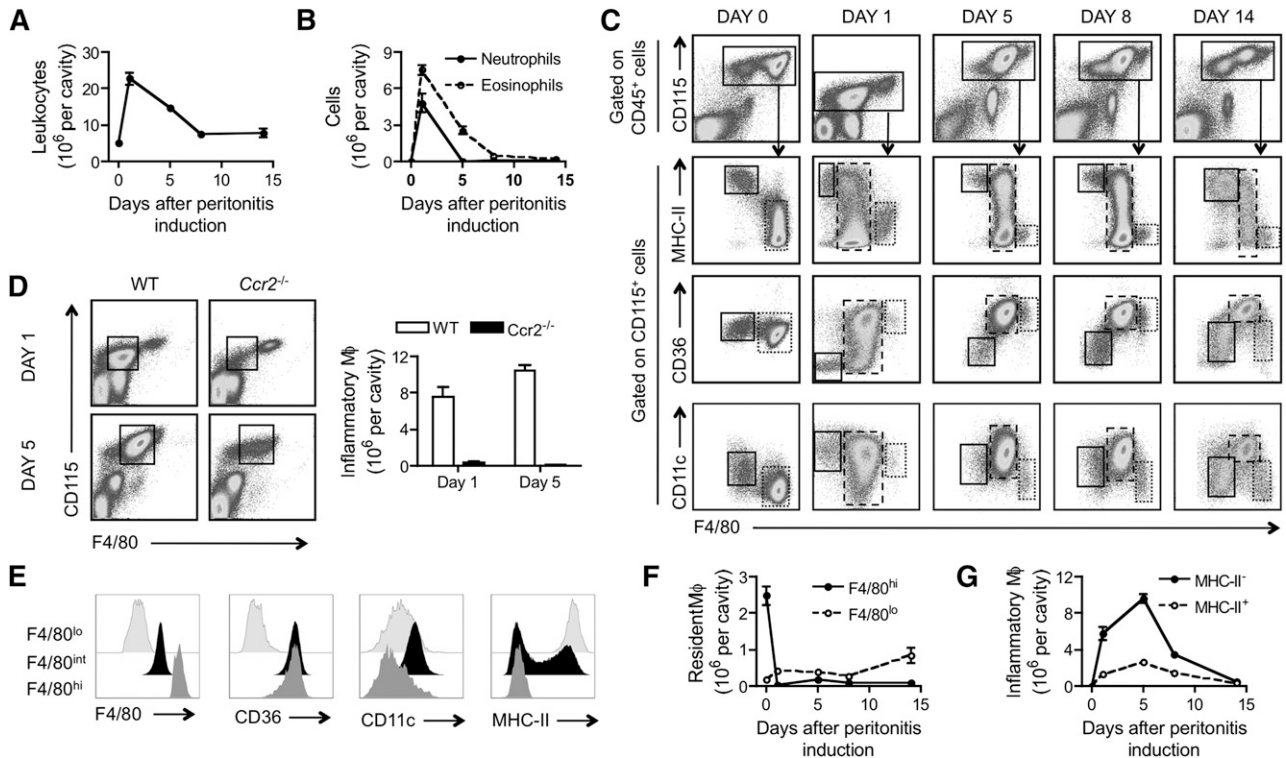


Figure 1. Phenotype and dynamics of resident and inflammatory macrophages during acute peritonitis. Quantification of total leukocytes (A) or granulocytes (neutrophils and eosinophils) (B) in the peritoneal cavity at the steady state and during a 14-day period after i.p. administration of thioglycollate. (C) Fluorescence-activated cell sorter (FACS) plots illustrating the gating strategy used for identification of macrophages at the steady state and during a 14-day period after i.p. administration of thioglycollate. Macrophages were found in CD115^{hi} gates (first row) at all times; CD115 expression on these cells was reduced at day 1. Three macrophage populations were discerned on the basis of F4/80 intensity, and these populations are depicted in the second through the fourth rows of the dot plots. F4/80^{low} macrophages (solid-line gates) and F4/80^{hi} macrophages (finely dotted gates) were resident macrophages, and inflammatory peritoneal macrophages (wide-dashed gates) appeared only in response to thioglycollate. (D) FACS plots illustrating the gating of inflammatory macrophages and corresponding cell counts in the peritoneum of CCR2-deficient mice and controls 1 and 5 days after initiation of peritonitis. (E) Comparison of F4/80, CD36, CD11c, and MHC-II cell surface expression levels between resident (F4/80^{low} and F4/80^{hi}) and inflammatory (F4/80^{int}) macrophages 5 days after initiation of peritonitis. (F) Quantification of resident macrophages and (G) inflammatory macrophages in the peritoneal cavity during the steady state and a 14-day period after i.p. administration of thioglycollate. Data are derived from at least 2 experiments performed with 5 replicates per experimental condition.

CD36. Approximately 20% to 30% of them displayed cell surface expression of MHC-II (Figure 1C). The gene expression profile of these inflammatory cells classified them as macrophages,²⁶ despite expression of CD11c and MHC-II that are also associated with dendritic cells (DCs) and despite their similar secretome to in vitro cultured granulocyte macrophage-CSF-derived DCs.²⁷ Inflammatory F4/80^{int} macrophages likely arose from circulating Ly-6C^{hi} monocytes, as their numbers were approximately 70-fold decreased 5 days after injection of thioglycollate in CCR2^{-/-} mice with reduced circulating levels of Ly-6C^{hi} monocytes²⁸ and deficiency in monocyte trafficking into the inflamed peritoneum²⁹ (Figure 1D). Cell surface expression of CD36 and CD11c distinguished inflammatory macrophages (F4/80^{int}) from the 2 populations of resident macrophages (F4/80^{lo} and F4/80^{hi}) (Figure 1E). Thus, F4/80^{hi} and F4/80^{lo} cells represent tissue resident macrophage populations; whereas F4/80^{int} cells are Ly-6C^{hi} monocyte-derived inflammatory macrophages recruited after induction of peritonitis. As expected,³⁰ the dominant CD115⁺ F4/80^{hi} resident macrophages almost disappeared after initiation of inflammation (Figure 1F). Their numbers remained low at all times studied after thioglycollate was administered, including as long as days 8 to 14, when total leukocyte counts had returned to baseline (Figure 1F). In contrast, CD115⁺ F4/80^{low} resident macrophages were overall unchanged over time (Figure 1F). Both MHC-II⁺ and MHC-II⁻ inflammatory macrophages peaked in magnitude 5 days after injection of thioglycollate. Then they were cleared rapidly between day 5 and 8 (Figure 1G), as

expected.^{15,31} Thus, we chose the window of time between day 5 and 8 as the interval to study events associated with the clearance of inflammatory macrophages.

Gene expression signature of inflammatory macrophages

CD115⁺ F4/80^{int} MHC-II⁻ and CD115⁺ F4/80^{int} MHC-II⁺ cells were separately sorted from mice injected 5 days earlier with thioglycollate and further analyzed using microarray. In addition, gene expression profiles were carried out on resident CD115⁺ F4/80^{lo} MHC-II⁺ and CD115⁺ F4/80^{hi} MHC-II⁻ macrophages during the steady state and 5 days after thioglycollate injection. Clustering analysis of these populations revealed that CD115⁺ F4/80^{lo} macrophages clustered closely together with or without thioglycollate treatment (Figure 2A), and CD115⁺ F4/80^{hi} macrophages also clustered together with or without thioglycollate (Figure 2A). Furthermore, both MHC-II⁺ and MHC-II⁻ monocyte-derived inflammatory macrophages were more similar to each other than to the resident macrophages (Figure 2A). These clustering patterns confirm that cell surface levels of F4/80 for the different macrophage populations remain stable, and therefore are useful as means to track given populations after induction of inflammation with thioglycollate.

Focusing on the recruited inflammatory macrophages, the MHC-II⁺ and MHC-II⁻ populations were overall highly similar. Fewer than 200 genes were significantly different when the 2

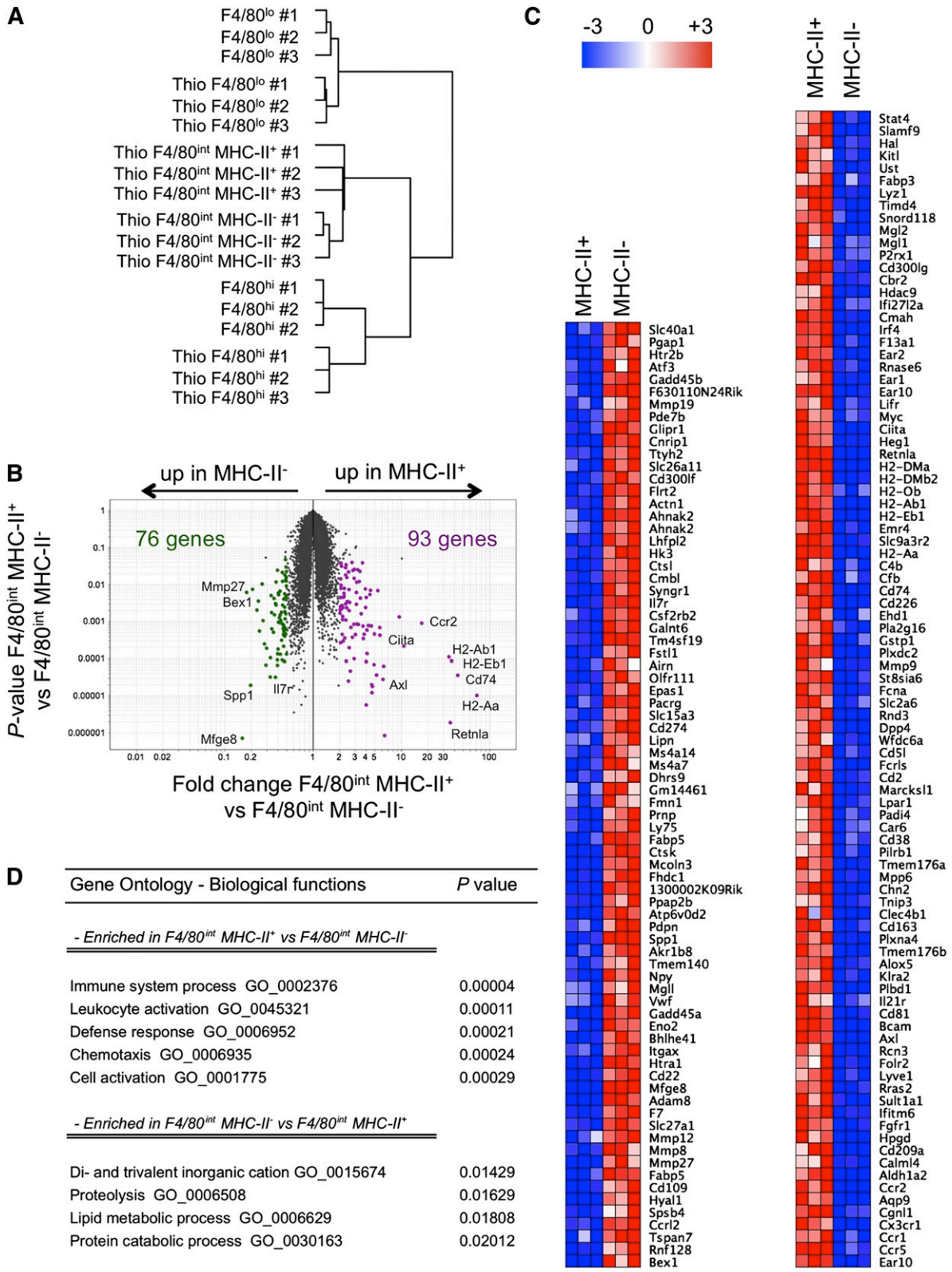


Figure 2. Gene expression analysis of inflammatory macrophage populations. (A) Hierarchical clustering of steady-state and inflammatory macrophage populations (3 replicates for each populations) based on the 15% of genes with the greatest variability. (B) Volcano plot (P value vs fold change, with each dot representing 1 probe set), highlighting the 76 genes upregulated in MHC-II⁻ inflammatory macrophages (green) and the 93 genes upregulated in MHC-II⁺ inflammatory macrophages (purple). Some differentially expressed probe sets were tagged with the gene name they correspond to. (C) Heat map depicting genes differentially expressed ($P < .05$; fold > 2) between MHC-II⁻ and MHC-II⁺ inflammatory macrophages. (D) Pathways associated with differentially expressed genes between MHC-II⁻ and MHC-II⁺ inflammatory macrophages.

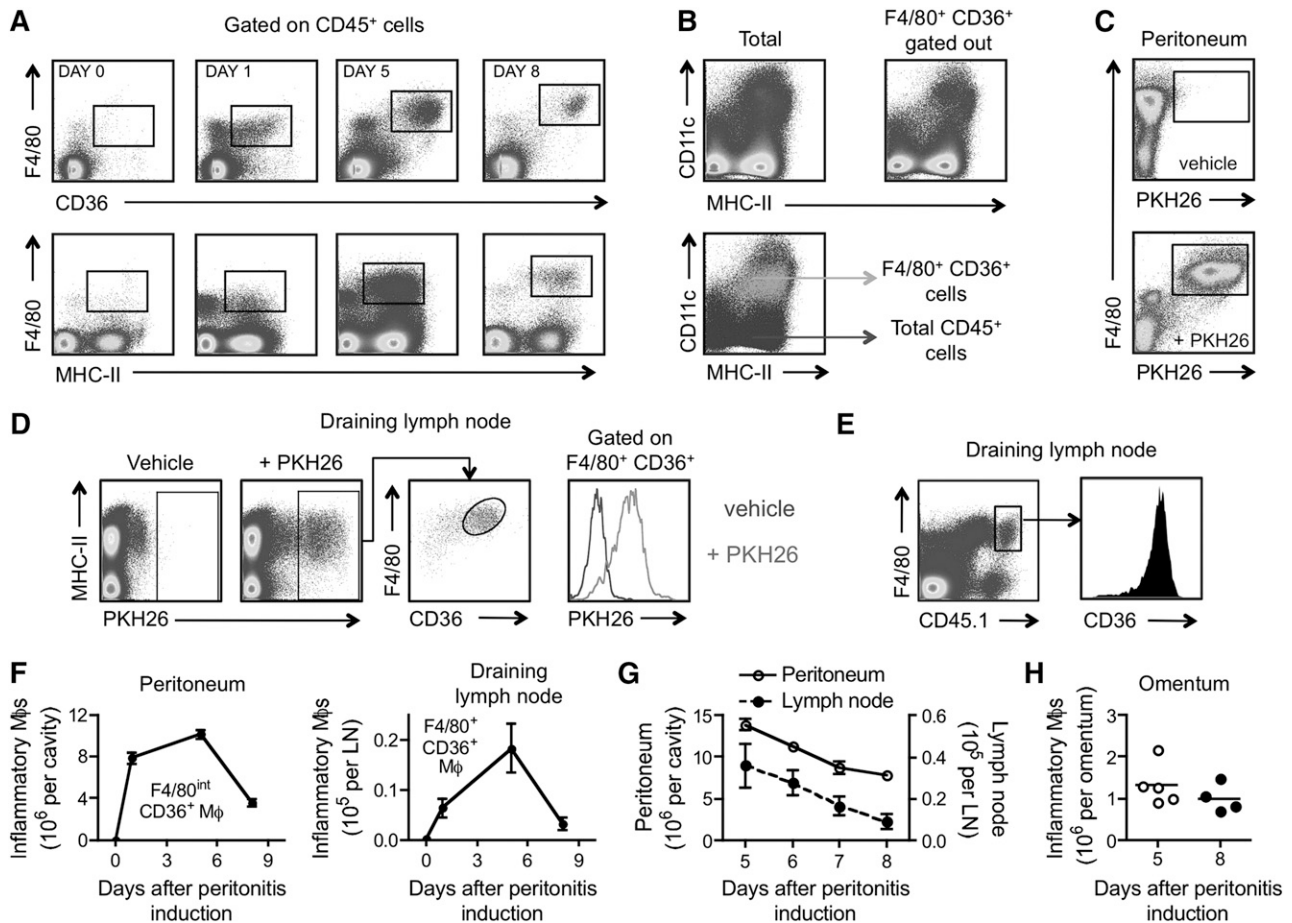


Figure 3. Kinetics of inflammatory macrophage migration to LNs. (A) FACS plots depicting the appearance of inflammatory macrophages ($F4/80^+CD36^+$ or $F4/80^+$ MHC-II $^+$) in the mediastinal LN over the course of 8 days after i.p. administration of thioglycollate. (B) Total LN cells (Left) or total cells with $F4/80^+CD36^+$ cells gated out (Right) based on cell surface expression of CD11c and MHC-II. Bottom plot overlays $F4/80^+CD36^+$ gated cells on total LN cells, plotted to show CD11c vs MHC-II. (C) Labeling of peritoneal inflammatory macrophages 24 hours after i.p. injection of the phagocytic tracer dye PKH26 in mice inflamed 2 days earlier by thioglycollate injection. (D) PKH26 $^+$ cells were identified in the mediastinal LN 3 days after injection of PKH26 in the peritoneal cavity (5 days after inflammation was induced by thioglycollate) and analyzed for F4/80 and CD36 cell surface expression. Far-right plot shows PKH26 levels after gating on all $F4/80^+CD36^+$ LN cells. (E) Inflammatory macrophages were retrieved from CD45.1 mice that had been injected 1 day earlier with thioglycollate. These macrophages were adoptively transferred into CD45.2 mice at the same stage of inflammation. CD45.1 $^+$ inflammatory macrophages were gated in the mediastinal LN cell suspension (left FACS plot) 4 days later and analyzed for CD36 expression. (F-H) Inflammatory macrophages in the peritoneum, draining LN and omentum at different times after induction of peritonitis by thioglycollate ($n = 4-6$ mice per group per time point). Data are representative of at least 2 independent experiments.

populations were compared (\geq twofold; $P < .05$; Figure 2B). The differentially regulated genes between the 2 populations are shown in Figure 2C, and included *Stat4*, *Mgl1* and *Mgl2*, *Irf4*, *Ii21r*, and *Lyve1* upregulated in the MHC-II $^+$ macrophages (Figure 2C), whereas proteases were especially prominent in MHC-II $^-$ macrophages, including genes encoding cathepsins L and K and several metalloproteases (Figure 2C). Pathway analysis of the differentially expressed genes revealed pathways associated with proteolysis and lipid catabolic processes in $F4/80^{int}$ MHC-II $^-$ macrophages (Figure 2D), whereas pathways associated with T-cell activation and migratory behavior were enriched in $F4/80^{int}$ MHC-II $^+$ macrophages (Figure 2D). These data raised the possibility that MHC-II $^+$ inflammatory macrophages were poised to emigrate.

Emigration of macrophages to the LN peaks before the phase of clearance

We used flow cytometry to assess arrival of inflammatory macrophages in the mediastinal LN that drains the peritoneal cavity^{15,32} after intraperitoneal thioglycollate administration. $F4/80^+CD11c^+CD36^+$ MHC-II $^+$ macrophages resembling the inflammatory MHC-II $^+$ macrophages from the peritoneum could be recovered

from the inflamed, but not steady state, mediastinal LN (Figure 3A). The population was also absent from any other LNs tested (inguinal, mesenteric, and brachial) 5 days after induction of peritonitis. These cells fell into a typical DC gate with regard to their expression of CD11c and MHC-II (Figure 3B), but they expressed higher levels of F4/80 and CD36 compared with classical DCs. To confirm that these cells were from the inflamed peritoneal cavity, we injected the phagocytic tracer PKH26 into the thioglycollate-treated peritoneum (Figure 3C). Nearly all PKH26 $^+$ cells in the mediastinal LN were $F4/80^+CD36^+$ (and also CD11c $^+$ and MHC-II $^+$) (Figure 3D). Conversely, when we first gated on $F4/80^+CD36^+$ cells in the LN, they were almost all PKH26 $^+$ (Figure 3D). Transfer of peritoneal cells from thioglycollate-inflamed CD45.1 congenic mice into the peritoneum of thioglycollate-inflamed CD45.2 recipients also demonstrated that inflammatory CD45.1 macrophages migrated from the peritoneum to the mediastinal LN (Figure 3E), and all $F4/80^+CD45.1^+$ cells bore the CD36 $^+$ phenotype (Figure 3E). Thus, $F4/80^+CD36^+$ cells appearing in the mediastinal LN after thioglycollate injection in the peritoneum indeed originated from the peritoneum, and this phenotype included the majority of phagocytic cells that emigrated from the peritoneum.

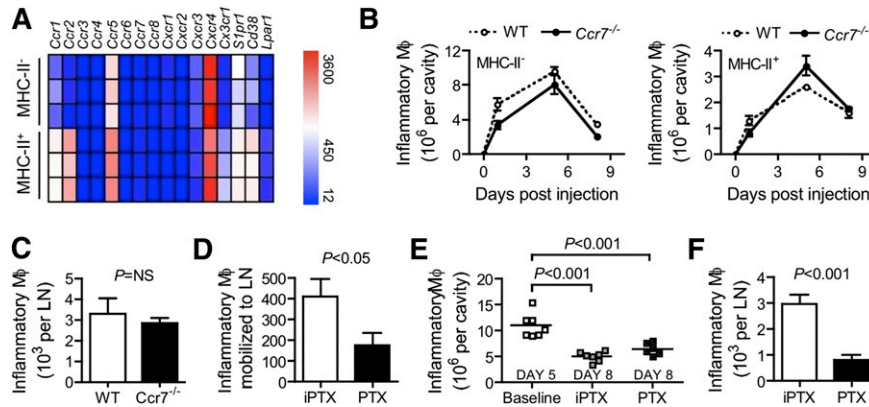


Figure 4. Blocking inflammatory macrophages migration marginally impaired their clearance during resolution. (A) Heat map showing the expression of chemotactic receptors in MHC-II⁻ and MHC-II⁺ inflammatory macrophages 5 days after intraperitoneal administration of thioglycollate (3 replicates were generated for each population). (B) Kinetics of MHC-II⁻ and MHC-II⁺ inflammatory macrophages during the course of thioglycollate-induced peritonitis in CCR7-deficient mice and controls ($n = 5$ mice per group per time). (C) Accumulation of inflammatory macrophage in the mediastinal LN of CCR7-deficient mice and controls 5 days after intraperitoneal administration of thioglycollate ($n = 5$ mice per group). (D) Mobilization to the mediastinal LN of adoptively transferred F4/80⁺ CD36⁺ CD45.1⁺ inflammatory macrophages in CD45.2 mice injected with thioglycollate in the presence of PTX or iPTX ($n = 5$ -7 per group). To maximize the recovery, cells and PTX or iPTX (single injection) were injected in the peritoneum at day 2, and LNs were analyzed at day 5. (E) Baseline number of inflammatory macrophage in the peritoneum at day 5 and numbers of macrophages recovered at day 8 after treatment with either active (PTX) or inactive (iPTX) pertussis toxin at day 5. Each symbol represents one mouse. (F) The number of F4/80⁺ CD36⁺ macrophages recovered in the mediastinal LN at day 8 from the experiment in panel E, illustrating that active pertussis toxin prevented emigration to the LN.

When we used this strategy to identify and quantify macrophages in the mediastinal LN, the magnitude of inflammatory macrophage accumulation in the LN did not increase during the 5- to 8-day window when macrophages were clearing from the peritoneum, as would be expected if the LN were the major depot for the clearing cells. Instead, the accumulation of inflammatory macrophages in the LN roughly paralleled the kinetics of accumulated macrophages in the peritoneum, with a peak accumulation in the LN at day 5 before macrophage clearance from the peritoneum began (Figure 3F). A day-by-day analysis of inflammatory macrophages in the peritoneum and the LN from day 5 to day 8 further revealed a similar rate of decrease at both sites (Figure 3G). There was also no increase in macrophage numbers in the omentum during days 5 to 8, when macrophage numbers contracted (Figure 3H). Thus, macrophages emigrate to mediastinal LNs from the inflamed peritoneum, but the kinetics suggest that the LN is simply sampling a portion of the cells in the peritoneum, rather than serving as the target for removal of macrophages. If the latter were true, a transient increase in macrophages in the LN (or omentum) during the window from day 5 to day 8, when macrophages are lost from the peritoneum, would be expected.

Blocking CCR7-independent, G α i-sensitive macrophage migration to the LN has a quantitatively minimal effect on macrophage contraction

LN-migrating macrophages were uniformly MHC-II⁺. In the peritoneum, we observed a significant enrichment in genes involved in migratory behavior such as chemokine receptors (*Ccr2*, *Ccr5*, *Ccr1*, and *Cx3cr1*), the cyclic adenosine 5'-diphosphate-ribose hydrolase (*Cd38*), and sphingosine-1-phosphate receptor (*Slpr1*), selectively, in F4/80^{int} MHC-II⁺ macrophages (Figures 2C and 4A). *Ccr7* mRNA was not detected in inflammatory macrophages in the peritoneum (Figure 4A). However, *Ccr7* is, with little exception, universally associated with the migration of leukocytes to LNs through lymphatic vessels,^{33,34} including the emigration of monocyte-derived cells from skin to draining LNs.³⁵ Discussion has argued that it would be essential for resolution of inflammation.²⁰ Thus, we tested whether CCR7 was needed for inflammatory macrophage migration to the mediastinal LN and resolution.

We injected CCR7-deficient animals with thioglycollate and followed the accumulation and clearance of inflammatory macrophages in the peritoneum. Other than a minor shift toward a slightly increased proportion of MHC-II⁺ macrophages (Figure 4B), there was no difference in their accumulation or clearance during the resolution phase compared with WT controls (Figure 4B). The numbers of inflammatory macrophages that migrated to the LN at day 5 (peak macrophage accumulation) were similar between *Ccr7*^{-/-} mice and WT controls (Figure 4C), illustrating that CCR7 is unnecessary for LN homing of macrophages. Chemokine receptors selectively expressed by MHC-II⁺ inflammatory macrophages (Figure 4A) are all coupled to G α i proteins, and are thus subject to inhibition by PTX. Thus, we adoptively transferred CD45.1⁺ inflammatory macrophages in CD45.2⁺ recipients and locally injected PTX or PTX inactivated by boiling (iPTX) simultaneously. PTX treatment substantially reduced migration of inflammatory macrophages to the mediastinal LN compared with treatment with iPTX (Figure 4D), revealing that emigration to the draining LN was dependent on G α i-mediated signals. However, cohorts of mice injected with PTX (compared with iPTX) at the onset of resolution at day 5 had only a nonsignificant trend toward an increased number of inflammatory macrophages (+25%) that were retained in the peritoneum at day 8 (Figure 4E), even though we recovered few inflammatory macrophages from the LNs of the same PTX-treated mice (Figure 4F). PTX did not affect inflammatory macrophage survival, but it increased their proliferation (supplemental Figure 1A-B, available on the Blood Web site), although the percentage of proliferating cells was very low, which might explain the nonsignificant 25% increase in the number of inflammatory macrophages observed at day 8 in the PTX-treated group (Figure 4E). Thus, macrophage migration to LNs fails to account quantitatively for removal of macrophages during the phase of intense macrophage contraction observed during resolution.

Apoptosis of inflammatory macrophages promotes macrophage clearance during resolution

A key step necessary to allow for macrophage clearance is the shut-down in the recruitment of their precursors, circulating Ly-6C^{hi} monocytes.³¹ The recruitment of neutrophils (gate ii, defined as Gr-1⁺

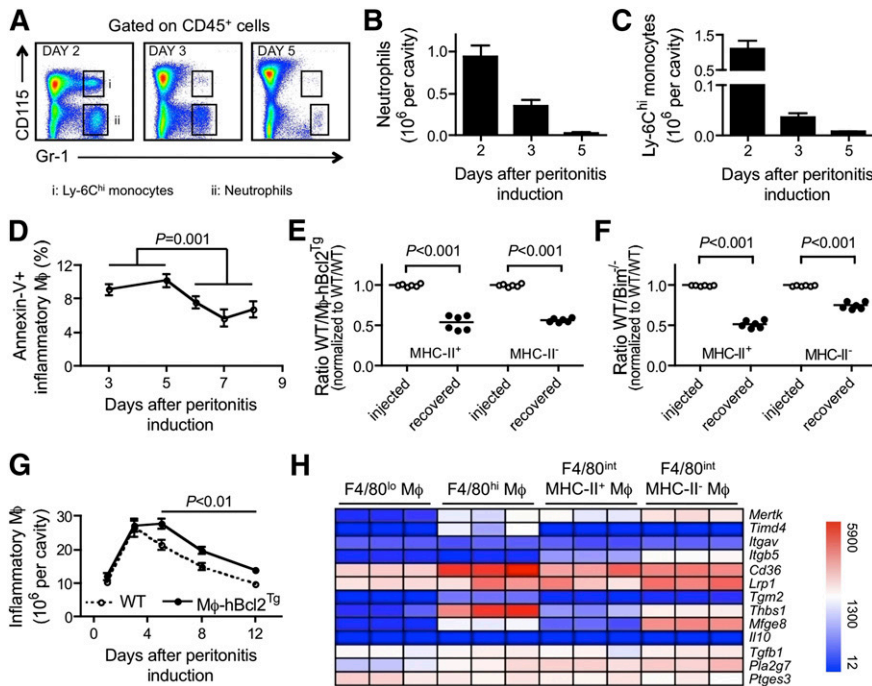


Figure 5. Inflammatory macrophages contraction during resolution is controlled by apoptotic cell death. (A) FACS plot illustrating the gating strategy used for neutrophils (ii, CD115⁻ Gr-1⁺) and Ly-6C^{hi} monocytes (i, CD115⁺ Gr-1⁺) in the inflamed peritoneal cavity. Quantification of neutrophils (B) as well as Ly-6C^{hi} monocytes (C) up to day 5 in mice injected with thioglycollate (n = 5 per time). (D) Quantification of annexin V staining in inflammatory macrophages from day 3 to 8 after intraperitoneal administration of thioglycollate (n = 5 per time). (E-F) Ratios of F4/80^{int} inflammatory macrophages competed after injection into thioglycollate-inflamed peritoneum at day 5 (injected) and recovered at day 8 (recovered). Two competitions are shown: between macrophages derived from WT and CD68-Bcl2 (Mφ-hBcl2^{Tg}) transgenic mice and between macrophages derived from WT and Bim^{-/-} mice. Each symbol represents data from 1 mouse. (G) Quantification of F4/80^{int} inflammatory macrophages in the peritoneum of irradiated recipient mice transplanted with bone marrow from CD68-Bcl2 (Mφ-hBcl2^{Tg}) or WT mice during a 12-day period after i.p. administration of thioglycollate. (H) Heat maps depict gene expression patterns of mRNA transcripts that mediate or are induced in response to efferocytosis. Three replicates are shown for different macrophage populations.

CD115⁻) and Ly-6C^{hi} monocytes (gate i, Gr-1⁺ CD115⁺) gradually and dramatically decreased before the onset of resolution (Figure 5B-C). However, cessation in recruitment alone could not account for a net reduction in macrophage numbers. Thus, we wondered whether concomitant local death would promote macrophage contraction between days 5 and 8 after intraperitoneal thioglycollate injection. F4/80^{int} inflammatory macrophages recovered from the peritoneum showed annexin V⁺–positive cells, even as early as day 3 (Figure 5D). Annexin V⁺–reactive macrophages were relatively more abundant, approaching 8% at early stages of resolution, and were present at all times studied (Figure 5D). To obtain a quantitative sense of the importance of apoptosis in macrophage clearance, we induced peritonitis using thioglycollate in 3 cohorts of mice, including CD45.1 WT mice, CD45.1 × CD45.2 F1 hybrid WT mice, and CD45.2 transgenic mice containing a transgene in which the CD68 promoter was used to drive expression of the antiapoptotic factor Bcl2.²² In all cohorts of mice, we injected thioglycollate and maintained the mice until day 5. Macrophages from CD45.1 WT and CD45.2 CD68-Bcl2 transgenic mice were mixed 1:1 and then immediately transferred to the site of peritonitis in the CD45.1 × CD45.2 F1 WT mice. We then recovered cell suspensions of the peritoneal lavage at day 8. CD68-Bcl2 transgenic (Tg) macrophages cleared less efficiently from the peritoneum than nontransgenic WT cells, such that in the 3-day period of analysis, the Bcl2-overexpressing macrophages represented two-thirds of the transferred macrophages (Figure 5E). As expected, overexpression of Bcl2 reduced, although it did not eliminate, death by apoptosis (supplemental Figure 2A); it did not affect proliferation (supplemental Figure 2B).

When we repeated this experiment, substituting CD68-Bcl2 transgenic macrophages with macrophages from Bim^{-/-} mice, the same outcome was observed, in which Bim^{-/-} macrophages that are partially resistant to death³⁶ came to dominate over WT macrophages locally in the peritoneum during resolution of thioglycollate-induced peritonitis (Figure 5F).

Next we performed a detailed kinetic analysis of F4/80^{int} inflammatory macrophages in irradiated recipient mice repopulated with bone marrow from CD68-Bcl2 transgenic or WT mice during

the course of thioglycollate-induced peritonitis. The appearance of inflammatory macrophages in the peritoneal cavity was comparable between both genotypes until day 3 (Figure 5G). However, macrophages from CD68-Bcl2 transgenic mice showed no contraction between days 3 and 5, whereas WT macrophages contracted (Figure 5G). Accumulation in draining LNs was comparable in both groups (supplemental Figure 2C). Together, these data indicate that local death accounts for macrophage contraction during inflammatory resolution and further suggest that efficient efferocytosis would be crucial to promote resolution. Using our microarray data, we probed the expression of key molecules involved in apoptotic cell clearance, as well as the response to efferocytosis (*Tgfb1*, *Pla2g7*, *Ptges3*) (Figure 5H). These data revealed that F4/80^{hi} resident and F4/80^{int} inflammatory macrophages seemed overall better-equipped than F4/80^{lo} resident macrophage for efferocytic function, as they expressed higher levels of *Merk*, *Cd36*, and *Lrp1*, for example. This was confirmed experimentally after injecting exogenous apoptotic cells in vivo into the peritoneum of mice injected 5 days earlier with thioglycollate (supplemental Figure 3). Finally, all macrophages expressed *Tgfb1*, but not *Il10*, as well as the genes encoding the enzymes producing the lipid mediators PAF (*Pla2g7*) and PGE2 (*Ptges3*), all of which are induced in response to efferocytosis.³⁷

Discussion

Resolution of inflammation is a key homeostatic response to injury critical to maintain tissue integrity. However, the fundamental mechanisms underlying the removal of infiltrated inflammatory macrophages are poorly understood. For decades, different studies have argued variably for local apoptosis,^{11–13} akin to the mechanism removing neutrophils,^{6,7} vs emigration to LNs¹⁵ as the fundamental process governing macrophage removal. Although in fact only a single study, that of Bellingan and colleagues,¹⁵ concluded that emigration to LNs dominated, the conclusions of this single study have taken hold in the field. Others argued that CD36³⁸ and CD11b^{39–41}

could modulate macrophage migration and thereby delay resolution, but they used a model of “resolution” that is triggered by lipopolysaccharide (LPS),³⁹ even though LPS promotes, rather than resolves, inflammation. The current study used the model of thioglycollate-induced peritoneal inflammation similarly to Bellingan and colleagues, although seemingly minor differences existed, including volume of injection and supplier. Local apoptosis can be difficult to appreciate because of the rapid and seemingly “invisible” clearance of apoptotic cells at the histological level. We got around this problem using a competitive approach that would allow us to quantify whether the ability to die (or emigrate) conferred a disadvantage to resolution. Although a low level of emigration of macrophages to LNs occurred during resolution of thioglycollate-induced inflammation, the concept that emigration is either required for or serves as the major mechanism in removing macrophages from resolving inflammatory lesions failed to stand. Instead, the fundamental mechanism most relevant to removal of macrophages is none other than local apoptosis. This revised concept will now need to be tested, using other models of inflammation in the peritoneum as well as in other tissues, but resolution of inflammation in the heart already appears to follow the paradigm we observe here.⁴²

We argue that the straightforward body of work presented here is of fundamental importance with respect to how data in the literature are interpreted. Indeed, the Bellingan study has strongly influenced the interpretation and design of many studies. For example, a few years ago, we discussed how impaired macrophage emigration out of atherosclerotic vessels could negatively affect the course of atherosclerosis if this process were required for resolution.^{17,43,44} However, when we observed that macrophage contraction from plaques did not depend on emigration out of the plaque environment,²¹ we were prompted to return to studies of acute inflammation and reconsider how macrophages are cleared therein. We then recognized that the study of Bellingan and colleagues was generally qualitative and needed further investigation. Other studies have discussed their own findings with interpretations that reflect an assumption that the conclusions of Bellingan and colleagues are correct.^{10,20} Indeed, lipid mediators that promote resolution, including resolvins and protectins, increase migration of macrophages to LNs,¹⁰ but it remains unclear whether this is their dominant means for controlling macrophage burden. It would now be valuable to evaluate the role of resolvins and protectins, along with other mediators associated with macrophage removal, such as CD36³⁸ and netrin,²⁰ in monocyte recruitment and macrophage apoptosis. Although the implications of our work include the idea that macrophage apoptosis is beneficial to the resolution of inflammation, we recognize that in a context such as atherosclerosis, the failure to clear apoptotic bodies through efferocytosis can significantly diminish the benefits of macrophage death and even contribute to disease pathology.⁴⁵ Along these lines, developing mouse models that would allow for inducible and macrophage-specific alterations of efferocytic pathways would be useful to probe the requirement of this process in favoring silent inflammatory macrophage removal during resolution.

Even if the majority of macrophages are not cleared via emigration to LNs through lymphatic vessels, lymphatic transport is still a vital route for resolution of inflammation. For example, the decoy chemokine receptor D6 that sequesters and removes proinflammatory chemokines⁴⁶ is primarily expressed on lymphatic vessels.⁴⁷ Furthermore, removal of soluble mediators through the lymph is critical for resolution.⁴⁷ Indeed, in the context of atherosclerosis, our recent work revealed that cholesterol loaded onto high-density lipoproteins is cleared from tissues, including the artery wall, through the lymphatic vasculature, suggesting that

intact lymphatic drainage would be important in the resolution of atherosclerosis.⁴⁸ Furthermore, although our data suggest that macrophage emigration to LNs during resolution of inflammation does not quantitatively prevail in the removal of macrophages and that the emigration is not otherwise essential for resolution (or else pertussis toxin would have delayed resolution), there is likely some relevance to the migration that does occur. For instance, emigratory macrophages may contribute to antigen presentation in the LN that, for pathogens, may be essential for mounting a response needed to ultimately eradicate the cause of the inflammatory response in the first place.³¹ All emigratory macrophages in the LNs were MHC-II⁺, and their MHC-II⁺ counterparts in the peritoneum expressed many genes relevant to antigen presentation. Although they bore many features of dendritic cells, these cells could not be classified as dendritic cells by gene expression profiling^{26,49}; instead, their expression profile is that of a macrophage. Genes associated with migration to LNs strongly cluster with dendritic cells, including CCR7, and did not appear to be induced in inflammatory macrophages. CCR7 also mediates the emigration of T cells³⁴ and neutrophils⁵⁰ to LNs from the skin, so we suspected it would govern the emigration of inflammatory macrophages from the peritoneum to the mediastinal LN. However, surprisingly, we found that CCR7 was not required for this step. This instance is one of only few documented cases in which emigration to LNs is CCR7-independent. Another example is found in chronic skin inflammation.³⁴ Thus, if CCR7 is a mediator of resolution, as recently proposed,^{19,20} its role in resolution may not directly relate to a role in macrophage migration from the inflammatory environment.

In closing, this study revises the conclusions of an earlier influential body of work and, in so doing, alters the fundamental cellular pathways that merit strong consideration for therapeutics that seek to recapitulate the events that promote resolution of inflammation.

Acknowledgments

The authors thank Gerald Morris for help with mice irradiation. The authors thank the Immunological Genome Project for microarray and bioinformatics support associated with this work.

This work was funded by National Institutes of Health grants R01 AI049653 and R01 AI061741 (to G.J.R.). E.G. was supported in part by a postdoctoral fellowship from the American Heart Association, Heritage Affiliate (10POST4160140). The Immunological Genome Project is funded by R24 AI072073 to Christophe Benoist (Harvard Medical School). Earlier studies included in this body of work were carried out at the Mount Sinai School of Medicine before the laboratory relocated to the Washington University School of Medicine.

Authorship

Contribution: E.L.G. designed and conducted experiments and wrote the manuscript; S.I. conducted experiments; P.L. provided key reagents; and G.J.R. designed and supervised experiments and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Gwendalyn J. Randolph, Department of Pathology & Immunology, Washington University School of Medicine, 660 South Euclid Ave, Campus Box 8118, St. Louis, MO 63110; email: grandolph@path.wustl.edu.

References

- Buckley CD, Gilroy DW, Serhan CN, Stockinger B, Tak PP. The resolution of inflammation. *Nat Rev Immunol*. 2013;13(1):59-66.
- Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*. 2010;140(6):900-917.
- Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140(6):883-899.
- Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. *Cell*. 2010;140(6):918-934.
- Nathan C, Ding A. Nonresolving inflammation. *Cell*. 2010;140(6):871-882.
- Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat Rev Immunol*. 2002;2(10):787-795.
- Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol*. 2008;8(5):349-361.
- Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest*. 2001;108(7):957-962.
- Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF- β 1 secretion and the resolution of inflammation. *J Clin Invest*. 2002;109(1):41-50.
- Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature*. 2007;447(7146):869-874.
- Tidball JG, St Pierre BA. Apoptosis of macrophages during the resolution of muscle inflammation. *J Leukoc Biol*. 1996;59(3):380-388.
- Lan HY, Mitsuhashi H, Ng YY, Nikolic-Paterson DJ, Yang N, Mu W, Atkins RC. Macrophage apoptosis in rat crescentic glomerulonephritis. *Am J Pathol*. 1997;151(2):531-538.
- Kuhlmann T, Bitsch A, Stadelmann C, Siebert H, Brück W. Macrophages are eliminated from the injured peripheral nerve via local apoptosis and circulation to regional lymph nodes and the spleen. *J Neurosci*. 2001;21(10):3401-3408.
- Janssen WJ, Barthel L, Muldrow A, et al. Fas determines differential fates of resident and recruited macrophages during resolution of acute lung injury. *Am J Respir Crit Care Med*. 2011;184(5):547-560.
- Bellingan GJ, Caldwell H, Howie SE, Dransfield I, Haslett C. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol*. 1996;157(6):2577-2585.
- Merched AJ, Ko K, Gotlinger KH, Serhan CN, Chan L. Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. *FASEB J*. 2008;22(10):3595-3606.
- Randolph GJ. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. *Curr Opin Lipidol*. 2008;19(5):462-468.
- Llodrá J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci USA*. 2004;101(32):11779-11784.
- Trojan E, Feig JE, Dogan S, et al. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci USA*. 2006;103(10):3781-3786.
- van Gils JM, Derby MC, Fernandes LR, et al. The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. *Nat Immunol*. 2012;13(2):136-143.
- Potteaux S, Gautier EL, Hutchison SB, et al. Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of ApoE^{-/-} mice during disease regression. *J Clin Invest*. 2011;121(5):2025-2036.
- Gautier EL, Huby T, Witztum JL, et al. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation*. 2009;119(13):1795-1804.
- Gautier EL, Huby T, Ouzilleau B, et al. Enhanced immune system activation and arterial inflammation accelerates atherosclerosis in lupus-prone mice. *Arterioscler Thromb Vasc Biol*. 2007;27(7):1625-1631.
- Lachmann A, Ma'ayan A. Lists2Networks: integrated analysis of gene/protein lists. *BMC Bioinformatics*. 2010;11:87.
- Ghosn EE, Cassado AA, Govoni GR, et al. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci USA*. 2010;107(6):2568-2573.
- Gautier EL, Shay T, Miller J, et al; Immunological Genome Consortium. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol*. 2012;13(11):1118-1128.
- Becker L, Liu NC, Averill MM, et al. Unique proteomic signatures distinguish macrophages and dendritic cells. *PLoS ONE*. 2012;7(3):e33297.
- Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol*. 2006;7(3):311-317.
- Tsou CL, Peters W, Si Y, et al. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest*. 2007;117(4):902-909.
- Barth MW, Hendrzak JA, Melnicoff MJ, Morahan PS. Review of the macrophage disappearance reaction. *J Leukoc Biol*. 1995;57(3):361-367.
- Gautier EL, Chow A, Spanbroek R, et al. Systemic analysis of PPAR γ in mouse macrophage populations reveals marked diversity in expression with critical roles in resolution of inflammation and airway immunity. *J Immunol*. 2012;189(5):2614-2624.
- Kool M, Soullié T, van Nimwegen M, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*. 2008;205(4):869-882.
- Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol*. 2005;5(8):617-628.
- Brown MN, Fintushel SR, Lee MH, et al. Chemoattractant receptors and lymphocyte egress from extralymphoid tissue: changing requirements during the course of inflammation. *J Immunol*. 2010;185(8):4873-4882.
- Qu C, Edwards EW, Tacke F, et al. Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med*. 2004;200(10):1231-1241.
- Bouillet P, Metcalf D, Huang DC, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 1999;286(5445):1735-1738.
- Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- β , PGE $_2$, and PAF. *J Clin Invest*. 1998;101(4):890-898.
- Park YM, Febbraio M, Silverstein RL. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest*. 2009;119(1):136-145.
- Kataru RP, Jung K, Jang C, et al. Critical role of CD11b⁺ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood*. 2009;113(22):5650-5659.
- Gomez IG, Tang J, Wilson CL, et al. Metalloproteinase-mediated Shedding of Integrin β 2 promotes macrophage efflux from inflammatory sites. *J Biol Chem*. 2012;287(7):4581-4589.
- Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. *Eur J Immunol*. 2011;41(2):366-379.
- Leuschner F, Rauch PJ, Ueno T, et al. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopenia. *J Exp Med*. 2012;209(1):123-137.
- Qin C, Nagao T, Grosheva I, Maxfield FR, Pierini LM. Elevated plasma membrane cholesterol content alters macrophage signaling and function. *Arterioscler Thromb Vasc Biol*. 2006;26(2):372-378.
- Pagler TA, Wang M, Mondal M, et al. Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circ Res*. 2011;108(2):194-200.
- Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*. 2010;10(1):36-46.
- Fra AM, Locati M, Otero K, et al. Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol*. 2003;170(5):2279-2282.
- Lee KM, Nibbs RJ, Graham GJ. D6: the 'crowd controller' at the immune gateway. *Trends Immunol*. 2013;34(1):7-12.
- Martel C, Li W, Fulp B, et al. Lymphatic vasculature mediates macrophage reverse cholesterol transport in mice. *J Clin Invest*. 2013;123(4):1571-1579.
- Miller JC, Brown BD, Shay T, et al; Immunological Genome Consortium. Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol*. 2012;13(9):888-899.
- Beauvillain C, Cunin P, Doni A, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood*. 2011;117(4):1196-1204.