Neutrophil and Macrophage Cell Surface Colony-Stimulating Factor 1 Shed by ADAM17 Drives Mouse Macrophage Proliferation in Acute and Chronic Inflammation

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ABSTRACT Macrophages are prominent cells in acute and chronic inflammatory diseases. Recent studies highlight a role for macrophage proliferation post-monocyte recruitment under inflammatory conditions. Using an acute peritonitis model, we identify a significant defect in macrophage proliferation in mice lacking the leukocyte transmembrane protease ADAM17. The defect is associated with decreased levels of macrophage colony-stimulating factor 1 (CSF-1) in the peritoneum and is rescued by intraperitoneal injection of CSF-1. Cell surface CSF-1 (csCSF-1) is one of the substrates of ADAM17. We demonstrate that both infiltrated neutrophils and macrophages are major sources of csCSF-1. Furthermore, acute shedding of csCSF-1 following neutrophil extravasation is associated with elevated expression of iRhom2, a member of the rhomboid-like superfamily, which promotes ADAM17 maturation and trafficking to the neutrophil surface. Accordingly, deletion of hematopoietic iRhom2 is sufficient to prevent csCSF-1 release from neutrophils and macrophages and to prevent macrophage proliferation. In acute inflammation, csCSF-1 release and macrophage proliferation are self-limiting due to transient leukocyte recruitment and temporally restricted csCSF-1 expression. In chronic inflammation, such as atherosclerosis, the ADAM17-mediated lesional macrophage proliferative response is prolonged. Our results demonstrate a novel mechanism whereby ADAM17 promotes macrophage proliferation in states of acute and chronic inflammation.

KEYWORDS ADAM17, iRhom2, inflammation, CSF-1, macrophages, cell proliferation

Macrophages are among the most prominent innate immune cells in chronic inflammatory diseases, as well as in acute inflammation (1–7). Studies of chronic inflammation using murine atherosclerosis models have revealed that macrophage proliferation within atherosclerotic lesions, rather than monocyte recruitment from the blood, dominates macrophage accumulation in established lesions (5–8). In obesity-associated adipose tissue inflammation, and in an acute zymosan-induced peritonitis model, significant macrophage proliferation was also evident (6, 7). These studies all highlight a role for macrophage proliferation as an important mechanism for expansion of the macrophage population in tissue inflammation.

Macrophage colony-stimulating factor 1 (CSF-1) is a potent stimulator of macrophage survival, proliferation, and differentiation (4), and accordingly, intravenous injection of CSF-1 into mice increases circulating monocyte numbers and macrophages

Received 1 March 2018 **Returned for modification** 26 March 2018 **Accepted** 6 June 2018

Accepted manuscript posted online 11 June 2018

Citation Tang J, Frey JM, Wilson CL, Moncada-Pazos A, Levet C, Freeman M, Rosenfeld ME, Stanley ER, Raines EW, Bornfeldt KE. 2018. Neutrophil and macrophage cell surface colony-stimulating factor 1 shed by ADAM17 drives mouse macrophage proliferation in acute and chronic inflammation. *Mol Cell Biol* 38:e00103-18. <https://doi.org/10.1128/MCB.00103-18>.

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in various peripheral areas (9, 10). CSF-1 exists in 3 biologically active isoforms: a membrane-spanning cell surface form (csCSF-1) and 2 secreted forms, namely, the glycoprotein CSF-1 (sgCSF-1) and the proteoglycan CSF-1 (spCSF-1), which circulate in the bloodstream (11). It is generally believed that circulating CSF-1 is synthesized primarily by endothelial cells and selectively maintains certain macrophage populations, including Kupffer cells, whereas csCSF-1 is synthesized locally in tissues by stromal, epithelial, and endothelial cells to regulate macrophage populations in these tissues (11, 12). *Csf1^{op}/Csf1^{op}* mice, which exhibit an inactivating mutation in the *Csf1* gene, have gross deficiencies in macrophage numbers and effector functions (13, 14). CSF-1 exerts its biological functions through the CSF-1 receptor (CSF-1R, or CD115), a type III receptor tyrosine kinase encoded by the *Csf1r* (*c-fms*) gene (15). Gene targeting of the *Csf1r* locus essentially phenocopies the deficiencies of the *Csf1^{op}/Csf1^{op}* mouse (16). The CSF-1R is preferentially expressed on cells of the mononuclear phagocyte system, and CSF-1 binding to the CSF-1R triggers receptor dimerization and autophosphorylation, CSF-1 internalization, and activation of key downstream signaling pathways, leading to cell survival and proliferation (17, 18). The extent of CSF-1-dependent local macrophage proliferation and its contributions to peripheral tissue macrophage accumulation seem to be tissue dependent and are not fully understood (7, 8, 10, 19–21).

The protease ADAM17 is a member of a disintegrin and metalloprotease (ADAM) family that has been shown to cleave and activate many cell surface proteins involved in inflammatory responses (22–25). Identified ADAM17 substrates include adhesion molecules, chemokines, cytokines, and their receptors, such as tumor necrosis factor alpha (TNF- α), TNF receptor 1 (TNF-R1), TNF-R2, csCSF-1, and CSF-1R (26–30). Thus, ADAM17 could be an important regulator of inflammatory processes, as well as of macrophage proliferation, through the generation of soluble TNF- α and soluble CSF-1 (sCSF-1) and/or by regulating their respective receptor densities. ADAM17 is constitutively expressed by most cells, and global deletion of ADAM17 is embryonically lethal in mice (24). Therefore, conditional-knockout mice have served as essential tools to assess ADAM17 functions in inflammation, tissue remodeling, and regenerative responses (31, 32). By using hematopoietic cell-specific deletion of ADAM17, we have previously reported that ADAM17 plays important roles in multiple stages of inflammatory responses, including the regulation of initial neutrophil influx into the peritoneal cavity after thioglycolate injection (27), monocyte transmigration under different inflammatory conditions (33, 34), and the regulation of macrophage uptake of apoptotic cells (35). We have shown that these regulatory functions of ADAM17 are mediated by cleavage of different substrates, such as L-selectin, integrins, and the scavenger receptor CD36, but mechanisms controlling ADAM17 proteolysis of specific substrates under different inflammatory conditions are still poorly understood. Recent studies have identified the rhomboid-like protein iRhom2, encoded by *Rhbf2*, as an essential regulator of the maturation of ADAM17 in hematopoietic cells and of the rapid activation of ADAM17 proteolytic cleavage of some substrates, such as TNF- α (36–41).

We therefore explored the roles of ADAM17 and iRhom2 in macrophage proliferation in the acute thioglycolate-induced peritonitis model, as well as in a chronic inflammatory atherosclerosis model. Our results show that inflammatory neutrophils and macrophages are both sources of csCSF-1 and that csCSF-1 is shed by ADAM17 in an iRhom2-dependent manner during inflammation. This ADAM17-mediated release of soluble CSF-1 plays an important role in driving macrophage proliferation under inflammatory conditions.

RESULTS

Leukocyte ADAM17 promotes macrophage proliferation in acute inflammation. To test the hypothesis that leukocyte surface proteins released by ADAM17 regulate inflammatory macrophage proliferation, we used the thioglycolate-induced acute peritonitis model in hematopoietic chimeric mice repopulated by wild-type (WT) or ADAM17-null bone marrow. We compared leukocyte accumulation after injection of

FIG 1 Hematopoietic deletion of ADAM17 causes a decrease in macrophage accumulation and a decrease in transient macrophage proliferation in thioglycolate-induced acute peritonitis. (A) Number of peritoneal macrophages from *Adam17*^{+/+} or *Adam17*^{-/-} hematopoietic chimeras collected 48 h after injection of thioglycolate; *n* = 5. The experiment was repeated 5 times. (B) Peritoneal macrophages with or without administration of BrdU 1 h before harvest were evaluated for BrdU incorporation and surface expression of different markers. The gating scheme to eliminate neutrophils and eosinophils is shown. Macrophages that were positive or negative for BrdU were further evaluated by surface markers F4/80, CD11b, CD115, Ly6C, and 7-aminoactinomycin D (7-AAD). (C) Time course of macrophage proliferation (BrdU incorporation) in elicited peritoneal macrophages. *n* = 8 at 24 h; *n* = 9 at 40 h; *n* = 10 at 48 h; *n* = 5 at 64 and 72 h. (D) Percentages of macrophages from wild-type (*Adam17*^{+/+}) and *Adam17*^{-/-} chimeras in S phase at 24, 40, and 64 h after thioglycolate injection. *, *P* < 0.01 versus wild-type controls. (E) Percentages of S phase macrophages in 50/50 mixed hematopoietic chimeras done as for panel D; *n* = 5. The experiment was repeated 3 times. Values are expressed as means ± SEM.

the sterile irritant thioglycolate into the peritoneal cavities of the chimeras. Our previous report demonstrated that ADAM17-dependent shedding of L-selectin regulates initial neutrophil influx but does not alter early monocyte recruitment in this model (27). We also showed that macrophage numbers in the peritoneum were comparable at 4, 12, and 24 h post-thioglycolate injection in wild-type and *Adam17*^{-/-} hematopoietic chimeras (27). However, in the present study, we found a 30% reduction in macrophage accumulation in *Adam17*^{-/-} chimeras at 48 h post-thioglycolate injection (Fig. 1A). We hypothesized that there might be significant local macrophage proliferation after initial infiltration at the site of inflammation in this model. Thus, we quantified macrophages in the S phase of the cell cycle at 24, 40, 64, and 72 h post-thioglycolate injection by administration of bromodeoxyuridine (BrdU) 1 h before harvest and analyzed the cells by flow cytometry. Peritoneal cells were stained for the markers Ly6G and Siglec F to gate out neutrophils and eosinophils, respectively, and

gated on F4/80⁺ single cells to assess BrdU incorporation in the macrophage population. Representative flow cytometry data collected 40 h after thioglycolate injection are presented in Fig. 1B. Our data show that while there are very few macrophages in S phase at 24 h, at 40 h after thioglycolate injection, ~30% of macrophages are in S phase in wild-type chimeras (Fig. 1C). In this acute inflammation model, macrophage proliferation is transient, peaking at 40 h post-thioglycolate injection and diminishing rapidly to baseline by 72 h (Fig. 1C). In the absence of hematopoietic ADAM17, this proliferation peak is reduced by 40.5% (Fig. 1D). Consistent with the known characteristics of inflammatory macrophages (5, 8), the proliferating cells are F4/80^{hi}, CD115^{hi}, Ly6C^{hi}, and CD11b^{hi} (Fig. 1B). Next, we examined whether the decrease in macrophage proliferation in *Adam17*^{-/-} chimeras is cell autonomous. We generated mixed hematopoietic chimeras in which lethally irradiated recipients were repopulated with 50% wild-type (CD45.1) and 50% ADAM17-null (CD45.2) bone marrow cells to test the two genotypes in the same local milieu. Our data show that the percentages of wild-type and ADAM17-null S phase macrophages in mixed chimeras did not differ (Fig. 1E), indicating that wild-type cells had released a soluble factor that normalized the environment and that the reduced macrophage proliferation in ADAM17-null chimeras is not cell intrinsic. Thus, ADAM17 must mediate the transient release of a soluble factor that is critical in stimulating macrophage proliferation in acute inflammation.

Soluble CSF-1, a cleavage product of ADAM17, promotes macrophage proliferation in the peritonitis model. Since CSF-1 is a potent stimulus of macrophage proliferation and the cell surface isoform, csCSF-1, depends on ADAM17 cleavage to release its soluble form (29), we examined levels of sCSF-1 in peritoneal fluid at 4, 12, 24, and 48 h by enzyme-linked immunosorbent assay (ELISA). In wild-type hematopoietic chimeras, sCSF-1 peaked at 12 h after thioglycolate injection, and its level was still appreciable at 24 h, the time points that precede macrophage proliferation, while *Adam17*^{-/-} chimeras showed significantly lower sCSF-1 levels at each time point in their cavities (Fig. 2A). To test whether the significant decrease in sCSF-1 levels in the peritoneal cavities of *Adam17*^{-/-} chimeras was caused by local or systemic responses post-thioglycolate injection, serum sCSF-1 levels were measured by ELISA. In contrast to the decrease in local sCSF-1 concentrations in the peritoneal cavities of *Adam17*^{-/-} chimeras, sCSF-1 in circulation remained largely unchanged (Fig. 2B). Thus, the local sCSF-1 response, rather than circulating systemic CSF-1, is regulated by ADAM17 in this inflammatory model. To test whether soluble CSF-1 is the critical factor that promotes macrophage proliferation, we injected 10 ng recombinant CSF-1 per mouse cavity at 8 h after thioglycolate injection. Administering extraneous CSF-1 rescued not only the defect in macrophage accumulation at 48 h post-thioglycolate injection (Fig. 2C) but also macrophage proliferation at this time point (Fig. 2D) in ADAM17-null chimeras. Furthermore, to exclude the possibility of a role for CSF-1 in promoting macrophage survival in the peritoneal cavity, we assessed macrophage apoptosis in the CSF-1 rescue experiments. We detected very low levels of macrophage apoptosis with or without exogenous CSF-1 in the cavity, and there were no differences in the percentages of apoptotic macrophages in wild-type and *Adam17*^{-/-} chimeras, ruling out a major role of soluble CSF-1 in macrophage survival at this time point in this model (Fig. 2E). Because ADAM17 is also known to shed the CSF-1 receptor (30), we next assessed F4/80⁺ macrophage surface CSF-1R expression by CD115 staining and flow analysis at 4, 12, and 24 h post-thioglycolate injection, the time points prior to the peak in macrophage proliferation (Fig. 3A). ADAM17-deficient macrophages exhibited higher surface CD115 expression at all time points tested, but CD115 expression was barely detectable at 4 h and 12 h post-thioglycolate injection on both wild-type and ADAM17^{-/-} peritoneal macrophages. By 24 h post-thioglycolate injection, CD115 expression was markedly elevated on macrophages compared with the earlier time points, suggesting this could be the time when macrophages increase CSF-1R signaling and internalization of CSF-1. ADAM17-null macrophages showed 3-fold higher CD115 mean fluorescence intensity compared to the wild-type macrophages at this time point (Fig. 3A), confirming that ADAM17 is involved in shedding of surface CD115 and that the

FIG 2 Transient macrophage proliferation is dependent on ADAM17-mediated generation of soluble CSF-1 in acute inflammation. (A) Soluble CSF-1 in peritoneal fluid from wild-type (*Adam17*^{+/+}) and *Adam17*^{-/-} hematopoietic chimeras by ELISA. *n* = 11, *n* = 12, *n* = 6, and *n* = 5 for *Adam17*^{+/+} chimeras and *n* = 9, *n* = 5, *n* = 5, and *n* = 5 for *Adam17*^{-/-} chimeras, evaluated at 4, 12, 24, and 48 h. *, *P* < 0.02; **, *P* = 0.0012, ***, *P* < 0.0001 for *Adam17*^{+/+} versus *Adam17*^{-/-}. (B) CSF-1 in circulation in *Adam17*^{+/+} and *Adam17*^{-/-} hematopoietic chimeras. *n* = 6, *n* = 5, *n* = 5, *n* = 5, and *n* = 5 for *Adam17*^{+/+} chimeras and *n* = 11, *n* = 5, *n* = 5, *n* = 10, and *n* = 10 for *Adam17*^{-/-} chimeras evaluated at 0, 4, 12, 24, and 48 h post-thioglycolate injection. *, *P* < 0.02 by two-way ANOVA followed by Sidak's multiple-comparison test. (C to E) Effects of exogenous CSF-1 (10 ng/cavity 8 h post-thioglycolate i.p. injection) on *Adam17*^{+/+} and *Adam17*^{-/-} hematopoietic chimeras. (C) Macrophage number (48 h; *n* = 6). **, *P* = 0.0015 versus *Adam17*^{+/+}. (D) Percentages of cells in S phase (48 h). (E) Numbers of apoptotic cells (annexin V/propidium iodide at 40 h). Values are expressed as means ± SEM.

CSF-1/CSF-1R pathway is not innately defective in *Adam17*^{-/-} macrophages. In addition to csCSF-1 and CSF-1R, tumor necrosis factor alpha (TNF- α) is an important ADAM17 substrate that plays major roles in inflammatory responses (22, 23). Indeed, soluble TNF- α in the peritoneal cavity was reduced by over 90% in ADAM17-null chimeras (Fig. 3B), which is consistent with ADAM17 being the major sheddase of TNF- α . We considered whether the cytokine could indirectly alter macrophage proliferation. To address this possibility, we injected recombinant TNF- α into the peritoneal cavities of ADAM17 hematopoietic chimeras and assessed macrophage proliferation. Exogenous TNF- α was unable to rescue the proliferative defect in ADAM17-null chimeras (Fig. 3C). These findings demonstrate that ADAM17-mediated cleavage of csCSF-1 controls macrophage proliferation in thioglycolate-induced acute inflammation.

Neutrophils and macrophages are major transient sources of soluble CSF-1 in acute peritonitis. It is known that stromal, epithelial, and endothelial cells are major sources of CSF-1 (12), but our chimeras lack ADAM17 only in hematopoietic cells. Because ADAM17 cleavage appears to be restricted to substrates on the same cell (42), this suggests that hematopoietic cells express csCSF-1. It has been reported that CSF-1 mRNA can be detected in hematopoietic cells, including neutrophils, in mice (43). At 12 h post-thioglycolate injection, the most abundant cell type within the peritoneal cavity is the neutrophil (Fig. 4A), and this is also the time when maximal release of sCSF-1 occurs (Fig. 2A). We therefore investigated whether csCSF-1 protein is detectable on leukocytes in the cavity at 12 h post-thioglycolate injection. To analyze the cell surface

FIG 3 CSF-1 receptor is not decreased on ADAM17-null macrophages, and another ADAM17 substrate, TNF- α , is not involved in regulating macrophage proliferation. (A) Cell surface CD115 expression on F4/80⁺ peritoneal macrophages was determined by flow cytometry at different times post-thioglycolate injection. $n = 5$ per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus *Adam17*^{+/+} at the same time point. The data are representative of the results of 3 experiments. MFI, mean fluorescence intensity. (B) Peritoneal fluid was collected 4 h after thioglycolate injection from *Adam17*^{+/+} and *Adam17*^{-/-} hematopoietic chimeras, and TNF- α levels were determined by ELISA ($n = 5$). (C) *Adam17*^{+/+} and *Adam17*^{-/-} hematopoietic chimeras received either PBS or TNF- α (1 ng/cavity) 4 h after thioglycolate injection, and peritoneal cells were collected at 48 h and analyzed by flow cytometry; $n = 5$. The experiment was repeated 2 times. *, $P < 0.05$ versus *Adam17*^{+/+}. Values are expressed as means and SEM.

CSF-1 expression by flow cytometry, we first confirmed the CSF-1 antibody specificity by staining peritoneal cells of CSF-1^{-/-} chimeras. While the antibody clearly detected a positive CSF-1 signal over IgG control on both wild-type and ADAM17-null neutrophils and macrophages at 12 h post-thioglycolate injection, the CSF-1-null cells showed no positive cell surface staining (Fig. 4B), confirming the specificity of the anti-CSF-1 antibody. Among all the leukocytes tested, inflammatory neutrophils and macrophages exhibited marked positive cell surface CSF-1 immunoreactivity, whereas lymphocytes and eosinophils did not (Fig. 4C). As expected, ADAM17-null neutrophils showed higher cell surface CSF-1 expression than ADAM17 wild-type neutrophils 12 h post-thioglycolate injection (Fig. 4D). Thus, inflammatory neutrophils and macrophages are major sources of surface CSF-1 protein in the thioglycolate-induced peritonitis model.

As csCSF-1 can be stored in intracellular membrane compartments and transported to the cell surface to be cleaved by ADAM17 (29), we next asked whether intracellular CSF-1 could be detected in neutrophils and macrophages by flow cytometry (Fig. 5A to D). Comparison of cell surface CSF-1 levels with total CSF-1 levels following cellular permeabilization showed that peritoneal neutrophils contain significant intracellular CSF-1 (total CSF-1 was approximately 3 times that of csCSF-1) (Fig. 5C), while there was no difference between total and cell surface CSF-1 in macrophages (Fig. 5D). This finding suggests that the neutrophil is a potential continuous supplier of csCSF-1. Neutrophils contain granules to store proteinases and antimicrobial peptides, and upon

FIG 4 Neutrophils and macrophages are major sources of csCSF-1 in acute inflammation. (A) Types of peritoneal cells (neut, neutrophils; macs, macrophages; lymphs, lymphocytes; eos, eosinophils) in wild-type mice collected 12 h after thioglycolate injection. The data are representative of the results of 5 experiments. (B) The specificity of the CSF-1 antibody used in flow cytometry was verified by the absence of staining of CSF-1-null neutrophils and macrophages. (C) Levels of csCSF-1 on peritoneal cells from wild-type mice 12 h after thioglycolate injection (IgG, goat IgG). The ratio of the mean fluorescence intensity (MFI) of csCSF-1 to IgG is shown above each cell type; $n = 5$. The experiment was repeated 3 times. (D) Levels of csCSF-1 on *Adam17*^{+/+} and *Adam17*^{-/-} neutrophils 12 h after thioglycolate injection; $n = 5$. Values are expressed as means and SEM.

activation, neutrophils undergo regulated degranulation to exert their host defense functions (44). It is likely that CSF-1 could be stored in structures such as these granules that macrophages lack. Our data indicate that before thioglycolate injection, the resting-state neutrophils and monocytes in circulation do not express csCSF-1 (Fig. 5E). It is known that the noninflamed peritoneal cavity does not contain neutrophils, and we show that resident peritoneal macrophages do not express csCSF-1 (Fig. 5E). At 4 h post-thioglycolate injection, a majority of resident macrophages exit the cavity, and newly infiltrated neutrophils are the predominant cells. These newly infiltrated neutrophils also do not express detectable csCSF-1. At 12 h post-thioglycolate injection, there are significant numbers of infiltrated neutrophils and macrophages (Fig. 4A), and csCSF-1 expression reaches its peak (Fig. 5E). By 24 h, macrophage and neutrophil csCSF-1 levels are reduced to 10% and 18% of the 12-h levels, respectively, and is only barely detectable in neutrophils by 48 h (Fig. 5E). Thus, soluble CSF-1 shed from csCSF-1 by ADAM17 is restricted to early stages of acute inflammation by transient csCSF-1 expression and release, which is in direct accordance with the transient macrophage proliferation detected in this acute model of inflammation (Fig. 1C).

ADAM17-mediated release of CSF-1 from neutrophils is partly responsible for inflammatory macrophage proliferation. To evaluate the contribution of neutrophil-released sCSF-1, partial depletion of neutrophils was achieved by systemic injection of a neutrophil neutralization antibody against Ly6G (45, 46). A 62.5% depletion of

FIG 5 Expression of CSF-1 in inflammatory neutrophils and macrophages is transient in the acute peritonitis model. (A and B) Representative histograms for total CSF-1 and control IgG staining in permeabilized peritoneal cells from wild-type mice 12 h after thioglycolate injection for neutrophils (A) and macrophages (B). The anti-CSF-1/control IgG MFI ratios were 10.2 for neutrophils and 5.3 for macrophages. (C and D) Surface versus total CSF-1 in peritoneal cells. An overlay of a representative histogram of surface csCSF-1 (fixed cells) and total CSF-1 (permeabilized cells) is shown for peritoneal cells 12 h after thioglycolate injection. (C) Neutrophils. (D) Macrophages. (E) Kinetics of csCSF-1 expression on peritoneal neutrophils and macrophages from wild-type mice. Shown is the anti-CSF-1/control IgG MFI ratio, with a ratio of 1.0 representing no detectable expression (dashed line). The CSF-1-to-IgG ratio in circulating blood neutrophils and monocytes is indicated as T0 blood. *, $P < 0.05$; **, $P < 0.01$ compared to time zero. The data presented are for $n = 5$ per group, repeated in 3 different experiments. Values are expressed as means and SEM.

neutrophils by the Ly6G antibody injection, compared with a control IgG (Fig. 6A), was sufficient to decrease peritoneal sCSF-1 levels by 44% at 12 h post-thioglycolate injection (Fig. 6B). Furthermore, the reduction in sCSF-1 was accompanied by a 20% decrease in macrophage proliferation at 40 h (Fig. 6C). Thus, our data suggest that

FIG 6 Neutrophil ADAM17 is responsible for the generation of soluble CSF-1 and the regulation of macrophage proliferation. (A to C) Deletion of neutrophils by anti-Ly6G antibody injection in wild-type mice (150 μ g/mouse; retro-orbital administration 8 h prior to thioglycolate injection). (A) Number of peritoneal neutrophils (12 h after thioglycolate injection; 7/4 Ly6B antibody; flow analysis). (B) Soluble CSF-1 levels in peritoneal fluid (PLF) (ELISA). (C) Peritoneal macrophage proliferation (40 h after thioglycolate injection; Vybrant DyeCycle Violet stain). Values are expressed as means and SEM; $n = 5$ for panels A to C.

FIG 7 Absence of iRhom2 restrains ADAM17-mediated release of csCSF-1 and diminishes inflammatory macrophage proliferation. (A) Representative Western blots of iRhom2 and ADAM17 (20 μ g protein/lane) from neutrophils purified from bone marrow and the peritoneum. (B) Quantification of protein levels from panel A normalized to 24-h levels ($n = 3$). (C) CSF-1 levels (ELISA) in lysates of isolated neutrophils presented as the fold increase compared to the bone marrow lysates. The data are representative of the results of 3 experiments. (D) Numbers of peritoneal neutrophils and macrophages from *Adam17*^{+/+} and *Adam17*^{-/-} hematopoietic chimeras, along with *iRhom2*^{+/+} and *iRhom2*^{-/-} hematopoietic chimeras, at 12 h post-thioglycolate injection; $n = 5$. The data are representative of the results of at least 3 experiments. (E) TNF- α levels in peritoneal fluid from *iRhom2*^{+/+} and *iRhom2*^{-/-} chimeras 4 h after thioglycolate injection (ELISA); $n = 5$. (F) CSF-1 levels in peritoneal fluid from *iRhom2*^{+/+} and *iRhom2*^{-/-} chimeras at 4 h and 12 h after thioglycolate injection (ELISA); $n = 5$. (G) Peritoneal macrophage proliferation (BrdU incorporation) in *iRhom2*^{+/+} and *iRhom2*^{-/-} chimeras (40 h post-thioglycolate injection). Values are expressed as means and SEM.

ADAM17 in neutrophils is responsible for a significant component of the macrophage proliferation during acute inflammation, with the remaining part most likely contributed by ADAM17 in macrophages.

iRhom2 is required for the ADAM17-mediated CSF-1 cleavage that promotes macrophage proliferation. ADAM17 cleavage of certain substrates is dependent upon iRhom2 (36–41). To determine if iRhom2 is required for shedding of csCSF-1 from neutrophils, we first evaluated iRhom2 expression in neutrophil populations. We found that iRhom2 was barely detectable in bone marrow neutrophils, but it was elevated significantly once neutrophils trafficked into the inflammatory peritoneal cavity, especially at 12 and 24 h after thioglycolate injection (Fig. 7A and B). Concurrently, total CSF-1 protein in peritoneal neutrophil lysates was elevated post-thioglycolate injection compared to the unchallenged bone marrow neutrophil lysate assayed by ELISA (Fig. 7C). To test the function of iRhom2 *in vivo* in the same system, we generated iRhom2-null and control wild-type hematopoietic chimeras to analyze in the thioglycolate-induced peritonitis model. As the peritoneal TNF- α level peaks at 4 h post-thioglycolate injection and quickly returns to baseline at 8 h (47), we tested the soluble TNF- α in the cavity at 4 h in iRhom2 chimeras (Fig. 7E). Also, as soluble CSF-1 is detectable at 4 h and peaks at 12 h post-thioglycolate injection (Fig. 2A), we assayed CSF-1 at 4 h and 12 h (Fig. 7F) post-thioglycolate injection in iRhom2 chimeras.

FIG 8 *iRhomb2* is required for ADAM17 maturation and transit to the cell surface in neutrophils and macrophages. Total levels of pro-ADAM17 were evaluated in lysates without PNGase F treatment. To detect active cell surface ADAM17, peritoneal cells from *iRhomb2*^{+/+} or *iRhomb2*^{-/-} chimeras were surface biotinylated, followed by purification of biotinylated proteins on neutravidin and PNGase F treatment. Note that active ADAM17 is absent in *iRhomb2*^{-/-} hematopoietic chimeras. Analysis of lysates and surface biotinylation by Western analysis are shown for peritoneal neutrophils 12 h after thioglycolate injection (A) and peritoneal macrophages 96 h after thioglycolate injection (B). Peritoneal neutrophil lysates and surface biotinylation are shown separately because of different exposure times (3 and 10 min, respectively).

Despite comparable recruitment of neutrophils into the cavities of wild-type and *iRhomb2*-null chimeras (Fig. 7D), both peritoneal TNF- α and sCSF-1 levels (Fig. 7E and F) and macrophage proliferation (Fig. 7G) were significantly reduced in *iRhomb2*^{-/-} chimeras, recapitulating the defects seen in ADAM17-null hematopoietic chimeras. Furthermore, deletion of hematopoietic *iRhomb2* prevented cell surface expression of neutrophil and macrophage ADAM17 (Fig. 8), confirming the critical role of *iRhomb2* in regulating ADAM17 availability for shedding csCSF-1 in this model. Thus, our data show that *iRhomb2* is required for ADAM17-mediated release of sCSF-1 from neutrophils and macrophages and that this release in turn is required for macrophage proliferation.

ADAM17 is required for macrophage proliferation in lesions of atherosclerosis.

The expression of ADAM17 has been detected within atherosclerotic lesions in mice (48) and in humans (49). A recent study reported that when ADAM17 was deleted from myeloid cells in mice, there was a significant decrease in lesional macrophage area (50). We therefore decided to use mouse atherosclerosis as a chronic inflammation model to investigate the role of ADAM17 in macrophage proliferation. LDL receptor (LDLR)-deficient mice fed a high-fat diet develop atherosclerosis, and it has been reported that their lesion macrophages undergo proliferation (1, 2, 5). To evaluate the effect of ADAM17 deletion on macrophage proliferation in atherosclerotic lesions, lethally irradiated *Ldlr*^{-/-} mice were repopulated with either wild-type or *Adam17*^{-/-} bone marrow cells to generate hematopoietic chimeras. The *Ldlr*^{-/-} hematopoietic mice were fed a high-fat, high-cholesterol diet for 16 weeks to induce atherosclerotic lesion formation, as reported previously (5). Serum cholesterol levels did not differ between wild-type and ADAM17-null chimeras at the end of the experiment (Table 1). To analyze macrophages and neutrophils in the artery wall by flow cytometry, the entire mouse aortic tree from the root to the iliac bifurcation was used to generate single-cell preparations (51). Macrophages and neutrophils were analyzed in aortic cell preparations by flow cytometry. The gating strategy is shown in Fig. 9A. We observed no difference in arterial neutrophil numbers between wild-type and ADAM17-null chimeric mice (Fig. 9B). In contrast, macrophage numbers were decreased by 35% in ADAM17-null hematopoietic chimeras (Fig. 9C). Furthermore, using Vybrant DyeCycle Violet staining, we detected a 33% decrease in lesion macrophage proliferation in ADAM17-null hematopoietic chimeras (Fig. 9D). Our data therefore indicate that ADAM17 is also an important regulator in a chronic inflammatory condition such as atherosclerosis,

TABLE 1 Serum cholesterol levels in wild-type (WT) and ADAM17-null hematopoietic chimeras in LDLR-deficient mice fed a high-fat diet

Expt no.	<i>Ldlr</i> ^{-/-} hematopoietic ADAM17 chimera genotype	<i>n</i>	Cholesterol (mg/dl) $\pm$ SEM	<i>P</i> value
1	WT	4	520 $\pm$ 40	0.144
	ADAM17 null	4	437 $\pm$ 30	
2	WT	8	416 $\pm$ 25	0.864
	ADAM17 null	8	411 $\pm$ 16	
3	WT	4	685 $\pm$ 49	0.247
	ADAM17 null	4	536 $\pm$ 114	

likely through regulating macrophage proliferation. Due to the continued influx of inflammatory neutrophils and macrophages during atherogenesis, we postulate ADAM17-mediated cleavage of csCSF-1 is persistent rather than transient, as in the acute peritonitis model (Fig. 10).

Together, our data demonstrate that ADAM17-mediated release of CSF-1 from inflammatory neutrophils and macrophages drives macrophage proliferation in states of inflammation and that this regulatory function of ADAM17 is dependent on iRhom2.

DISCUSSION

Using the thioglycolate-induced acute peritonitis and the high-fat diet-induced chronic atherosclerosis models, we have identified significant defects in macrophage proliferation in mice lacking leukocyte ADAM17. We demonstrated that both neutrophils and macrophages are major sources of csCSF-1 during acute inflammation and, furthermore, that shedding of csCSF-1 by ADAM17 following neutrophil extravasation is associated with elevated expression of iRhom2, a member of the rhomboid-like superfamily, which promotes ADAM17 maturation and trafficking to the neutrophil surface. Accordingly, deletion of hematopoietic iRhom2 is sufficient to prevent csCSF-1 release from neutrophils and macrophages and recapitulates the macrophage proliferation defect seen in ADAM17 deficiency. In acute inflammation, csCSF-1 release and macrophage proliferation are self-limiting due to transient leukocyte recruitment and temporally restricted csCSF-1 expression. In chronic inflammation, such as atherosclerosis, the ADAM17-mediated lesional macrophage proliferative response is likely prolonged. The results of this study reveal a novel mechanism whereby ADAM17 promotes macrophage proliferation in states of inflammation through the shedding of csCSF-1 from neutrophils and macrophages.

Although ADAM17 has been previously reported to play important roles in multiple stages of inflammation, the finding that ADAM17 promotes macrophage proliferation via its cleavage of cell surface CSF-1 expressed on neutrophils and macrophages in inflammation is novel and somewhat unexpected. While the major sources of functional CSF-1 protein in steady state are believed to be stromal, epithelial, and endothelial cells (12), other sources appear to be involved in inflammation. *Csf1* RNA has been detected in neutrophils from mouse bone marrow, blood, and arthritis synovial fluid; in the peritoneal cavity in thioglycolate- and uric acid-induced peritonitis (43); and in activated monocytes (52). A recent study in a mouse UV-induced skin injury model demonstrated that while Langerhans cells (a myeloid cell) require the other CSF-1R ligand, interleukin 34 (IL-34), to continually self-renew under steady state, these cells require CSF-1 to regenerate during damage repair (53). In the UV-induced skin injury model, neutrophils are the major cells to infiltrate the injured skin, and *Csf1* mRNA peaks with neutrophil infiltration. Depleting neutrophils by antibody injection significantly decreased *Csf1* mRNA and dampened Langerhans cell regeneration, indicating a strong correlation between neutrophil-derived CSF-1 and Langerhans cell regeneration during skin inflammation. Our study shows that while resting bone marrow neutrophils and resident macrophages do not express appreciable amounts of CSF-1 protein, inflammatory neutrophils, as well as macrophages, express CSF-1 protein on their

FIG 9 ADAM17 promotes atherosclerotic lesion macrophage proliferation and accumulation. *Adam17*^{+/+} and *Adam17*^{-/-} hematopoietic chimeras on the *Ldlr*^{-/-} background were fed a high-fat diet for 16 weeks starting at 4 weeks post-bone marrow transplant. Cells were isolated from pooled digested aortas from 2 mice to create each sample point. The gating scheme is shown in panel A. Aortic cells were gated on CD45⁺ cells first. Macrophages were defined as CD11b⁺ Mac-3⁺ cells, while neutrophils were defined as CD11b⁺ Ly6G⁺ cells. Lesion macrophages in S/G₂/M phases of the cell cycle were detected with Vybrant DyeCycle Violet stain. (B) Neutrophil numbers, with fluorescent calibration beads added as an internal control to determine absolute cell numbers. (C) Macrophage numbers, determined as for panel B. (D) Percentages of Mac-3⁺ macrophages in S/G₂/M phases of the cell cycle (Vybrant DyeCycle Violet stain) from *Adam17*^{+/+} and *Adam17*^{-/-} *Ldlr*^{-/-} hematopoietic chimeras.

surfaces and intracellularly. These results are consistent with previous studies indicating CSF-1 release from atherosclerotic lesion macrophages in rabbits and humans (54, 55). Our data provide direct evidence that inflammatory neutrophils and macrophages are important sources of functional CSF-1 during inflammation and that ADAM17-mediated cleavage and release of CSF-1 are required for an optimal proliferative response.

The thioglycolate-induced acute peritonitis model has helped dissect mechanisms of multiple key steps during acute inflammation, from the initiation of neutrophil

FIG 10 Diagram of regulatory mechanisms. Shown are proposed regulatory mechanisms by ADAM17-mediated release of csCSF-1 from neutrophils and macrophages during acute inflammation and with chronic hypercholesterolemia leading to atherosclerosis.

infiltration, followed by monocyte influx, to neutrophil apoptosis and efferocytosis by macrophages, to the clearance of inflammatory macrophages, which ultimately leads to the resolution of inflammation (27, 56–58). During the resolution stage, typically beyond 72 h post-thioglycolate injection, inflammatory macrophage numbers are reduced through a combination of apoptosis and migration to draining lymph nodes (56, 57). Because CSF-1 promotes not only macrophage proliferation but also survival (11), we analyzed apoptosis in macrophages. Our data demonstrate that apoptotic macrophages are rare (around 2% of total peritoneal macrophages) at 40 h post-thioglycolate injection and are not affected by CSF-1 injection. Furthermore, soluble-CSF-1 levels are low after 48 h, suggesting that ADAM17-mediated shedding of csCSF-1 does not play a major role at this time point in preventing macrophage apoptosis in the thioglycolate-induced peritonitis model.

In addition to csCSF-1, ADAM17 is known to cleave CSF-1 receptor CD115 (30). We confirmed the higher cell surface CD115 level by flow cytometry in *Adam17*^{-/-} macrophages in the acute peritonitis model. However, our data with mixed *Adam17*^{-/-} chimeras and the CSF-1 rescue experiment indicate that, despite higher CD115 levels, ADAM17-null macrophages respond similarly to wild-type macrophages both to endogenously released csCSF-1 and to injection of exogenous CSF-1. Several factors could explain the failure of elevated CSF-1R expression to compensate for the loss of soluble CSF-1 in the ADAM17-null chimeric mice. First, low levels of receptor occupancy may be sufficient for the maximum proliferative response, so that the observed elevation of cell surface CSF-1R has no effect on the proliferative response. Indeed, levels of cell surface CSF-1R need not correlate with the proliferative response. For example, resident peritoneal macrophages, with a relatively high receptor density, fail to proliferate in response to CSF-1, whereas peripheral blood monocytes, with less than half the number of cell surface receptors, proliferate (59, 60). However, most importantly, in the present setting, cell surface CSF-1R is also responsible for internalizing and destroying CSF-1 at a high rate. This is the fate of 86% or more of the cell

surface CSF-1R–CSF-1 complexes, irrespective of the proliferative response of the cells (61–63). This fact, coupled with the requirement for CSF-1 for almost the entire 12-h G₁ period for entry of cells into S phase (64), is important when considering the transient response of cells to the transient elevation of CSF-1 observed in the acute inflammatory response reported here. Cells with higher cell surface expression of the CSF-1R will more rapidly deplete the available CSF-1 required to maintain the entry of cells into S phase, so rather than compensating for the decreased CSF-1, ADAM17-null cells are expected to more rapidly deplete the limited CSF-1 pool during a critical period.

ADAM17 activity is dependent on a class of polytopic proteins, the iRhoms, which are noncatalytic relatives of rhomboid intramembrane proteases (36). It has been reported that iRhom2 binds to ADAM17 and promotes its exit from the endoplasmic reticulum (ER). In the absence of iRhom2, ADAM17 fails to mature and traffic to the cell surface (36) and is thus unable to cleave its substrates on the same cell. Studies in mouse embryonic fibroblasts support a role for iRhom2 in the rapid activation of ADAM17 shedding of some, but not all, of its substrates (40, 41). We demonstrate here that ADAM17-mediated CSF-1 cleavage from inflammatory neutrophils and macrophages is iRhom2 dependent. We show that iRhom2-null hematopoietic chimeras display phenotypes similar to those of ADAM17-null chimeras in that both soluble TNF- α and CSF-1 levels are greatly reduced in the inflamed peritoneal cavity, as is inflammatory macrophage proliferation. This was accompanied by nondetectable cell surface ADAM17 in the absence of iRhom2. We also show the concurrent increase of both iRhom2 and ADAM17 proteins during inflammation in wild-type mice. Thus, csCSF-1 is one of the ADAM17 substrates that requires iRhom2 for its release from inflammatory neutrophils and macrophages and subsequently regulates macrophage proliferation. We have shown that the orchestrated activation of ADAM17 by iRhom2 and the resulting csCSF-1 shedding generate a synchronous wave of soluble CSF-1 in the inflamed peritoneal cavity that promotes macrophage proliferation.

The multitude of ADAM17 substrates that play important roles in inflammation have made ADAM17 an attractive candidate for studies of inflammatory diseases, including atherosclerosis, adipose tissue metabolism, insulin resistance, and diabetes (48, 65). Since its initial detection within atherosclerotic lesions in mice (48) and in humans (49), ADAM17 expression has been modulated to investigate its role in atherogenesis under a variety of circumstances. Recently, a study by van der Vorst et al. (50) presented two contrasting effects of ADAM17 on atherogenesis. When ADAM17 was deleted from myeloid cells, there was a significant decrease in lesion macrophage area; when ADAM17 was deleted from endothelial cells, however, no such effect was detected (50). In agreement with their finding, we observed decreased lesion macrophage numbers and proliferation in LDLR-deficient mice with ADAM17 deletion in all hematopoietic cells, and we provide the mechanisms underlying this process. Furthermore, our data indicate cell-type-specific functions of ADAM17 that provide a critical reference for comparison with other studies involving the manipulation of ADAM17 levels in different cell types. A study using ADAM17 hypomorphic LDLR-deficient mice, in which residual ADAM17 is expressed in all tissues, demonstrated a 1.5-fold increase in total lesion area, with a concurrent increase in macrophages and vascular smooth muscle cells and a constitutive activation of TNF receptor 2 signaling (66). This study illustrates the complex nature of ADAM17 regulation of shedding *in vivo*. Since there are a large number of potential substrates, ADAM17 may regulate inflammatory responses via different substrate pathways that are unique to the specific conditions.

Overall, we conclude that although there are multiple soluble forms of CSF-1, shedding of cell surface CSF-1 by iRhom2-dependent ADAM17 cleavage is required for local macrophage proliferation *in vivo*. The realization that ADAM17 has cell-type-specific functions, substrates, and modes of regulation could help develop therapeutic modulators that more precisely target ADAM17 activity in different states and phases of inflammation.

MATERIALS AND METHODS

Animals. (i) Adam17 hematopoietic chimeras. *Adam17^{ΔEx5/ΔEx5}* (*Adam17^{-/-}*) or WT hematopoietic chimeras were generated as previously described (24). All animals used for studies were second-generation hematopoietic chimeras on the C57BL/6J background (Jackson Laboratory; 000664) or second-generation chimeras in *Ldlr^{-/-}* mice (C57BL/6J background; Jackson Laboratory; 002207). Fetal livers were prepared from *Adam17^{ΔEx5/ΔEx5}* and WT mice to repopulate the first-generation chimeras. *Ldlr^{-/-}* chimeras were placed on a high-fat diet (20% fat and 1.25% cholesterol; Research Diets Inc.; D12108Ci) for 16 weeks starting 4 weeks after transplantation to induce atherosclerosis.

(ii) ADAM17 mixed hematopoietic chimeras. Bone marrow cells from Ly5.1-expressing C57BL/6J (B6.SJL-PTprc^a Pepc^b/BoyJ; Jackson Laboratory; stock 002014) and *Adam17^{-/-}* cells (Ly5.2) were mixed 1:1 to repopulate lethally irradiated C57BL/6J recipients.

(iii) iRhom2 hematopoietic chimeras. Bone marrow cells from WT or *iRhom2^{-/-}* mice on the C57BL/6 background were used to repopulate C57BL/6 recipients. *iRhom2^{-/-}* mice were generated as described previously (36). All experiments involving *iRhom2^{-/-}* animals were undertaken with the approval of the UK Home Office under project license PPL 80/2584.

(iv) CSF-1 hematopoietic chimeras. CSF-1-deficient (*Csf1^{op}/Csf1^{op}*) mice and their wild-type littermate controls were generated by heterozygous mating of B6;C3Fe a/a-Csf1^{op}/J mice (Jackson Laboratory; 000231). The CSF-1-deficient mice were fed crushed (powdered) chow due to their lack of teeth. Bone marrow cells of wild-type and CSF-1-deficient mice were used to repopulate lethally irradiated C57BL/6J recipients. Due to the absence of marrow cavities in the bones of CSF-1-deficient mice, bone marrow cells were isolated from bones that were ground using a pestle and mortar (12).

Experimental procedures. All the mice used were male. The sample size ($n \geq 5$) was based on preliminary data and power calculations using GraphPad Instat 3 ($\alpha = 0.05$; $\beta = 0.05$ [where α is the probability of false positive error and β is the probability of false negative error]) to give us adequate power to detect a 20% difference. Similar power calculations were made for lesion cell isolation based on recently published data (5). Preestablished animal exclusion (~4% for all experiments shown) eliminated mice only if there were problems with withdrawal of peritoneal fluid (e.g., if the peritoneal fluid was bloody, the gastrointestinal [GI] tract was inadvertently punctured, or there was no leukocyte infiltration, implying problems with thioglycolate injection). All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Sterile-peritonitis model. Thioglycolate-induced peritonitis was initiated by intraperitoneal (i.p.) injection of 1 ml of 4% sterile thioglycolate (BD Diagnostic; 221398). Peritoneal leukocytes were collected at different time points after administration of thioglycolate by injection and removal of 3 ml phosphate-buffered saline (PBS)–5 mM EDTA, and the lavage fluid was saved following centrifugation to pellet the cells.

Determination of cell proliferation and apoptosis. Proliferating cells were determined using two approaches: BrdU incorporation and Vybrant DyeCycle Violet stain. To determine the time course of BrdU incorporation in the sterile-peritonitis model, BrdU (1 mg i.p.; BD PharMingen BrdU flow kit; 559619) was injected into the peritoneal cavity 1 h before harvest, and cells were collected as described above and stained with an anti-BrdU antibody (allophycocyanin [APC] or fluorescein isothiocyanate [FITC] labeled), according to the manufacturer's instructions. For some experiments, Vybrant DyeCycle Violet stain (ThermoFisher; V35003) was used according to the manufacturer's instructions. Vybrant DyeCycle Violet is a cell-permeable dye added after cell isolation. The dye allows DNA profiling in live cells, thus overcoming a major disadvantage of BrdU, which requires use of strong denaturing reagents to expose the BrdU epitope, leading to altered cell profiles with flow cytometry. Peritoneal neutrophil and macrophage apoptosis was evaluated with an annexin V-FITC apoptosis detection kit (Calbiochem).

Injection of TNF- α or CSF-1 to evaluate their roles in the proliferative defect of ADAM17-null hematopoietic chimeras. To investigate whether TNF- α or CSF-1 was able to rescue the decrease in ADAM17-null macrophage proliferation in the thioglycolate model, injections of PBS or TNF- α (1 ng or 500 ng/cavity) or of PBS or CSF-1 (10 ng/cavity) were given 4 h or 8 h after thioglycolate injection, respectively. Cells were collected and analyzed as described above. Intraperitoneal injection of CSF-1 at this dose should not affect the serum CSF-1 level, based on our previous studies (67).

Partial neutrophil depletion. To evaluate the effect of partial neutrophil depletion, mice were injected via the retro-orbital plexus with either rat anti-mouse neutrophil Ly6G (150 μ g/mouse; 1A8; BioXCell) or control rat IgG (150 μ g/mouse; 2A3; BioXCell) 8 h prior to thioglycolate injection. Peritoneal cells and fluid were collected as described above 12 h after thioglycolate injection to determine the extent of neutrophil depletion by flow cytometry and sCSF-1 levels by ELISA. Neutrophil depletion was assessed by gating on Ly6B⁺ F4/80⁻ cells. This gating detects a similar population identified using the more standard Ly6G⁺ Ly6B⁺ population, but Ly6G cannot be used to detect neutrophils following 1A8 treatment, since 1A8 blocks the epitope.

Mouse cell isolation. (i) Leukocyte isolation from bone marrow and peritoneum for analysis of cell lysates. Bone marrow cells were flushed from femurs and tibias with PBS and 0.2% bovine serum albumin (BSA), dispersed with a 25-gauge needle, and passed through a 70- μ m filter to ensure a single-cell suspension. Peritoneal cells were collected from naive mice or at different times after thioglycolate injection for flow analysis. Neutrophil lysates were generated from purified neutrophils by a rapid negative-selection system (Stemcell Technologies, Inc.), according to the manufacturer's instructions.

(ii) Atherosclerotic lesion cell isolation. Cells were isolated from lesions as previously described by digesting the entire aorta (from the root to the iliac bifurcation) following perfusion with PBS (51). The cleaned aortic tree was cut into 1- to 2-mm pieces and incubated with an enzyme cocktail containing 450

TABLE 2 Antibodies used for flow cytometry and Western blot analysis

Antigen ^a	Label	Source	No.	Clone	Concn (flow/5 × 10 ⁵ cells) ^a
Flow cytometry					
ms B220	FITC	Becton Dickinson	553087	RA3-6B2	0.5
ms CD3	PE	Becton Dickinson	555275	17A2	0.5
ms CD4	FITC	eBioscience	GK1.5	GK1.5	0.5
ms CD8a	FITC	Becton Dickinson	553030	53-6.7	0.5
ms CD11b	PE-Cy7	eBioscience	25-0112	M1/70	0.5
ms CD16/32	None	Becton Dickinson	553142	2.4G2	0.5
ms CD19	FITC	Becton Dickinson	557398	1D3	0.5
ms CD19	APC-Cy7	BioLegend	115530	6D5	0.5
ms CD45	APC	eBioscience	17-0451	30-F11	0.5
ms CD45.1	FITC	eBioscience	11-0453	A20	0.5
ms CD45.2	PE	eBioscience	12-0454	104	0.5
ms CD115	PE	eBioscience	12-1152	AFS98	0.5
ms CSF-1	LightningLink-PE	R&D Systems	AF416	AP-goat IgG	1
AP goat IgG	LightningLink-PE	R&D Systems	AB-108-C	AP-goat IgG	1
ms F4/80	FITC	AbD Serotec	MCA497FB	Cl:A3-1	0.5
ms F4/80	PE	eBioscience	12-1152	AFS98	0.5
ms F4/80	PE-Cy5	eBioscience	15-4801	BM8	0.5
ms Ly6G	FITC	Becton Dickinson	551460	1A8	0.5
ms Ly6G	APC-Cy7	Becton Dickinson	560600	1A8	0.5
ms Ly6B.2 (7/4)	FITC	AbD Serotec	MCA771FB	7/4	0.5
ms Ly6C	PerCP-Cy5.5	eBioscience	45-5932	HK1.4	0.5
ms Mac-3	PE	Becton Dickinson	553324	M3/84	0.5
Mac-3 IgG control	PE	BD	554685	R3-34	0.5
ms SiglecF	PE	Becton Dickinson	552126	E50-2440	0.5
Western blot analysis					
ADAM17	None	Cell Sciences	PX084A	AP-rabbit	1 µg/ml
iRhom2 (<i>Rhbd2</i>)	None	Abcode	R3142-1	AP-rabbit	1:3,000

^aIn micrograms unless otherwise indicated.^bAP, affinity purified; ms, mouse.

U/ml type I collagenase (Worthington Bio; 4197), 125 U/ml type XI collagenase (Sigma; C7657), 60 U/ml hyaluronidase (Sigma; H3506), and 60 U/ml DNase (Sigma; D-4527) for 1 h at 37°C. The digested aortic tissue was passed through a 70-µm cell strainer to collect cells. Lesion cells were analyzed by flow cytometry.

Flow cytometry. The antibodies used for flow cytometry are shown in Table 2. All flow analyses utilized directly conjugated antibodies, and nonspecific binding was blocked with anti-CD16/32 (BD Pharmingen). Mouse CSF-1 staining was detected using affinity-purified goat anti-mouse CSF-1 and control IgG that were directly labeled with phycoerythrin (PE) LightningLink (Novus Biologicals) according to the manufacturer's directions. The fluorescence-activated cell sorter (FACS) buffer for staining was PBS with 0.2% BSA. For analysis of cCSF-1 staining, cells were either fixed on ice with 1% paraformaldehyde for 20 min to evaluate cell surface levels or fixed and permeabilized with Cytofix/Cytoperm (BD Sciences) for 20 min on ice to assess total levels of CSF-1. Samples were analyzed using either a BD FACScan or LSRII, as noted. For most lesion cell analyses, the final cell suspension included addition of Sphero AccuCount Ultra Rainbow fluorescent particles (3.8 µm; Spherotech; ACURFP-38-5) to facilitate determination of absolute cell numbers. Lesion macrophage proliferation was determined by Vybrant DyeCycle Violet staining (ThermoFisher; V35003). Flow data were analyzed using FlowJo 7.5 software (TreeStar).

ELISA for CSF-1. To detect soluble-CSF-1 levels in mouse peritoneal fluid, a DY416 kit with both antibodies recognizing the extracellular domain of CSF-1 was purchased from R&D Systems. Ninety-six-well plates (Nunc; Immuno 446612) were coated overnight at room temperature (RT) with 100 µl/well of 4-µg/ml anti-CSF-1 capture antibody (MAB416; R&D Systems) in PBS. The wells were blocked with 220 µl of 1% BSA in PBS at RT. CSF-1 was detected using 0.1 µg/ml biotinylated antibody (BAF416; R&D Systems). Following addition of streptavidin-horseradish peroxidase (HRP) and developing reagent (tetramethylbenzidine; DY999; R&D Systems), absorbance was measured using a SpectraMax 2Me spectrophotometer. Peritoneal fluid collected in 3 ml PBS with 5 mM EDTA buffer was evaluated without dilution. Mouse serum CSF-1 levels were measured using a Quantikine ELISA kit (R&D; MMC00) according to the manufacturer's instructions.

Western blot characterization of neutrophil lysates. Neutrophils from different sources and time points were isolated by negative selection, macrophages were isolated by adhesion on tissue culture plates for 2 h, and 15 × 10⁶ cells were lysed in 200 µl of NP-40 lysis buffer (150 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 8.0, 1% NP-40 containing protease inhibitors at a final concentration of 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride [PMSF]). All samples were run on 7.5% SDS gels under reducing conditions, loading 20 µg of protein/lane as determined by the bicinchoninic acid (BCA) assay. The antibodies used for Western analysis are listed in Table 2. Blots were developed using ECLplus (Life Technologies).

Cell surface biotinylation to monitor ADAM17 surface expression. Peritoneal neutrophils and macrophages were purified as described above, and cell surface biotinylation of suspended neutrophils and adherent macrophages utilized a cell surface isolation kit (Pierce). Following labeling, cells were lysed in NP-40 lysis buffer as described above plus 50 μ M GM6001 protease inhibitor to prevent ADAM17 autoproteolysis (68). The lysates were added to neutravidin-agarose and incubated end over end for 2 h. Following 4 washes with lysis buffer supplemented with 300 mM NaCl, the biotinylated proteins were eluted using peptide-N-glycosidase F (PNGase F) denaturation buffer with boiling. Samples were treated with 500 units of PNGase F (New England BioLabs) and incubated for 1 h at 37°C, followed by SDS gel separation and Western blot analysis of ADAM17. The same samples were evaluated for total levels of pro-ADAM17 in lysates without PNGase F treatment or neutravidin capture.

Statistics. Values are expressed as means $\pm$ standard errors of the mean (SEM). Data were evaluated using unpaired two-tailed Student *t* tests with InStat 3 (GraphPad Software) or with one-way or two-way analysis of variance (ANOVA). Normal distribution was verified. An estimate of variation within each group and similarity of variance between the groups were established for group comparisons. If a sample population did not follow a Gaussian distribution, a nonparametric Mann-Whitney test was utilized. Significance was concluded when the *P* value was <0.05 .

ACKNOWLEDGMENTS

We thank Xiaoping Wu, Puget Sound Blood Center, for her assistance with the LSRII flow cytometry; Clinton Robbins, University of Toronto, for discussion of his use of Vybrant DyeCycle Violet stain for evaluating macrophage proliferation within lesions; and C. C. (Lynn) Hedrick for her perspectives on gating strategies for mouse aortic lesion digests.

This work was supported in part by an American Heart Association Innovation Award (12IRG9040007) to E.W.R.; National Institutes of Health grants R01HL067267 and P01HL018645 to E.W.R., R01HL127694 to E.W.R. and K.E.B., R21AI135447 to K.E.B., and R01CA032551, R01NS091519, P01CA100324, and P30CA13330 to E.R.S.; and Wellcome Trust grant 101035/Z/13/Z and UK Medical Research Council Programme U105178780 to M.F.

Author contributions were as follows. J.T. and E.W.R. designed the studies and analyzed and interpreted the data; J.T. performed most of the experiments. J.M.F. assisted with many of the biochemical and immunochemical analyses, including Western blots to evaluate ADAM17 regulatory mechanisms and quantification of CSF-1; coordinated the generation of study mice; and contributed to analysis and interpretation of data. C.L.W. coordinated the generation of study mice, contributed to analysis and interpretation of data, and edited the manuscript. A.M.-P., C.L., and M.F. provided bone marrow from iRhomb2-null mice, assisted with data interpretation, and edited the manuscript. M.E.R. contributed to discussion and edited the manuscript. E.R.S. provided unpublished observations from his laboratory and reagents generated by his laboratory to characterize CSF-1 isoforms, helped in the design of experiments and interpretation of data, and edited the manuscript. J.T. and E.W.R. wrote the manuscript. K.E.B. interpreted the data and edited the manuscript.

We declare that we have no competing financial interests.

REFERENCES

- Moore KJ, Tabas I. 2011. Macrophages in the pathogenesis of atherosclerosis. *Cell* 145:341–355. <https://doi.org/10.1016/j.cell.2011.04.005>.
- Moore KJ, Sheedy FJ, Fisher EA. 2013. Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol* 13:709–721. <https://doi.org/10.1038/nri3520>.
- Tabas I, Glass CK. 2013. Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* 339:166–172. <https://doi.org/10.1126/science.1230720>.
- Chitu V, Stanley ER. 2006. Colony-stimulating factor-1 in immunity and inflammation. *Curr Opin Immunol* 18:39–48. <https://doi.org/10.1016/j.coi.2005.11.006>.
- Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo JL, Gorbato R, Sukhova GK, Gerhardt LM, Smyth D, Zavitz CC, Shikata EA, Parsons M, van Rooijen N, Lin HY, Husain M, Libby P, Nahrendorf M, Weissleder R, Swirski FK. 2013. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med* 19:1166–1172. <https://doi.org/10.1038/nm.3258>.
- Amano SU, Cohen JL, Vangala P, Tencerova M, Nicoloso SM, Yawe JC, Shen Y, Czech MP, Aouadi M. 2014. Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation. *Cell Metab* 19:162–171. <https://doi.org/10.1016/j.cmet.2013.11.017>.
- Davies LC, Rosas M, Jenkins SJ, Giles PJ, Liao C, Scurr MJ, O'Donnell VB, Brombacher F, Fraser DJ, Allen JE, Jones SA, Taylor PR. 2012. Distinct bone marrow-derived and tissue resident macrophage-lineages proliferate at key stages during inflammation. *Immunology* 137:1–2.
- Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, MacDonald AS, Allen JE. 2011. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 332:1284–1288. <https://doi.org/10.1126/science.1204351>.
- Hume DA, Pavli P, Donahue RE, Fidler IJ. 1988. The effect of human recombinant macrophage colony-stimulating factor (CSF-1) on the murine mononuclear phagocyte system in vivo. *J Immunol* 141:3405–3409.
- Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H, Chisholm O, Hofstetter W, Pollard JW, Stanley ER. 1994. Role of colony

- stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* 120:1357–1372.
11. Pixley FJ, Stanley ER. 2004. CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol* 14:628–638. <https://doi.org/10.1016/j.tcb.2004.09.016>.
 12. Ryan GR, Dai XM, Dominguez MG, Tong W, Chuan F, Chisholm O, Russell RG, Pollard JW, Stanley ER. 2001. Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1^{op}/Csf1^{op}) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood* 98:74–84. <https://doi.org/10.1182/blood.V98.1.74>.
 13. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 345:442–444. <https://doi.org/10.1038/345442a0>.
 14. Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Jr, Ahmed-Ansari A, Sell KW, Pollard JW, Stanley ER. 1990. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A* 87:4828–4832. <https://doi.org/10.1073/pnas.87.12.4828>.
 15. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. 1985. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 41:665–676. [https://doi.org/10.1016/S0092-8674\(85\)80047-7](https://doi.org/10.1016/S0092-8674(85)80047-7).
 16. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, Sylvestre V, Stanley ER. 2002. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* 99:111–120. <https://doi.org/10.1182/blood.V99.1.111>.
 17. Stanley ER, Chitu V. 2014. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb Perspect Biol* 6:a021857. <https://doi.org/10.1101/cshperspect.a021857>.
 18. Chitu V, Stanley ER. 2017. Regulation of embryonic and postnatal development by the CSF-1 receptor. *Curr Top Dev Biol* 123:229–275. <https://doi.org/10.1016/bs.ctdb.2016.10.004>.
 19. Hamilton JA, Cook AD, Tak PP. 2016. Anti-colony-stimulating factor therapies for inflammatory and autoimmune diseases. *Nat Rev Drug Discov* 16:53–70. <https://doi.org/10.1038/nrd.2016.231>.
 20. Louis C, Cook AD, Lacey D, Fleetwood AJ, Vlahos R, Anderson GP, Hamilton JA. 2015. Specific contributions of CSF-1 and GM-CSF to the dynamics of the mononuclear phagocyte system. *J Immunol* 195:134–144. <https://doi.org/10.4049/jimmunol.1500369>.
 21. Jenkins SJ, Ruckerl D, Thomas GE, Hewitson JP, Duncan S, Brombacher F, Maizels RM, Hume DA, Allen JE. 2013. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J Exp Med* 210:2477–2491. <https://doi.org/10.1084/jem.20121999>.
 22. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* 385:729–733. <https://doi.org/10.1038/385729a0>.
 23. Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton AK, Schoenen F, Seaton T, Su JL, Warner J, Willard D, Becherer JD. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor- α . *Nature* 385:733–736. <https://doi.org/10.1038/385733a0>.
 24. Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, Boyce RW, Nelson N, Kozlosky CJ, Wolfson MF, Rauch CT, Cerretti DP, Paxton RJ, March CJ, Black RA. 1998. An essential role for ectodomain shedding in mammalian development. *Science* 282:1281–1284. <https://doi.org/10.1126/science.282.5392.1281>.
 25. Garton KJ, Gough PJ, Raines EW. 2006. Emerging roles for ectodomain shedding in the regulation of inflammatory responses. *J Leukoc Biol* 79:1105–1116. <https://doi.org/10.1189/jlb.0106038>.
 26. Murphy G, Murphy A, Khokha R. 2008. Clipping, shedding and RIPping keep immunity on cue. *Trends Immunol* 29:75–82. <https://doi.org/10.1016/j.it.2007.10.009>.
 27. Tang J, Zarbock A, Gomez I, Wilson CL, Lefort CT, Stadtmann A, Bell B, Huang LC, Ley K, Raines EW. 2011. Adam17-dependent shedding limits early neutrophil influx but does not alter early monocyte recruitment to inflammatory sites. *Blood* 118:786–794. <https://doi.org/10.1182/blood-2010-11-321406>.
 28. Scholzen T, Gerdes J. 2000. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 182:311–322. [https://doi.org/10.1002/\(SICI\)1097-4652\(200003\)182:3<311::AID-JCP1>3.0.CO;2-9](https://doi.org/10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9).
 29. Horiuchi K, Miyamoto T, Takaishi H, Hakozaaki A, Kosaki N, Miyauchi Y, Furukawa M, Takito J, Kaneko H, Matsuzaki K, Morioka H, Blobel CP, Toyama Y. 2007. Cell surface colony-stimulating factor 1 can be cleaved by TNF- α converting enzyme or endocytosed in a clathrin-dependent manner. *J Immunol* 179:6715–6724. <https://doi.org/10.4049/jimmunol.179.10.6715>.
 30. Becker AM, Walcheck B, Bhattacharya D. 2015. ADAM17 limits the expression of CSF1R on murine hematopoietic progenitors. *Exp Hematol* 43:44–52 e1–3. <https://doi.org/10.1016/j.exphem.2014.10.001>.
 31. Wilson CL, Gough PJ, Chang CA, Chan CK, Frey JM, Liu Y, Braun KR, Chin MT, Wight TN, Raines EW. 2013. Endothelial deletion of ADAM17 in mice results in defective remodeling of the semilunar valves and cardiac dysfunction in adults. *Mech Dev* 130:272–289. <https://doi.org/10.1016/j.mod.2013.01.001>.
 32. Chalaris A, Adam N, Sina C, Rosenstiel P, Lehmann-Koch J, Schirmacher P, Hartmann D, Cichy J, Gavrilova O, Schreiber S, Jostock T, Matthews V, Hasler R, Becker C, Neurath MF, Reiss K, Saftig P, Scheller J, Rose-John S. 2010. Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice. *J Exp Med* 207:1617–1624. <https://doi.org/10.1084/jem.20092366>.
 33. Gomez IG, Tang J, Wilson CL, Yan W, Heinecke JW, Harlan JM, Raines EW. 2012. Metalloproteinase-mediated shedding of integrin β 2 promotes macrophage efflux from inflammatory sites. *J Biol Chem* 287:4581–4589. <https://doi.org/10.1074/jbc.M111.321182>.
 34. Tsubota Y, Frey JM, Tai PW, Welikson RE, Raines EW. 2013. Monocyte ADAM17 promotes diapedesis during transendothelial migration: identification of steps and substrates targeted by metalloproteinases. *J Immunol* 190:4236–4244. <https://doi.org/10.4049/jimmunol.1300046>.
 35. Driscoll WS, Vaisar T, Tang J, Wilson CL, Raines EW. 2013. Macrophage ADAM17 deficiency augments CD36-dependent apoptotic cell uptake and the linked anti-inflammatory phenotype. *Circ Res* 113:52–61. <https://doi.org/10.1161/CIRCRESAHA.112.300683>.
 36. Adrain C, Zettl M, Christova Y, Taylor N, Freeman M. 2012. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science* 335:225–228. <https://doi.org/10.1126/science.1214400>.
 37. Mcllwain DR, Lang PA, Maretzky T, Hamada K, Ohishi K, Maney SK, Berger T, Murthy A, Duncan G, Xu HC, Lang KS, Haussinger D, Wakeham A, Itie-Youten A, Khokha R, Ohashi PS, Blobel CP, Mak TW. 2012. iRhom2 regulation of TACE controls TNF-mediated protection against *Listeria* and responses to LPS. *Science* 335:229–232. <https://doi.org/10.1126/science.1214448>.
 38. Issuree PD, Maretzky T, Mcllwain DR, Monette S, Qing X, Lang PA, Swendeman SL, Park-Min KH, Binder N, Kalliolias GD, Yarlina A, Horiuchi K, Ivashkiv LB, Mak TW, Salmon JE, Blobel CP. 2013. iRhom2 is a critical pathogenic mediator of inflammatory arthritis. *J Clin Invest* 123:928–932. <https://doi.org/10.1172/JCI66168>.
 39. Christova Y, Adrain C, Bambrough P, Ibrahim A, Freeman M. 2013. Mammalian iRhoms have distinct physiological functions including an essential role in TACE regulation. *EMBO Rep* 14:884–890. <https://doi.org/10.1038/embor.2013.128>.
 40. Maretzky T, Mcllwain DR, Issuree PD, Li X, Malapeira J, Amin S, Lang PA, Mak TW, Blobel CP. 2013. iRhom2 controls the substrate selectivity of stimulated ADAM17-dependent ectodomain shedding. *Proc Natl Acad Sci U S A* 110:11433–11438. <https://doi.org/10.1073/pnas.1302553110>.
 41. Grieve AG, Xu H, Kunzel U, Bambrough P, Sieber B, Freeman M. 2017. Phosphorylation of iRhom2 at the plasma membrane controls mammalian TACE-dependent inflammatory and growth factor signalling. *Elife* 6:e23968. <https://doi.org/10.7554/eLife.23968>.
 42. Blobel CP. 2005. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* 6:32–43. <https://doi.org/10.1038/nrm1548>.
 43. Ericson JA, Duffau P, Yasuda K, Ortiz-Lopez A, Rothamel K, Rifkin IR, Monach PA, ImmGen Consortium. 2014. Gene expression during the generation and activation of mouse neutrophils: implication of novel functional and regulatory pathways. *PLoS One* 9:e108553. <https://doi.org/10.1371/journal.pone.0108553>.

44. Mayadas TN, Cullere X, Lowell CA. 2014. The multifaceted functions of neutrophils. *Annu Rev Pathol* 9:181–218. <https://doi.org/10.1146/annurev-pathol-020712-164023>.
45. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. 2009. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16:183–194. <https://doi.org/10.1016/j.ccr.2009.06.017>.
46. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* 83:64–70. <https://doi.org/10.1189/jlb.0407247>.
47. Matsukawa A, Kudo S, Maeda T, Numata K, Watanabe H, Takeda K, Akira S, Ito T. 2005. Stat3 in resident macrophages as a repressor protein of inflammatory response. *J Immunol* 175:3354–3359. <https://doi.org/10.4049/jimmunol.175.5.3354>.
48. Canault M, Peiretti F, Kopp F, Bonardo B, Bonzi MF, Coudeyre JC, Alessi MC, Juhan-Vague I, Nalbone G. 2006. The TNF alpha converting enzyme (TACE/ADAM17) is expressed in the atherosclerotic lesions of apolipoprotein E-deficient mice: possible contribution to elevated plasma levels of soluble TNF alpha receptors. *Atherosclerosis* 187:82–91. <https://doi.org/10.1016/j.atherosclerosis.2005.08.031>.
49. Satoh M, Ishikawa Y, Itoh T, Minami Y, Takahashi Y, Nakamura M. 2008. The expression of TNF-alpha converting enzyme at the site of ruptured plaques in patients with acute myocardial infarction. *Eur J Clin Invest* 38:97–105. <https://doi.org/10.1111/j.1365-2362.2007.01912.x>.
50. van der Vorst EP, Zhao Z, Rami M, Holdt LM, Teupser D, Steffens S, Weber C. 2017. Contrasting effects of myeloid and endothelial ADAM17 on atherosclerosis development. *Thromb Haemostasis* 117:644–646. <https://doi.org/10.1160/TH16-09-0674>.
51. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. 2006. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med* 203:1273–1282. <https://doi.org/10.1084/jem.20052205>.
52. Roth P, Stanley ER. 1992. The biology of CSF-1 and its receptor. *Curr Top Microbiol Immunol* 181:141–167.
53. Wang Y, Bugatti M, Ulland TK, Vermi W, Gilfillan S, Colonna M. 2016. Nonredundant roles of keratinocyte-derived IL-34 and neutrophil-derived CSF1 in Langerhans cell renewal in the steady state and during inflammation. *Eur J Immunol* 46:552–559. <https://doi.org/10.1002/eji.201545917>.
54. Rosenfeld ME, Yla-Herttuala S, Lipton BA, Ord VA, Witztum JL, Steinberg D. 1992. Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. *Am J Pathol* 140:291–300.
55. Chang MY, Olin KL, Tsoi C, Wight TN, Chait A. 1998. Human monocyte-derived macrophages secrete two forms of proteoglycan-macrophage colony-stimulating factor that differ in their ability to bind low density lipoproteins. *J Biol Chem* 273:15985–15992. <https://doi.org/10.1074/jbc.273.26.15985>.
56. Gautier EL, Ivanov S, Lesnik P, Randolph GJ. 2013. Local apoptosis mediates clearance of macrophages from resolving inflammation in mice. *Blood* 122:2714–2722. <https://doi.org/10.1182/blood-2013-01-478206>.
57. Bellingan GJ, Caldwell H, Howie SE, Dransfield I, Haslett C. 1996. 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* 157:2577–2585.
58. Serhan CN, Chiang N, Van Dyke TE. 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 8:349–361. <https://doi.org/10.1038/nri2294>.
59. Byrne PV, Guilbert LJ, Stanley ER. 1981. Distribution of cells bearing receptors for a colony-stimulating factor (CSF) in murine tissues. *J Cell Biol* 91:848–853. <https://doi.org/10.1083/jcb.91.3.848>.
60. Stanley ER, Chen DM, Lin H-S. 1978. Induction of macrophage production and proliferation by a purified colony stimulating factor. *Nature* 274:168–170. <https://doi.org/10.1038/274168a0>.
61. Guilbert LJ, Stanley ER. 1980. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. *J Cell Biol* 85:153–159. <https://doi.org/10.1083/jcb.85.1.153>.
62. Guilbert LJ, Tynan PW, Stanley ER. 1986. Uptake and destruction of 125I-CSF-1 by peritoneal exudate macrophages. *J Cell Biochem* 31:203–216. <https://doi.org/10.1002/jcb.240310303>.
63. Guilbert LJ, Stanley ER. 1986. The interaction of 125I-colony stimulating factor-1 with bone marrow-derived macrophages. *J Biol Chem* 261:4024–4032.
64. Tushinski RJ, Stanley ER. 1985. The regulation of mononuclear phagocyte entry into S phase by the colony stimulating factor CSF-1. *J Cell Physiol* 122:221–228. <https://doi.org/10.1002/jcp.1041220210>.
65. Menghini R, Fiorentino L, Casagrande V, Lauro R, Federici M. 2013. The role of ADAM17 in metabolic inflammation. *Atherosclerosis* 228:12–17. <https://doi.org/10.1016/j.atherosclerosis.2013.01.024>.
66. Nicolaou A, Zhao Z, Northoff BH, Sass K, Herbst A, Kohlmaier A, Chalaris A, Wolfrum C, Weber C, Steffens S, Rose-John S, Teupser D, Holdt LM. 2017. Adam17 deficiency promotes atherosclerosis by enhanced TNFR2 signaling in mice. *Arterioscler Thromb Vasc Biol* 37:247–257. <https://doi.org/10.1161/ATVBAHA.116.308682>.
67. Wiktor-Jedrzejczak W, Urbanowska E, Aukerman SL, Pollard JW, Stanley ER, Ralph P, Ansari AA, Sell KW, Szperl M. 1991. Correction by CSF-1 of defects in the osteopetrotic op/op mouse suggests local, developmental, and humoral requirements for this growth factor. *Exp Hematol* 19:1049–1054.
68. Schlondorff J, Becherer JD, Blobel CP. 2000. Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). *Biochem J* 347:131–138.