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ORIGINAL ARTICLE

Control of macrophage lineage populations by CSF-1 receptor and GM-CSF in homeostasis and inflammation

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There is recent interest in the role of monocyte/macrophage subpopulations in pathology. How the hemopoietic growth factors, macrophage-colony stimulating factor (M-CSF or CSF-1) and granulocyte macrophage (GM)-CSF, regulate their *in vivo* development and function is unclear. A comparison is made here on the effect of CSF-1 receptor (CSF-1R) and GM-CSF blockade/depletion on such subpopulations, both in the steady state and during inflammation. In the steady state, administration of neutralizing anti-CSF-1R monoclonal antibody (mAb) rapidly (within 3–4 days) lowered, specifically, the number of the more mature Ly6C^{lo} peripheral blood murine monocyte population and resident peritoneal macrophages; it also reduced the accumulation of murine exudate (Ly6C^{lo}) macrophages in two peritonitis models and alveolar macrophages in lung inflammation, consistent with a non-redundant role for CSF-1 (or interleukin-34) in certain inflammatory reactions. A neutralizing mAb to GM-CSF also reduced inflammatory macrophage numbers during antigen-induced peritonitis and lung inflammation. In GM-CSF gene-deficient mice, a detailed kinetic analysis of monocyte/macrophage and neutrophil dynamics in antigen-induced peritonitis suggested that GM-CSF was acting, in part, systemically to maintain the inflammatory reaction. A model is proposed in which CSF-1R signaling controls the development of the macrophage lineage at a relatively late stage under steady state conditions and during certain inflammatory reactions, whereas in inflammation, GM-CSF can be required to maintain the response by contributing to the prolonged extravasation of immature monocytes and neutrophils. A correlation has been observed between macrophage numbers and the severity of certain inflammatory conditions, and it could be that CSF-1 and GM-CSF contribute to the control of these numbers in the ways proposed.
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Macrophage numbers in tissues can correlate with disease severity, for example, in rheumatoid arthritis and tumor lesions.[1,2] Tissue macrophages in the adult can derive, particularly during inflammation, from blood monocytes, which in turn derive from precursors in the bone marrow, by the processes of proliferation and differentiation. One cytokine which appears to govern macrophage lineage development during normal homeostasis is macrophage-colony stimulating factor (M-CSF or CSF-1), which was originally defined by its ability to give rise *in vitro* to macrophage colonies when added to precursor bone marrow cells.[3,4] Subsequently, a mouse (*Csf1^{op}/Csf1^{op}*), which had a deficiency in the numbers of certain tissue macrophage populations including the peritoneum, was discovered to have an inactivating mutation in the *Csf1* gene;[5,6] targeted disruption of the CSF-1 receptor (CSF-1R; c-Fms; CD115) gene resulted in a mouse (*Csf1r^{-}/Csf1r^{-}*) with a slightly more severe phenotype.[7] The precise point in the macrophage development pathway at which CSF-1 is critical is debated, and the information from the *Csf1^{op}/Csf1^{op}* and *Csf1r^{-}/Csf1r^{-}* mice is conflicting.[6–12] Another recently discovered ligand for the CSF-1R, interleukin (IL)-34,[13] also needs now to be considered. A different CSF, namely granulocyte macrophage-CSF (GM-CSF), was found in the same type of semi-solid assay, with bone marrow cells to generate both granulocyte and macrophage colonies; however, unlike CSF-1, GM-CSF deficiency does not seem to compromise steady state myelopoiesis to a significant extent with the major phenotype being a lung condition, namely pulmonary alveolar proteinosis, due to a defect in alveolar macrophage maturation.[14] Other studies have implicated both of these CSFs in inflammation, as their depletion can

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suppress a number of inflammatory/autoimmune conditions.[15] However, based on differences in, for example, their expression, receptor expression, structure and *in vitro* action, it is likely that they will have different modes of action and functions in such conditions, as well as in the steady state.[15] In regard to macrophage lineage control by the CSFs, what is needed is information using concomitant neutralization/depletion of both of these CSFs in suitable model systems, both under steady state conditions and in pathology, so that comparisons can be made of their respective actions.

It would appear from recent studies that there is a great deal of macrophage lineage heterogeneity as assessed by macrophage activation states or phenotypes, as well as by the monocyte subpopulations, which can give rise to tissue macrophages.[16–20] With respect to activation or polarization states, macrophages have begun to be divided, on the one hand, into M1 or 'classically activated' macrophages, and into M2 or 'alternatively activated' macrophages, on the other.[18,19] As a generalization, M1 macrophages have been considered to be 'proinflammatory', whereas M2 populations have been considered to be less so and to be more involved in trophic or tissue repair processes. *In vitro*, GM-CSF- and CSF-1-treated monocytes/macrophages have been considered to some extent to be like M1 and M2 populations, respectively.[21–23] With respect to blood monocyte subpopulations in the human and the mouse, two major monocyte subpopulations have been defined based on differences in surface marker expression and proposed relative significance for inflammatory reactions. Using surface marker delineation, one murine monocyte ('classical')[20] subpopulation is Ly6C(Gr-1)^{hi} CX_3CR1^{lo} $CCR2^{hi}$ $CD62L^{hi}$, which is considered to be relatively less mature and to have a greater propensity to enter rapidly into sites of injury or infection;[16,17] in contrast, another more mature subpopulation ('non-classical')[20] is Ly6C(Gr-1)^{lo} CX_3CR1^{hi} $CCR2^{lo}$ $CD62L^{lo}$ and was considered originally to generate tissue macrophages in the steady state; however, it is possible that this subpopulation arrives more slowly in lesions and contributes to the gradual appearance of Ly6C^{lo} macrophages with possible scavenger and tissue repair functions.[24–27] There is some recent, albeit limited, literature on the *in vivo* involvement of endogenous GM-CSF and CSF-1 in the control of these populations,[28,29] but no information from comparative studies. The precise links between these monocyte subpopulations and the above-mentioned macrophage activation or polarization states are still being delineated.

In order to compare the effects of the neutralization/depletion of the activities of the two CSFs on macrophage lineage dynamics in inflammation, a convenient and short-term model is needed, where extravasating macrophage populations can be easily isolated; also some evidence for CSF dependence is essential. We have previously established a methylated bovine serum albumin (mBSA)-driven peritonitis model, whose severity was lessened in GM-CSF−/− mice.[30] In this paper, we monitored macrophage lineage population numbers in the peritoneum, blood and bone marrow, in the steady state and during this antigen-induced peritoneal inflammation, under conditions of CSF-1R neutralization and GM-CSF neutralization, as well as in a GM-CSF−/− mouse. We report that CSF-1R controls monocytopoiesis during both homeostasis and inflammation, perhaps surprisingly, at a relatively late stage at the level of monocyte/macrophage generation, whereas during antigen-induced peritoneal inflammation and perhaps in other inflammatory conditions, it is suggested that GM-CSF is required to sustain the infiltration of monocytes (and neutrophils) into the inflamed site.

RESULTS

Effect of neutralizing anti-CSF-1R and anti-GM-CSF antibodies on monocyte numbers in the steady state

We examined initially the effect in the steady state of neutralizing monoclonal antibody (mAbs) to c-Fms (CSF-1R; CD115) and to GM-CSF on monocyte population numbers in peripheral blood and also in bone marrow of C57BL/6 mice. Peripheral blood monocytes were identified (Figure 1a), as previously described.[17] Intraperitoneal (i.p.) administration of anti-CSF-1R mAb (AFS98), but not of anti-GM-CSF mAb (22E9), daily for 4 days, resulted in a slight but significant decrease in total blood monocyte numbers (Figure 1b). The effect of the two mAbs on monocyte subpopulations was also assessed by using the monocyte maturation marker, Ly6C, as the readout; murine monocytes are believed to downregulate this surface marker as they mature, exiting the bone marrow as Ly6C^{hi} cells and maturing to a Ly6C^{lo} population in the blood.[17] The anti-CSF-1R mAb-treated mice, but not the anti-GM-CSF mAb-treated cohort, had significantly lower levels only of the less abundant and putatively more mature Ly6C^{lo} subpopulation (Figure 1c), suggesting, by this approach at least, that CSF-1 but not GM-CSF could be having a role in controlling their numbers in the steady state; for the anti-CSF-1R treatment, no compensatory increase in the number of Ly6C^{hi} monocytes was noted. This decrease in the less abundant Ly6C^{lo} subpopulation would seem to account for the small reduction in total monocytes following anti-CSF-1R administration (Figure 1b).

There was no change in the bone marrow monocyte numbers (Figure 1d) and percentage (data not shown), following either CSF-1R or GM-CSF neutralization, suggesting that neither CSF controls monocytopoiesis at this relatively early stage, at least when assessed by this approach; these observations in the bone marrow are also consistent with the lack of effect of each Ab on the number of Ly6C^{hi} blood monocytes noted above (Figure 1c), which, as mentioned, are believed to arise from the corresponding bone marrow population.[17] It would not appear that the lack of reduction in Ly6C^{hi} bone marrow monocytes following AFS98 administration is due to lack of access of the mAb to the site, as their detectable surface CSF-1R was reduced (Figure 1e), presumably due to steric hindrance, as was also reported some time ago following AFS98 injection.[32] There was also no effect of either mAb on numbers of blood neutrophils (Figure 1f) or lymphocytes (data not shown).

Rapid depletion of Ly6C^{lo} monocytes and peritoneal macrophages by neutralizing anti-CSF-1R antibody

The data presented in Figure 1c were obtained after i.p. injections of 300 μg AFS98, given once daily for 4 days, with monocyte subpopulation numbers being quantified 1 day after the final mAb administration; in other words, Ly6C^{lo} monocyte numbers were reduced within 4 days from the first injection. In separate experiments, we found that Ly6C^{lo} monocyte depletion was optimal at doses of 100–300 μg per mouse with this protocol (data not shown). We monitored how soon the drop in the number of Ly6C^{lo} monocytes was occurring with AFS98, following a single 300 μg injection. As can be seen in Figure 2a, we could observe a significant reduction in Ly6C^{lo} monocytes with this particular protocol, also within 4 days. Intravenous administration gave similar kinetics (data not shown). Thus, in our studies, anti-CSF-1R mAb can reduce Ly6C^{lo} monocyte numbers within 4 days, unlike the relatively slow depletion reported recently (>1 week) with another anti-CSF-1R mAb.[29] As an indication of how fast anti-CSF-1R was acting, circulating CSF-1 levels were elevated even within 1 day (Figure 2b), possibly due to the rapid reduction in CSF-1R-mediated CSF-1 internalization by macrophage lineage cells.[33,34]

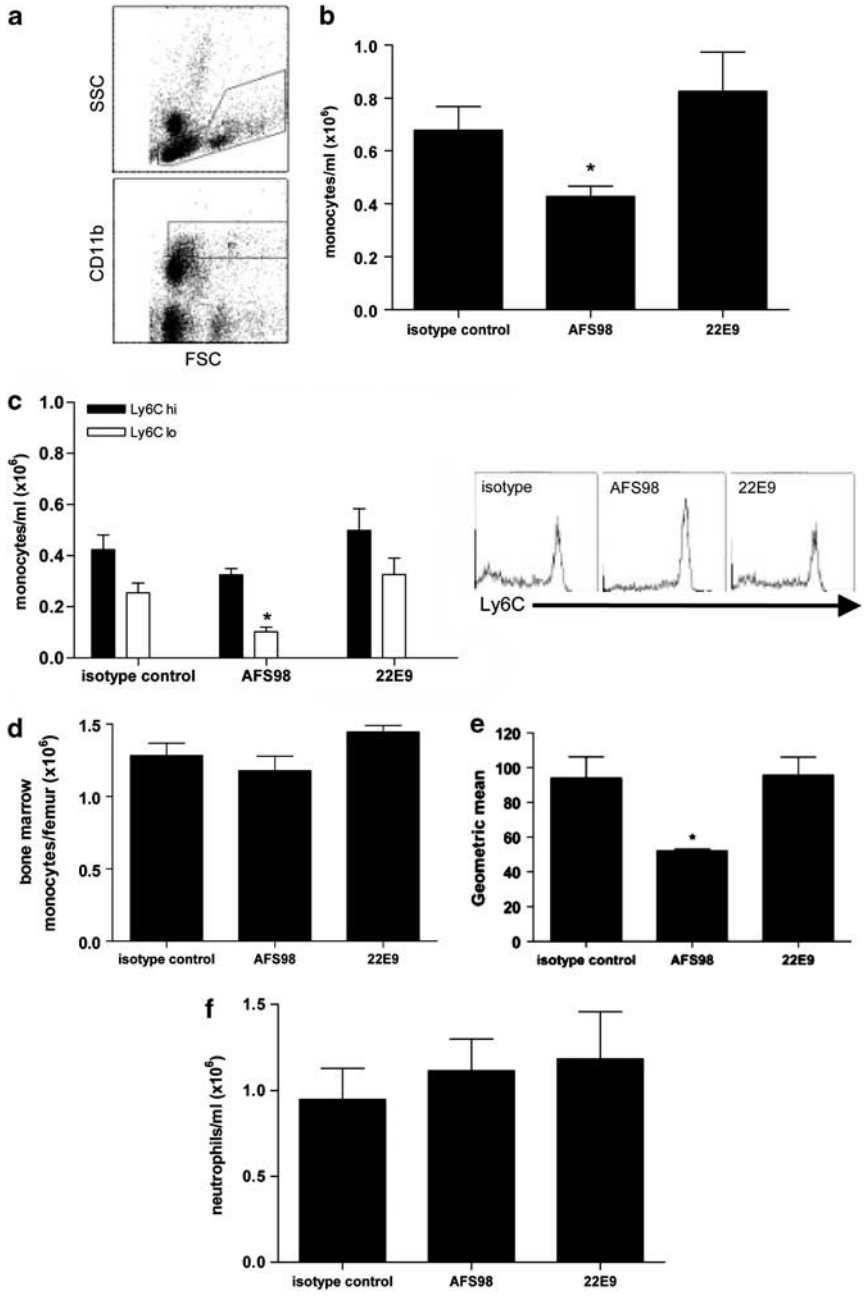

Figure 1 Anti-CSF-1R antibody but not anti-GM-CSF antibody lowers the Ly6C^{lo} blood monocyte numbers in the steady state. (**a**) Peripheral blood leukocytes from C57BL/6 mice were labeled with anti-CD11b and analyzed on a flow cytometer. Monocytes were identified sequentially as SSC^{lo} CD11b^{hi} cells.[17] (**b**) C57BL/6 mice were given four daily doses (300 μg) i.p. of the mAbs, AFS98 (anti-CSF-1R), 22E9 (anti-GM-CSF) or an isotype control (anti-β-galactosidase). Blood was harvested 24 h after the final Ab administration and monocyte numbers per ml monitored. (**c**) Subpopulations in the monocytes from (**b**) were stained for Ly6C expression, Ly6C^{hi}: filled columns, Ly6C^{lo}: open columns, (**d**) Numbers of bone marrow monocytes (CD11b^{hi} Ly6C^{hi} CD115^{+})[29,27,35,77] were measured in mice treated as in (**b**). (**e**) The geometric mean of CSF-1R expression was measured on bone marrow monocytes in mice treated as in (**b**). (**f**) Blood neutrophils (Ly6G^{+})[40] were measured in mice treated as in (**b**). Data are expressed as monocytes per ml, bone marrow monocytes/femur or neutrophils per ml (mean ± s.e.m.); n=10 mice. (**b**, **c** and **e**) *$P<0.05$, AFS98 versus isotype control.

We see in Figure 2c that reduced numbers of the resident peritoneal (Ly6C^{lo}; see below) macrophage population could be observed rapidly within 3 days of AFS98 administration; dramatically elevated peritoneal fluid CSF-1 levels were noted within 1 day (Figure 2d), again possibly due to reduced ligand uptake (by peritoneal macrophages).

One possible explanation for the surprising selectivity of anti-CSF-1R mAb towards the Ly6C^{lo} monocyte subpopulation could be relative CSF-1R expression. It has recently been reported using Macgreen mice, in which an EGFP reporter gene is driven by the *csf1r* promoter, that the Gr-1^{-} (that is, Ly6C^{lo}) monocyte subpopulation had three- to fourfold higher expression of the *csf1r*–EGFP transgene.[29] However, in addition to comparable levels of surface CSF-1R expression in these mice (Figure 2e), we found similar levels of *csf1r*–EGFP expression in the same mice for the two monocyte subpopulations (Figure 2f); also,

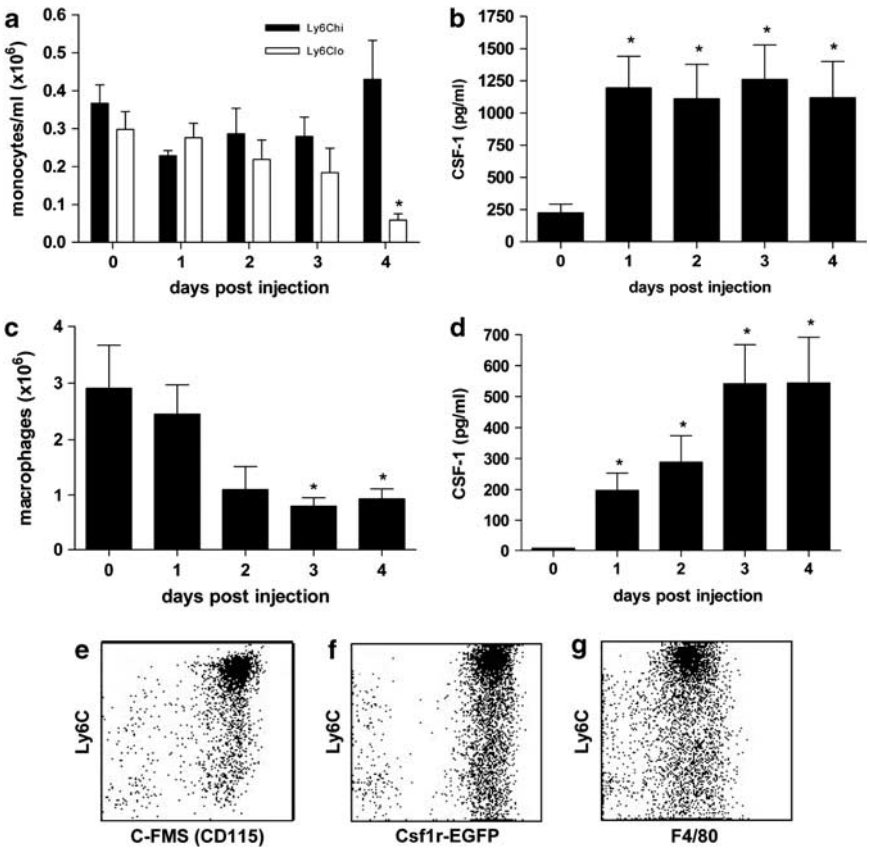

Figure 2 Rapid depletion of Ly6C^{lo} blood monocytes and peritoneal macrophages by neutralizing CSF-1R mAb in the steady state. (**a**–**d**) C57BL/6 mice were treated with a single 300 μg i.p. injection of neutralizing CSF-1R mAb and (**a**) blood monocyte subpopulations, Ly6C^{hi}: filled columns, Ly6C^{lo}: open columns, (**b**) blood CSF-1 levels, (**c**) peritoneal macrophages and (**d**) peritoneal fluid CSF-1 levels measured daily for 4 days. On *Csf1r-EGFP* Ly6C^{hi} and Ly6C^{lo} monocyte subpopulations (**e**) surface expression of CSF-1R (CD115), (**f**) *GFP* expression and (**g**) surface expression of F4/80. Data are expressed as mean ± s.e.m. n=10 mice, (**a** and **c**); n=8 mice, (**b** and **d**) *$P<0.05$, time point post injection versus day 0.

for these Macgreen mice, we found similar relative expression of the F4/80 surface antigen (Figure 2g) again in disagreement with the data in MacDonald *et al.*,[29] which reported also a three- to fourfold higher expression in the Gr-1$^-$ (Ly6C^{lo}) monocytes.

Effect of neutralizing anti-CSF-1R and anti-GM-CSF antibodies on monocyte/macrophage populations during an inflammatory response

We showed above that a single injection of AFS98 lowered resident C57BL/6 peritoneal macrophage numbers within 3 days (Figure 2c). The resident peritoneal macrophage numbers were also monitored following i.p. or subcutaneous injections of the two individual mAbs over a 4-day period (same injection protocol as in Figure 1); by both routes, as for the Ly6C^{lo} monocytes, CSF-1R neutralization, but not that of GM-CSF, reduced the peritoneal macrophage number in the steady state (data not shown). We next assessed the effect of the two mAbs on monocyte/macrophage populations during peritoneal and lung inflammatory reactions.

Thioglycolate-induced peritonitis. For the widely studied thioglycolate-elicited peritonitis involving i.p. injection of the non-specific irritant, thioglycolate broth, macrophages were harvested 24 h after the last antibody treatment, that is, at 96 h post challenge, as this is the period when peritoneal macrophage numbers peaked.[31] We show in Figure 3a that anti-CSF-1R mAb, but not anti-GM-CSF mAb, suppressed the increase in exudate macrophage numbers in this model, an observation distinct from what was reported recently with another neutralizing anti-CSF-1R mAb.[29] Consistent with the lack of effect of 22E9, we showed previously that GM-CSF−/− mice did not have reduced elicited macrophage numbers (day 4) in this model.[30]

Antigen-induced peritonitis. In contrast to thioglycolate-elicited peritonitis,[30] in an mBSA peritonitis model that we established,[31] involving i.p. challenge to a mouse primed with this antigen, there were fewer exudate macrophages in the GM-CSF−/− mice and they were deficient in a number of properties.[30] As we wanted to compare the effects of blockade/depletion of CSF-1R and GM-CSF on monocyte/macrophage (and neutrophil) populations during an inflammatory reaction, we used this latter model for such comparison.

C57BL/6 mice were primed and boosted with mBSA and adjuvant intradermally, then challenged with mBSA i.p.;[31] AFS98, 22E9 or the isotype control mAb was administered i.p. or subcutaneously before and after the mBSA challenge (at days −2, −1, 0, 1, 2, 3). Total peritoneal macrophage numbers (identified as in Figure 3a) were significantly reduced in both the anti-CSF-1R mAb- and the anti-GM-CSF mAb-treated groups following i.p. injection (Figure 3b), with only the former mAb having an effect subcutaneously (data not shown). For this reason, the i.p. route of administration was chosen for subsequent studies.

One possible explanation for the reduced peritoneal exudate macrophages following mAb injection could be that monocytes are not able to traffick and hence may accumulate in the blood. For blood

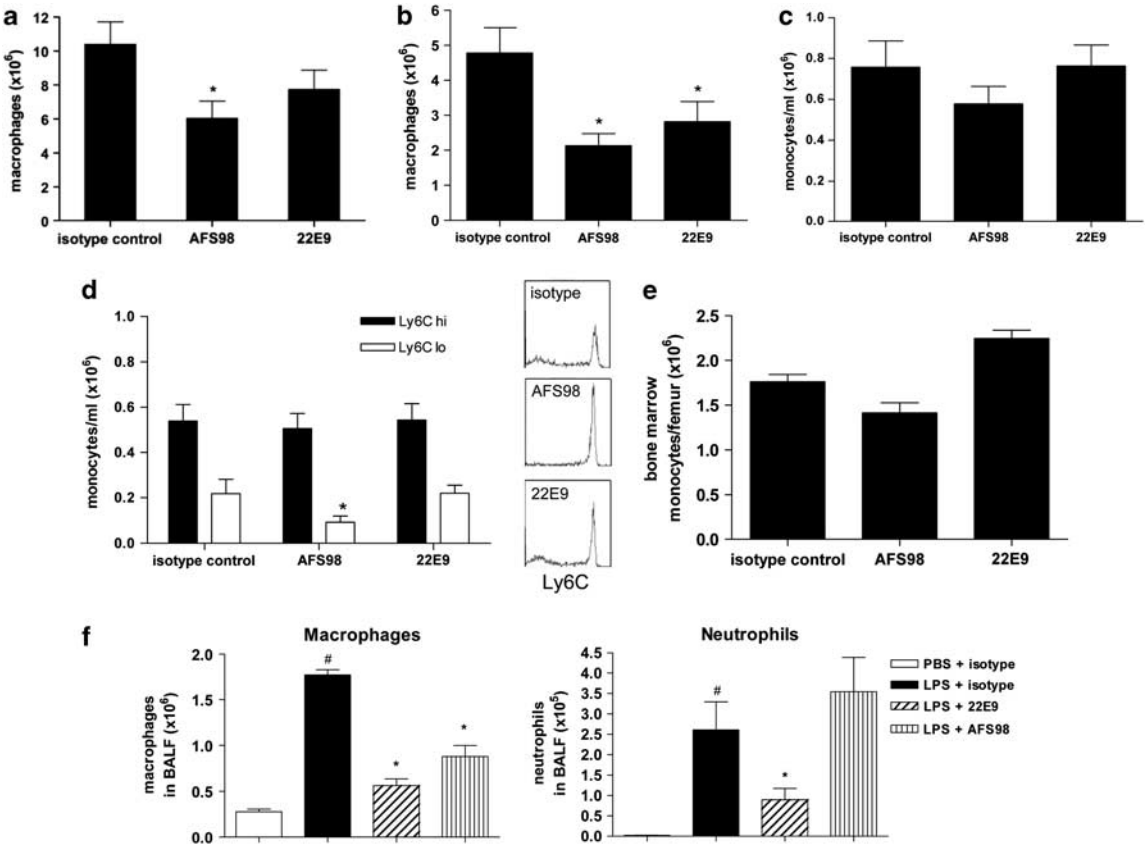

Figure 3 Effect of neutralizing anti-CSF-1R and anti-GM-CSF antibodies on monocyte/macrophage numbers in inflammation. (**a**–**e**) C57BL/6 mice were injected with either (**a**) thioglycolate medium i.p. or (**b**–**e**) primed with mBSA then challenged i.p. to induce peritoneal inflammation (Methods). 22E9, AFS98 or isotype control mAb was given i.p. before and after thioglycolate-medium injection or mBSA challenge (at days −2, −1, 0, 1, 2, 3). Peritoneal exudate cells, blood and bone marrow were harvested on day 4 post challenge. (**a**) Peritoneal macrophage numbers at day 4 from thioglycolate medium-treated mice (identified by FSC/SSC gating and as $CD11b^{hi}$ $Ly6G^{-}$ cells).[30] (**b**) Peritoneal macrophage numbers at day 4 from mBSA-challenged mice. (**c**) Total blood monocyte numbers from mBSA-challenged mice (measured as in Figure 1). (**d**) Monocyte subpopulation numbers from mBSA-challenged mice (measured as in Figure 1). (**e**) Bone marrow monocyte number from mBSA-challenged mice (measured as in Figure 1). Data are expressed as total macrophage number, monocytes per ml or bone marrow monocytes/femur (mean ± s.e.m.); n=10 mice. $^{*}P<0.05$, mAb treatment versus isotype control. (**f**) BALB/c mice were pretreated with isotype control, AFS98 or 22E9 (100 μg per mouse) 3 h before LPS challenge (10 μg per mouse) intranasally. Control mice received PBS intranasally, instead of LPS, as well as isotype control. Bronchoalveolar lavage fluid macrophages and neutrophils were harvested by lavage 3 days after LPS administration or PBS. Data are expressed as total cell number (mean ± s.e.m.); n=6–8 mice. $^{*}P<0.05$, LPS/mAb versus LPS/isotype; $^{\#}P<0.05$, LPS/isotype versus PBS/isotype.

monocytes at the 4-day time point examined, their total numbers and those of the subpopulations in C57BL/6 mice treated with isotype control mAb were similar to the steady state values (Figures 3c and d versus 1b and c). Following AFS98 and 22E9 administration, there was no accumulation of monocyte populations to account for the reduced peritoneal exudate macrophage numbers, but rather similar data to what was noted in the steady state (Figure 1c) with the anti-CSF-1R mAb, but not the anti-GM-CSF mAb, reducing the $Ly6C^{lo}$ monocytes (Figure 3d). The number of $Ly6C^{hi}$ $CD11b^{+}$ bone marrow monocytes also did not increase at this time point in the isotype control group (Figures 3e versus 1d) and it was not altered in either of the neutralizing mAb-treated groups (Figure 3e).

Lung inflammation. In an LPS-induced lung inflammation model, it can be seen that blockade with AFS98 and with 22E9[35,36] reduces the LPS-induced increase in alveolar macrophage numbers (Figure 3f). Increased neutrophil numbers, in contrast, were suppressed only by 22E9 administration. In this model, a different anti-CSF-1R mAb was reported as failing to reduce macrophage numbers.[29]

Thus in our hands, CSF-1R blockade had both tissue and systemic manifestations on macrophage lineage numbers during an acute inflammatory response, suggesting a link between the two, whereas GM-CSF neutralization had its effects only locally (but see below and Discussion).

Monocyte/macrophage populations in blood and tissue of GM-CSF−/− mice during the course of an inflammatory reaction

In addition to GM-CSF neutralization by mAb, we assessed the effect of the complete absence of GM-CSF by using the GM-CSF−/− mouse (backcrossed onto the C57BL/6 background[14,37]), partly for convenience, because we wanted to use these mice to study the role of GM-CSF over the time course of a peritoneal inflammatory reaction. In the steady state, no significant difference in the total number of blood monocytes could be seen between wild-type (C57BL/6) and GM-CSF−/− mice (Figure 4a), in agreement with the literature.[12,14] However, on closer examination, unlike anti-GM-CSF mAb treatment (Figure 1c), a slight decrease in the number of the minor $Ly6C^{lo}$ monocyte subpopulation could in fact be measured (Figure 4b;

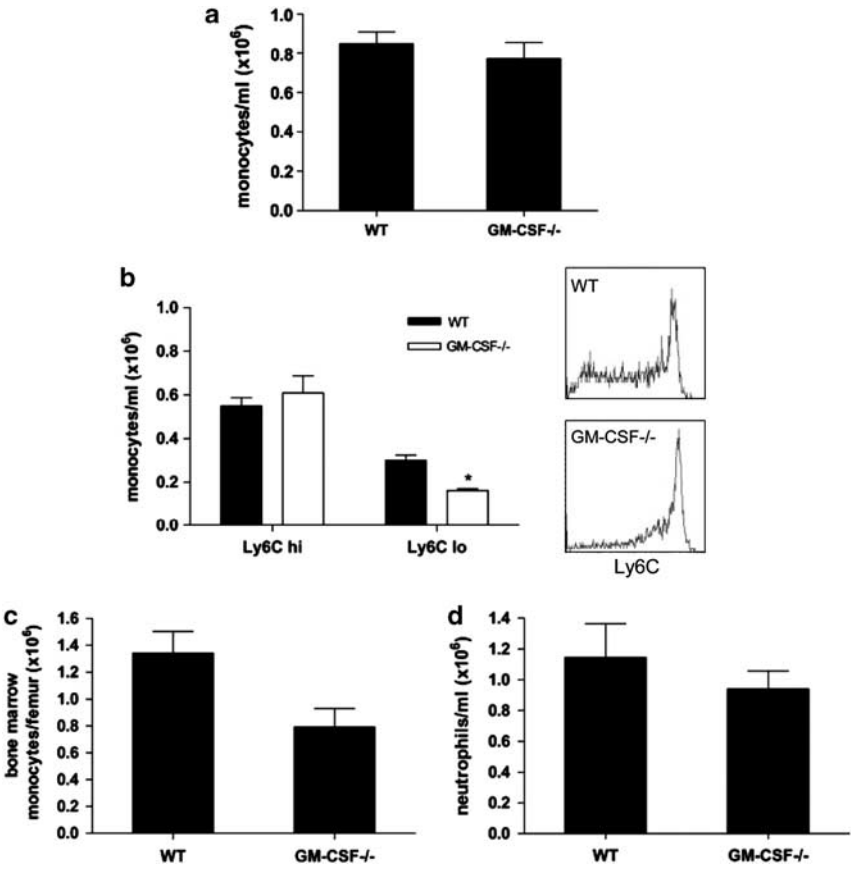

Figure 4 Myeloid cell numbers in GM-CSF−/− mice in the steady state. Myeloid cell populations from wild-type (C57Bl/6) and GM-CSF−/− mice were examined by flow cytometry as in Figure 1. (**a**) Number of blood monocytes. (**b**) Ly6C^{hi} and Ly6C^{lo} monocyte subpopulation numbers. (**c**) Bone marrow monocyte number. (**d**) Number of blood neutrophils. Data are expressed as monocytes per ml, bone marrow monocytes/femur or neutrophils per ml (mean ± s.e.m.); n=10 mice. *P<0.05, GM-CSF−/− versus wild type.

see Discussion). There was no decrease in the number of bone marrow monocytes (Figure 4c) or blood neutrophils (Figure 4d).

Data above assessed the CSF contribution to monocyte/macrophage development separately in blood, in bone marrow and in tissue. It would be useful to be able to link any CSF-dependent changes noted at these respective sites during the course of an inflammatory reaction. As such detailed kinetic analysis would require a significant supply of mAbs, for convenience we focused on assessing the time course of the GM-CSF dependence of mBSA-induced peritonitis using GM-CSF−/− mice; *Csf1r*$^{-}$/*Csf1r*$^{-}$ and *Csf1*$^{op/op}$ were again not used because of the compromised bone architecture.[5–7]

Peritoneal macrophages and neutrophils. For wild-type and GM-CSF−/− mice, reduced numbers of macrophages were initially seen in the exudate (up to around 12 h; Figure 5a); this is a well-documented occurrence during an inflammatory reaction in the peritoneum and is referred to as the 'macrophage disappearance reaction'.[38] An increase in recoverable macrophages was again observed for both strains from about 12–18 h post challenge (Figure 5a). We showed before[31] for wild-type mice that total macrophage numbers peaked around day 4, but were still elevated at least until day 7. A similar peak can be seen in Figure 5a, whereas in GM-CSF−/− mice, the numbers again remained low throughout,[31] indicating the need for GM-CSF in prolonging the macrophage component in this inflammation model.[30] These data are consistent with the 22E9-dependent suppression of peritoneal macrophage number in this model (Figure 3b).

Given the differences for the macrophage changes between the two mouse strains, we decided also to compare changes in neutrophil numbers. When peritoneal neutrophil numbers were followed in the same way, for the wild-type mice, as expected,[31] there was an earlier influx than for the macrophages, with a peak around 18–24 h (Figure 5b); in the GM-CSF−/− case, an early influx preceded an earlier decline at around 18 h, that is, the later phase of the influx was again not prolonged beyond this time point.

Peritoneal macrophage subpopulations. Peritoneal macrophage subpopulations were next examined, again using Ly6C expression. As before,[39] in wild-type mice, the resident macrophages were Ly6C^{lo} and were replaced by exudate macrophages, which initially were Ly6C^{hi}, but then were Ly6C^{lo}, as the mBSA-induced peritoneal inflammation proceeded (Figure 6a). It can be seen that this transition from Ly6C^{hi} to Ly6C^{lo} expression was gradual rather than being abrupt, indicating perhaps local conversion rather than separate trafficking kinetics being responsible (see Discussion). During the course of the reaction, neutrophils were also found in the same FSC/SSC region; however, these were removed for the macrophage analysis by gating out Ly6G^{+} cells, a widely used neutrophil marker.[40]

The comparative data for the kinetics of appearance of Ly6C^{hi} and Ly6C^{lo} macrophages in wild-type versus GM-CSF−/− mice are

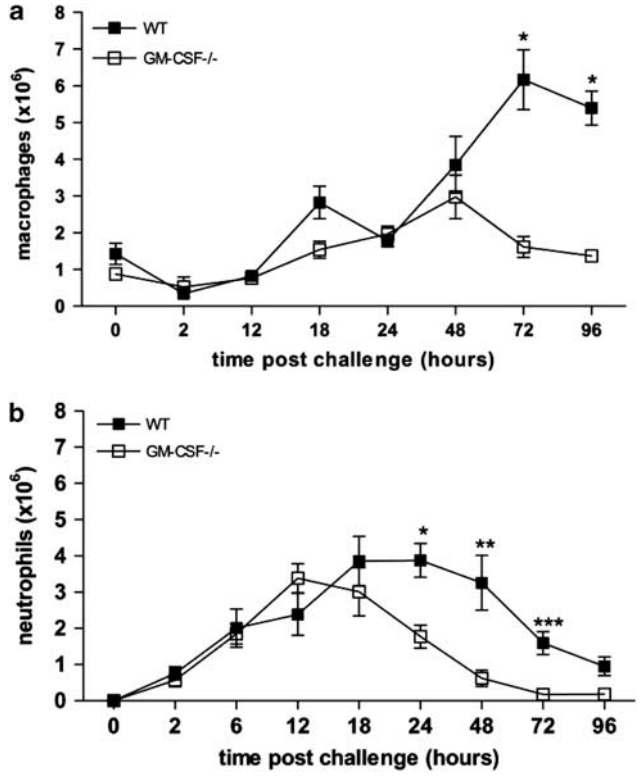

Figure 5 Kinetics of the changes in macrophage and neutrophil numbers during peritoneal inflammation in wild-type versus GM-CSF−/− mice. Wild-type and GM-CSF−/− mice were sensitized to mBSA and then challenged i.p. with mBSA to induce a peritoneal inflammatory reaction; (**a**) peritoneal exudate macrophage (FSC/SSC gating and CD11b$^+$ Ly6G$^-$) and (**b**) neutrophil (Ly6G$^+$) numbers were analyzed at various time points post challenge. Data are expressed as total cell number (mean ± s.e.m.); n=10 mice. (**a**) $^*P<0.0001$, wild type versus GM-CSF−/−; (**b**) $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, wild type versus GM-CSF−/−.

provided in Figures 6b and c, respectively. For the wild-type macrophage response, the presence of Ly6C^{hi} macrophages had a similar, but delayed kinetic profile to the neutrophils (Figures 6b versus 5b), with a peak around 48–72 h preceding a decline by 96 h; in GM-CSF−/− mice, as for the neutrophils (Figure 5b), the Ly6C^{hi} macrophage numbers declined earlier (Figure 6b). For the wild-type mice, the later macrophage response (around 72–96 h; Figure 5a) is in fact characterized by the appearance of Ly6C^{lo} macrophages (Figure 6c), as found before in the thioglycolate-elicited model,[39] which did not occur in the gene-deficient mice (Figure 6c).

Blood monocyte subpopulations and neutrophils. Blood monocyte subpopulation numbers in the two mouse strains were also monitored over the same time course; the major Ly6C^{hi} population fluctuated differently for the two strains (Figure 6d), whereas no obvious differences were detected in the less abundant Ly6C^{lo} population, which hardly altered if at all (Figure 6e). For the wild-type mice, around the time when (Ly6C^{hi}) macrophage numbers started to increase in the peritoneum (around 18 h; Figures 5a and 6b), there was also a peak in Ly6C^{hi} monocyte numbers (Figure 6d). It is tempting to suggest that the subsequent dip in Ly6C^{hi} monocyte numbers (Figure 6d) at about the time that the peritoneal macrophage numbers started to climb more dramatically (24–72 h; Figure 5a) is related to the high 'demand' occurring at the inflammatory site (see Discussion); perhaps, the different pattern at these time points in the GM-CSF−/− mice is related to the lack of this 'demand' (see Discussion). It could also be that the peak in the number of the bone marrow Ly6C^{hi} monocytes at around 48 h for the wild-type mice (Figure 6f) is due to this site, endeavouring to provide sufficient Ly6C^{hi} blood monocytes for the local inflammatory response (Figures 5a and 6d).

At around the time of peritoneal neutrophil influx in wild-type mice (2 h, Figure 5b), for blood neutrophil numbers, there was a transient peak, which for some reason was slightly delayed in GM-CSF−/− mice (Figure 6g). For both mouse strains, when polymorphonuclear neutrophils numbers rose in the inflamed peritoneum (around 6–18 h; Figure 5b), there was a dip in circulating polymorphonuclear neutrophils numbers (Figure 6g), suggesting a possible relationship; at times >18 h for wild-type mice, but not GM-CSF−/− mice, the polymorphonuclear neutrophils numbers were prolonged in the peritoneum (Figure 5b) and possibly in blood (Figure 6g).

DISCUSSION

We adopted strategies to study the contribution of CSF-1R and GM-CSF to monocyte/macrophage subpopulation numbers, both in the steady state and during inflammation. For CSF-1R involvement, we chose to use mAb neutralization in adult mice rather than gene-deficient mice on account of the compromised bone marrow cavity in these mice, leading to compensatory myelopoiesis elsewhere, and also because of the age-related recovery in macrophage lineage development[6,7] and the conflicting reports as to the extent of the monocytopenia.[6–8,10,12,41–44] This approach also has an advantage over CSF-1R kinase inhibitors, as such inhibitors have other targets that potentially complicate the interpretation of some of their effects.

We were able to observe a surprisingly specific and rapid ($\leqslant$4 days) CSF-1R-dependent reduction in the number of the Ly6C^{lo} blood monocyte subpopulation in the steady state (Figure 1c); we suggest that this rapid loss of Ly6C^{lo} monocytes is consistent with the *in vivo* evidence that Ly6C^{hi} monocytes mature quickly into Ly6C^{lo} monocytes within a 48-h period.[17] CSF-1 has been shown *in vitro* to modulate Ly6C expression in macrophage lineage cells,[45] but it has remained unclear as to the events that control this conversion *in vivo*.[17,46,47] Seeing that we did not find evidence of Ly6C^{hi} monocyte accumulation upon anti-CSF-1R administration, it is possible that, rather than there being a simple CSF-1-dependent blockade in the conversion from Ly6C^{hi} to Ly6C^{lo} cells, the Ly6C^{lo} population and not the immature Ly6C^{hi} monocytes depend upon CSF-1R activity for the maintenance of their numbers *in vivo* by enhancing their survival. The lack of effect of the anti-CSF-1R mAb on Ly6C^{hi} monocyte numbers would not seem to be due to lack of receptor signaling in this population, as they have similar CSF-1R surface expression (Figure 2e) and both monocyte populations can proliferate *in vitro* in the presence of CSF-1;[17] it could be that the Ly6C^{hi} population has another CSF-1R-independent mechanism to maintain their numbers. It is also possible, for some reason, that the Ly6C^{lo} monocytes are preferentially killed. This preferential depletion of the less abundant Ly6C^{lo} subpopulation is consistent with the partial monocytopenia observed in some, but not all studies with *Csf1r*$^{-}$/*Csf1r*$^{-}$ mice and *Csf1*$^{op/op}$.[6–8,10,12,41–44] The data suggests that CSF-1R activity, presumably resulting from CSF-1 (and/or IL-34) activity, is not required until relatively late in macrophage lineage development under homeostatic conditions, the stage of the CSF-1 requirement being a controversial issue.[48] Since the submission of this manuscript, it has also been shown[49] that in the steady state, AFS-98 lowers

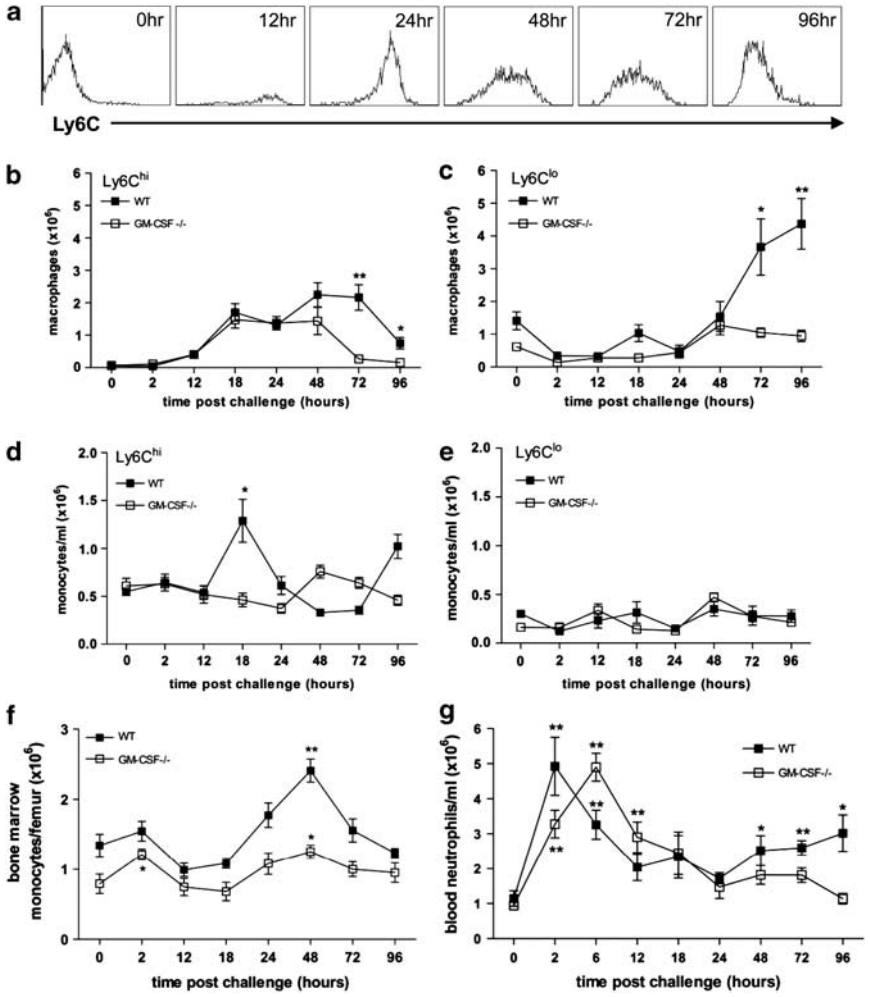

Figure 6 Kinetics of the changes in monocyte/macrophage subpopulation numbers during peritoneal inflammation in wild-type versus GM-CSF−/− mice. Wild-type and GM-CSF−/− mice were sensitized to mBSA and then challenged i.p. to induce a peritoneal inflammatory reaction. (**a**–**e**) Ly6C surface expression was measured on peritoneal macrophages and blood monocytes at various time points post challenge. (**a**) Peritoneal macrophages from wild-type mice. (**b** and **c**) Peritoneal macrophage subpopulation numbers in wild-type and GM-CSF−/− mice: (**b**) Ly6C^{hi}; (**c**) Ly6C^{lo}. (**d** and **e**) Peripheral blood monocyte subpopulation numbers in C57BL/6 and GM-CSF−/− mice: (**d**) Ly6C^{hi}; (**e**) Ly6C^{lo}. (**f**) Bone marrow monocyte number. (**g**) Peripheral blood neutrophil numbers. Data are expressed as total cell number, monocytes per ml, bone marrow monocytes/femur or neutrophils per ml (mean ± s.e.m.); n=10 mice per strain. (**b** and **c**) $^*P<0.05$, $^{**}P<0.001$, time point post challenge versus Day 0; (**d**) $^*P<0.01$, time point post challenge versus Day 0; (**f**) $^*P<0.05$, $^{**}P<0.001$, time point post challenge versus Day 0; (**g**) $^*P<0.05$, $^{**}P<0.0001$, time point post challenge versus Day 0.

preferentially the Ly6C^{lo} blood monocyte subpopulation, as well as F4/80$^+$ splenic macrophages.

Others failed to note a decline in total blood monocyte numbers after daily injections of AFS98 for 5 days,[50] possibly because subpopulations were not examined. Recently, following administration of a different neutralizing anti-CSF-1R mAb (M279, IgG1), it was reported in Macgreen mice that the Gr-1^{neg} (that is, Ly6C^{lo}) monocyte subpopulation was also specifically depleted with accumulation of the Gr-1^{hi} subset;[29] however, unlike our observations above, this reduction was slow and required more than 7 days after the last injection.[29] We also did not confirm a compensatory accumulation of the Ly6C^{hi} (that is, Gr-1^{hi}) subpopulation. The reason(s) for these differences with the respective anti-CSF-1R mAbs is unknown although the often-used AFS98 is IgG2a, whereas the new anti-CSF-1R mAb (M279) in MacDonald *et al.*[29] is IgG1 (see below). Differences in the properties of the respective IgG2a and IgG1 mAbs, possibly even due to a different mode of action, presumably account for the different kinetics of the Ly6C^{lo} monocyte depletion.

Whether the rapidly reduced peritoneal macrophage (Ly6C^{lo}) numbers in the steady state, following anti-CSF-1R mAb injection ($\leqslant$3 days) (Figure 2c), are due to a similar mechanism(s) in the peritoneum, as proposed above for the blood, is unknown. There is evidence for local control of macrophage numbers in the unstimulated peritoneal cavity,[51,52] in contrast to earlier conclusions,[53] and the evidence for the role of blood Ly6C^{lo} monocytes as precursors for resident tissue cells, is rather limited.[54] However, it is possible that the fewer resident peritoneal macrophages observed could be due to some extent to the reduced numbers of blood Ly6C^{lo} monocytes following anti-CSF-1R neutralization being available to migrate. Our resident peritoneal macrophage data is also compatible with the data for both *Csf1r*$^{-}$/*Csf1r*$^{-}$ and *Csf1*$^{op/op}$ mice,[6,7] and with the rapid loss of i.p. CSF-1R$^+$ osteoclast precursors 3 days after intravenous injection of AFS98.[55] Our data is also consistent with the rapid reduction (within 4 days) of steady state tissue macrophages following administration of an orally active CSF-1R tyrosine kinase inhibitor.[56]

As regards inflammation, we showed above that the widely used anti-CSF-1R mAb, AFS98, reduced the number of (Ly6C^{lo}) macrophages in two peritonitis models and of alveolar macrophages in LPS-induced lung inflammation; for thioglycolate-induced peritonitis, our data are similar to the effects of an orally active CSF-1R tyrosine kinase inhibitor,[57] but at variance with negative findings using a different CSF-1R mAb (M279) in this model and in the same lung inflammation model,[29] leading the authors to conclude that CSF-1 signaling does not have a non-redundant function in inflammation. Our different findings are consistent with those in atherosclerosis models with a reduction in lesion size upon AFS98 administration, correlating with a reduction in circulating monocyte number, as well as in *Csf1*$^{op/op}$ mice;[58,59] they are also consistent with the reduced inflammatory response to both CSF-1R and CSF-1 inhibition in different autoimmune/inflammatory settings.[9,15,50,57,58,60–62] Thus, the conclusions drawn about the role of CSF-1R, and by implication CSF-1 (and/or IL-34), in inflammation, following CSF-1R mAb administration, appear to depend on the particular anti-CSF-1R mAb administered.

Murine Ly6C^{lo} (Gr-1^{-}) monocytes are termed 'non-classical', 'resident' or 'patrolling' monocytes.[20] They express more of the proinflammatory cytokine, tumor necrosis factor, than the so-called 'classical' monocytes;[63] Ly6C^{lo} monocytes are considered to be analogous to the human CD16^{+} 'non-classical' monocyte subpopulation[20,64] and, interestingly, in the context of our findings, it is this subpopulation which is depleted in rheumatoid arthritis blood after treatment with neutralizing anti-CSF-1 mAb.[65] Thus, we suggest that in the mouse, depletion of non-classical Ly6C^{lo} monocytes may contribute to the anti-inflammatory effects of CSF-1R and CSF-1 inhibition.

For anti-GM-CSF mAb treatment, there were no discernable changes in total monocyte (Figure 1b), monocyte subpopulation (Figure 1c) or peritoneal macrophage numbers (data not shown) in the steady state. However, in GM-CSF−/− mice, whereas there was no significant reduction in total monocyte numbers in the steady state,[12,14] a more detailed analysis showed slightly fewer of the less abundant Ly6C^{lo} monocytes (Figure 4b). The reason(s) for the difference from the anti-GM-CSF data is unknown, although developmental effects in the GM-CSF−/− may be contributing. Even though no decrease in monocyte numbers was noted in the GM-CSF−/− mouse,[12,14] there was a compromised cytokine response to LPS, suggesting perhaps altered monocyte/macrophage function.[14,66] Our data showing reduced Ly6C^{lo} monocytes in the steady state in the GM-CSF−/− mouse is consistent with these lower cytokine levels and indicates that there may be other subtle differences not yet discovered in the GM-CSF-deficient mouse.[12,14,67,68] It is interesting that we could not demonstrate similar findings in the steady state with the neutralizing anti-GM-CSF mAb, possibly because the latter approach could not decrease the levels of GM-CSF sufficiently. In this connection, it has been shown that cell survival requires only low GM-CSF concentrations with higher levels required for additional myeloid cell functions, such as differentiation and activation.[69] Even though anti-GM-CSF mAb administration did not reduce steady state Ly6C^{lo} monocyte/macrophage cell numbers, it could be that other properties of monocytes/macrophages (and neutrophils) are altered.[70]

We previously showed for the first time in any inflammation model, using i.p. thioglycolate injection, that immature Ly6C^{hi} macrophages were observed early in the exudate to be replaced by Ly6C^{lo} macrophages.[39] To be able to compare directly in the same experiment the involvement of CSF-1R and GM-CSF on macrophage lineage populations in an inflammation model, we chose mBSA-dependent acute peritonitis established in our laboratory,[31] as unlike the widely used thioglycolate-induced peritonitis model, we had shown that the maintenance of the inflammatory reaction was GM-CSF-dependent.[30] Also, an antigen-driven model would serve as a useful one for immune-driven inflammation models in general. The lower (Ly6C^{lo}) macrophage numbers during mBSA-induced peritoneal inflammation upon CSF-1R neutralization (Figure 3b) could be due to their derivation from the Ly6C^{hi} macrophages in the inflamed peritoneum and/or be due to their independent migration from the depleted Ly6C^{lo} blood monocyte population (Figure 3d). Both mechanisms for the control of Ly6C^{lo} tissue macrophage numbers have been claimed;[16,24,25,27] our data (Figure 6a) showing a loss of Ly6C^{hi} over time on the exudate macrophages would appear to be more consistent for the former mechanism. For the acutely inflamed peritoneum in mice, in contrast to Ly6C^{hi} monocytes, it has been difficult to show Ly6C^{lo} blood monocyte migration.[16,17,54]

The neutralizing anti-GM-CSF mAb also was able to reduce the day 4 peritoneal exudate (Ly6C^{lo}) macrophage numbers when administered locally (Figure 3b), but not systemically (that is, subcutaneously). We previously used GM-CSF−/− mice to show that for sterile peritonitis models, a requirement for GM-CSF in the control of exudate cell numbers was stimulus dependent.[30] We also showed before, by this strategy in the mBSA-dependent model, that the exudate macrophages in the gene-deficient mice, while being present in lower numbers, were also defective in properties such as inflammatory cytokine production and phagocytosis.[30] During peritonitis development, in agreement with the 22E9 treatment (Figure 3b), in GM-CSF−/− mice, there were reduced numbers of exudate (Ly6C^{lo}) macrophages at day 4 (Figures 5a and 6c). When the kinetics of the reaction were followed, it appears that levels of peritoneal Ly6C^{hi} macrophages (Figure 6b) and neutrophils (Figure 5b) early in the response are relatively preserved which, however, are not prolonged due to GM-CSF deficiency.

Consistent with the possibility of there being a GM-CSF-dependent signal(s) from the peritoneum to the blood, there was suggestive evidence that Ly6C^{hi} monocyte levels in wild-type mice, but not in GM-CSF−/− mice, are reduced in the blood at 48–72 h (Figure 6d), that is, 1–2 days before the peak of the appearance of the exudate macrophages in the former mice (Figure 5a). A possible explanation for the lower numbers of blood Ly6C^{hi} monocytes in wild-type mice at the peak of the macrophage reaction in the inflamed tissue is that, the monocyte supply is briefly inadequate to meet the demand of providing large numbers of macrophages for this reaction—such a transient reduction in monocyte numbers has been observed in both thioglycolate and *L. monocytogenes*—elicited peritonitis.[17,71] We are proposing that the above mentioned GM-CSF-dependent signal(s) is missing in GM-CSF−/− mice, leading to a lack of continued demand for cellular influx into the peritoneal cavity, which is reflected systemically in the Ly6C^{hi} blood monocyte and neutrophil profiles (Figures 6d and g, respectively). The lack of an early effect on neutrophil (and macrophage) infiltration in GM-CSF−/− mice is consistent with findings for the acute neutrophil response in casein-induced peritonitis and with the observation that i.p. GM-CSF injection does not induce such an influx.[72] In addition to these direct or indirect systemic effects of GM-CSF, it is also likely that GM-CSF is required locally in the inflamed peritoneum, not only to 'activate' macrophages[15,30,73] and neutrophils, but also to directly maintain macrophage numbers, for example, by a pro-survival action.[1,74] The reduction in peritoneal exudate macrophages at day 4 in GM-CSF−/− mice (Figure 5a) is more pronounced than that achieved by anti-GM-CSF mAb injection (Figure 3b), possibly because GM-CSF is entirely absent throughout.

However, this difference may be simply due to incomplete GM-CSF depletion by 22E9.

Given what is known about the respective biologies of CSF-1 and GM-CSF,[15] it would be surprising if there were no different effects on macrophage lineage populations upon neutralization/depletion of CSF-1R and GM-CSF activity, both in the steady state and during an inflammatory reaction. CSF-1 circulates at detectable levels in the steady state and is constitutively produced *in vitro* by several cell types;[15] in contrast, the production of GM-CSF usually requires stimulation, for example by infection/inflammation, to be measured easily. Based on the literature and our data above, we propose a model in which CSF-1R activity, and by implication CSF-1 and/or the recently described CSF-1R ligand, IL-34,[13] is required for the generation of relatively more mature macrophage lineage populations in blood and tissue, both during homeostasis and inflammation; on the other hand, in inflammation, GM-CSF can be important for the prolonged mobilization of less mature monocytes, and also of polymorphonuclear neutrophils, from bone marrow to blood to tissue.[28,75] Obviously, additional studies are required to test the general relevance or not of this model.

METHODS

Mice

GM-CSF gene-deficient (GM-CSF−/−) mice, backcrossed onto the C57BL/6 background for 11 generations, were originally provided by the Ludwig Institute for Cancer Research (Parkville, Victoria, Australia). C57BL/6 mice were originally obtained from Central Animal Services, Monash University (Clayton, Victoria, Australia).[37] *Cfms-GFP* (Macgreen) mice carry a transgene encoding green fluorescent protein driven by the *csf1r* promoter.[76] These mice have been back crossed onto C57BL/6 for 12 generations. These strains of mice are bred in our on-site animal facility. BALB/c mice were obtained from the Animal Resource Centre Pty. Ltd (Perth, Australia). Mice were fed standard rodent chow and water *ad libitum* and were housed in sawdust-lined cages in groups of five. Mice of 8–12 week old were used in all experiments, which were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conducted in compliance with the guidelines of the National Health and Medical Research Council of Australia on animal experimentation.

Reagents

mAbs against the following antigens were used in this study: CD11b (Mac-1 α-chain; M1/70-allophycocyanin: BD Pharmingen, San Diego, CA, USA), Ly6C (AL-21-FITC: BD Pharmingen; HK1.4-PerCP-Cy5.5: eBioscience, San Diego, CA, USA), Ly6G (1A8-PE; BD Pharmingen), CD115 (c-Fms: AFS98 PE and biotin: eBioscience). The following hybridomas (IgG2a) were gifts: neutralizing anti-mouse GM-CSF (22E9.11: Dr J Abrams, Schering Biopharma, Palo Alto, CA, USA), anti-β-galactosidase (GL117.41: Schering Biopharma) and anti-(CSF-1R) c-Fms (AFS98: Dr S-I Nishikawa, Kobe, Japan). Streptavidin PerCP (BD Pharmingen) was used as a secondary, where appropriate.

Flow cytometric analysis

Cells were washed twice in phosphate-buffered saline (PBS), counted and resuspended in fluorescence activated cell sorting buffer (1% BSA in PBS containing 0.01% NaN_3). For phenotypic analysis, cells (1×10^6 cells/stain) were initially incubated with 10% mouse serum for 20 min at 4 °C. Subsequently, cells were incubated with the appropriately labeled primary Abs, followed by the appropriate secondary Ab. All incubations were performed on ice for 20 min and were followed with three washes with fluorescence activated cell sorting buffer. Appropriate isotype controls were used in all cases. For flow cytometric analysis, a typical forward and side-scatter gate was set to exclude dead cells and aggregates; a total of 10^4 events in the gate were collected and analyzed using a FACSort and software (BD Biosciences, San Jose, CA, USA).[31]

Peritonitis models

As before,[31] mice were immunized intradermally in the base of the tail with 100 μg of mBSA (Sigma-Aldrich, St Louis, MO, USA), emulsified in an equal volume of CFA containing 5 mg ml^{-1} heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco, Detroit, MI, USA); 14 days later the primary immunization protocol was repeated as a boost. 7 days later (that is, day 21 after the primary immunization), peritoneal exudate cells were elicited by i.p. injection of 100 μg of mBSA. Peritoneal exudate cells were also elicited by i.p. injection of 1 ml of Brewer's thioglycolate medium (Difco) as previously described.[39] Cells were harvested at various time points after injection, blood collected by cardiac puncture, bone marrow cells by flushing from mouse femurs, and peritoneal exudate cells by lavaging with 5 ml of ice-cold, sterile PBS.

LPS-induced lung inflammation

Mice were anesthetized lightly by inhalation of Penthrane vapor and 50 μl of (a) PBS and (b) maximally tolerated dose of LPS (10 μg of *Escherichia coli* serotype 026:B6: (Sigma) in 50 μl of PBS), administered intranasally as previously described.[35,36] After 72 h of LPS or PBS treatment, mice were killed by an i.p. overdose of anesthetic (sodium pentobarbital, 300 mg kg^{-1} i.p.). Lungs from each terminally anesthetized mouse were lavaged *in situ* with a 400 μl aliquot, followed by three 300 μl aliquots of PBS as before.[35,36,78] The total number of viable cells in the bronchoalveolar lavage fluid was determined, cytospins prepared using 50–200 μl bronchoalveolar lavage fluid and cells differentiated by standard morphologic criteria. A minimum of 500 cells per slide were counted.

mAb blockade

In steady state experiments, mice were usually injected daily, either subcutaneously or i.p., for 4 days, with 300 μg of AFS98 (anti-CSF-1R), 22E9 (anti-GM-CSF) or an isotype IgG2a control (anti-β-galactosidase). In the mBSA-induced and thioglycolate-induced peritonitis experiments, mice were injected i.p., daily from day −2 to 3, with day 0 being the time of initiation of the peritonitis. For the LPS-induced lung inflammation experiments, mice were anesthetized lightly by inhalation of Penthrane vapor and 30 μl of the same Abs (100 μg per mouse), administered intranasally. The mAbs were administered 3 h before LPS or PBS.

Quantitation of CSF-1

CSF-1 levels in peritoneal fluid and serum were assayed using ELISA according to manufacturer's instructions (PeproTech, Rocky Hill, NJ, USA).

Statistics

A *t*-test was usually used to determine statistical differences and $P<0.05$ was considered significant. For lung inflammation experiments, data was analyzed using the two-way analysis of variance and Bonferroni post-test.

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1 Hamilton JA, Tak PP. The dynamics of macrophage lineage populations in inflammatory and autoimmune diseases. *Arthritis Rheum* 2009; **60**: 1210–1221.
2 Pollard JW. Macrophages define the invasive microenvironment in breast cancer. *J Leukoc Biol* 2008; **84**: 623–630.
3 Stanley ER, Hansen G, Woodcock J, Metcalf D. Colony stimulating factor and the regulation of granulopoiesis and macrophage production. *Fed Proc* 1975; **34**: 2272–2278.
4 Metcalf D, Nicola NA. *Biochemistry of the CSFs*, Vol 1, 1st edn. Cambridge University Press, New York, USA, 1995.
5 Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H *et al.* The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 1990; **345**: 442–444.
6 Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H *et al.* Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* 1994; **120**: 1357–1372.
7 Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S *et al.* Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteope-

trosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* 2002; **99**: 111–120.
8 Bergmann CE, Hoefer IE, Meder B, Roth H, van Royen N, Breit SM *et al.* Arteriogenesis depends on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. *J Leukoc Biol* 2006; **80**: 59–65.
9 Chitu V, Stanley ER. Colony-stimulating factor-1 in immunity and inflammation. *Curr Opin Immunol* 2006; **18**: 39–48.
10 Dai XM, Zong XH, Sylvestre V, Stanley ER. Incomplete restoration of colony-stimulating factor 1 (CSF-1) function in CSF-1-deficient Csf1op/Csf1op mice by transgenic expression of cell surface CSF-1. *Blood* 2004; **103**: 1114–1123.
11 Ginhoux F, Tacke F, Angeli V, Bogunovic M, Loubeau M, Dai XM *et al.* Langerhans cells arise from monocytes *in vivo*. *Nat Immunol* 2006; **7**: 265–273.
12 Nilsson SK, Lieschke GJ, Garcia-Wijnen CC, Williams B, Tzelepis D, Hodgson G *et al.* Granulocyte-macrophage colony-stimulating factor is not responsible for the correction of hematopoietic deficiencies in the maturing op/op mouse. *Blood* 1995; **86**: 66–72.
13 Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E *et al.* Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science* 2008; **320**: 807–811.
14 Stanley E, Lieschke GJ, Grail D, Metcalf D, Hodgson G, Gall JA *et al.* Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci USA* 1994; **91**: 5592–5596.
15 Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol* 2008; **8**: 533–544.
16 Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003; **19**: 71–82.
17 Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA *et al.* Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* 2004; **172**: 4410–4417.
18 Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**: 953–964.
19 Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity* 2005; **23**: 344–346.
20 Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010; **116**: e74–e80.
21 Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J Immunol* 2007; **178**: 5245–5252.
22 Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 2009; **27**: 451–483.
23 Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E *et al.* Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci USA* 2004; **101**: 4560–4565.
24 Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A *et al.* Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 2007; **204**: 1057–1069.
25 Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007; **317**: 666–670.
26 Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* 2009; **27**: 669–692.
27 Landsman L, Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol* 2007; **179**: 3488–3494.
28 King IL, Dickendesher TL, Segal BM. Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. *Blood* 2009; **113**: 3190–3197.
29 MacDonald KP, Palmer JS, Cronau S, Seppanen E, Olver S, Raffelt NC *et al.* An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood* 2010; **116**: 3955–3963.
30 Cook AD, Braine EL, Hamilton JA. Stimulus-dependent requirement for granulocyte-macrophage colony-stimulating factor in inflammation. *J Immunol* 2004; **173**: 4643–4651.
31 Cook AD, Braine EL, Hamilton JA. The phenotype of inflammatory macrophages is stimulus dependent: implications for the nature of the inflammatory response. *J Immunol* 2003; **171**: 4816–4823.
32 Sudo T, Nishikawa S, Ogawa M, Kataoka H, Ohno N, Izawa A *et al.* Functional hierarchy of c-kit and c-fms in intramarrow production of CFU-M. *Oncogene* 1995; **11**: 2469–2476.
33 Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER. Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. *Cell* 1982; **28**: 71–81.
34 Bartocci A, Mastrogiannis DS, Migliorati G, Stockert RJ, Wolkoff AW, Stanley ER. Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc Natl Acad Sci USA* 1987; **84**: 6179–6183.
35 Bozinovski S, Jones JE, Vlahos R, Hamilton JA, Anderson GP. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFkappa B and AP-1 *in vivo*. *J Biol Chem* 2002; **277**: 42808–42814.
36 Bozinovski S, Jones J, Beavitt SJ, Cook AD, Hamilton JA, Anderson GP. Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. *Am J Physiol Lung Cell Mol Physiol* 2004; **286**: L877–L885.
37 Campbell IK, Rich MJ, Bischof RJ, Dunn AR, Grail D, Hamilton JA. Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. *J Immunol* 1998; **161**: 3639–3644.
38 Barth MW, Hendrzak JA, Melnicoff MJ, Morahan PS. Review of the macrophage disappearance reaction. *J Leukoc Biol* 1995; **57**: 361–367.
39 Chan J, Leenen PJ, Bertoncello I, Nishikawa SI, Hamilton JA. Macrophage lineage cells in inflammation: characterization by colony-stimulating factor-1 (CSF-1) receptor (c-Fms), ER-MP58, and ER-MP20 (Ly-6C) expression. *Blood* 1998; **92**: 1423–1431.
40 Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol* 1993; **151**: 2399–2408.
41 Lieschke GJ, Grail D, Hodgson G, Metcalf D, Stanley E, Cheers C *et al.* Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood* 1994; **84**: 1737–1746.
42 Ryan GR, Dai XM, Dominguez MG, Tong W, Chuan F, Chisholm O *et al.* 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* 2001; **98**: 74–84.
43 Wiktor-Jedrzejczak W, Ratajczak MZ, Ptasznik A, Sell KW, Ahmed-Ansari A, Ostertag W. CSF-1 deficiency in the op/op mouse has differential effects on macrophage populations and differentiation stages. *Exp Hematol* 1992; **20**: 1004–1010.
44 Wiktor-Jedrzejczak WW, Ahmed A, Szczylik C, Skelly RR. Hematological characterization of congenital osteopetrosis in op/op mouse. Possible mechanism for abnormal macrophage differentiation. *J Exp Med* 1982; **156**: 1516–1527.
45 de Bruijn MF, Slieker WA, van der Loo JC, Voerman JS, van Ewijk W, Leenen PJ. Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. *Eur J Immunol* 1994; **24**: 2279–2284.
46 Qu C, Edwards EW, Tacke F, Angeli V, Llodra J, Sanchez-Schmitz G *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**: 1231–1241.
47 Tacke F, Ginhoux F, Jakubzick C, van Rooijen N, Merad M, Randolph GJ. Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. *J Exp Med* 2006; **203**: 583–597.
48 Lagasse E, Weissman IL. Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. *Cell* 1997; **89**: 1021–1031.
49 Hashimoto D, Chow A, Greter M, Saenger Y, Kwan WH, Leboeuf M *et al.* Pretransplant CSF-1 therapy expands recipient macrophages and ameliorates GVHD after allogeneic hematopoietic cell transplantation. *J Exp Med* 2011; **208**: 1069–1082.
50 Le Meur Y, Tesch GH, Hill PA, Mu W, Foti R, Nikolic-Paterson DJ *et al.* Macrophage accumulation at a site of renal inflammation is dependent on the M-CSF/c-fms pathway. *J Leukoc Biol* 2002; **72**: 530–537.
51 Melnicoff MJ, Horan PK, Breslin EW, Morahan PS. Maintenance of peritoneal macrophages in the steady state. *J Leukoc Biol* 1988; **44**: 367–375.
52 Volkman A. Disparity in origin of mononuclear phagocyte populations. *J Reticuloendothel Soc* 1976; **19**: 249–268.
53 van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med* 1968; **128**: 415–435.
54 Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* 2006; **211**: 609–618.
55 Hayashi S, Miyamoto A, Yamane T, Kataoka H, Ogawa M, Sugawara S *et al.* Osteoclast precursors in bone marrow and peritoneal cavity. *J Cell Physiol* 1997; **170**: 241–247.
56 Manthey CL, Johnson DL, Illig CR, Tuman RW, Zhou Z, Baker JF *et al.* JNJ-28312141, a novel orally active colony-stimulating factor-1 receptor/FMS-related receptor tyrosine kinase-3 receptor tyrosine kinase inhibitor with potential utility in solid tumors, bone metastases, and acute myeloid leukemia. *Mol Cancer Ther* 2009; **8**: 3151–3161.
57 Conway JG, McDonald B, Parham J, Keith B, Rusnak DW, Shaw E *et al.* Inhibition of colony-stimulating-factor-1 signaling *in vivo* with the orally bioavailable cFMS kinase inhibitor GW2580. *Proc Natl Acad Sci USA* 2005; **102**: 16078–16083.
58 Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B *et al.* Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation* 2008; **117**: 1649–1657.
59 Smith JD, Trogan E, Ginsberg M, Grigaux C, Tian J, Miyata M. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc Natl Acad Sci USA* 1995; **92**: 8264–8268.
60 Campbell IK, Rich MJ, Bischof RJ, Hamilton JA. The colony-stimulating factors and collagen-induced arthritis: exacerbation of disease by M-CSF and G-CSF and requirement for endogenous M-CSF. *J Leukoc Biol* 2000; **68**: 144–150.
61 Paniagua RT, Chang A, Mariano MM, Stein EA, Wang Q, Lindstrom TM *et al.* c-Fms-mediated differentiation and priming of monocyte lineage cells play a central role in autoimmune arthritis. *Arthritis Res Ther* 2010; **12**: R32.
62 Segawa M, Fukada S, Yamamoto Y, Yahagi H, Kanematsu M, Sato M *et al.* Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. *Exp Cell Res* 2008; **314**: 3232–3244.
63 Burke B, Ahmad R, Staples KJ, Snowden R, Kadioglu A, Frankenberger M *et al.* Increased TNF expression in CD43++ murine blood monocytes. *Immunol Lett* 2008; **118**: 142–147.

64 Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 2007; **81**: 584–592.
65 Sardis S, Mukherjee A, Olson S, Dokmanovich M, Maher R, Cai C-H *et al.* Safety, pharmacokinetics, and pharmacodynamics of PD-0360324, a human monoclonal antibody to monocyte/macrophage colony stimulating factor, in healthy volunteers [abstract]. *Arthritis Rheum* 2009; **60**: 408.
66 Basu S, Dunn AR, Marino MW, Savoia H, Hodgson G, Lieschke GJ *et al.* Increased tolerance to endotoxin by granulocyte-macrophage colony-stimulating factor-deficient mice. *J Immunol* 1997; **159**: 1412–1417.
67 Bezbradica JS, Gordy LE, Stanic AK, Dragovic S, Hill T, Hawiger J *et al.* Granulocyte-macrophage colony-stimulating factor regulates effector differentiation of invariant natural killer T cells during thymic ontogeny. *Immunity* 2006; **25**: 487–497.
68 Plenz G, Eschert H, Beissert S, Arps V, Sindermann JR, Robenek H *et al.* Alterations in the vascular extracellular matrix of granulocyte macrophage colony-stimulating factor (GM-CSF) -deficient mice. *FASEB J* 2003; **17**: 1451–1457.
69 Hercus TR, Thomas D, Guthridge MA, Ekert PG, King-Scott J, Parker MW *et al.* The GM-CSF receptor: linking its structure to cell signaling and its role in disease. *Blood* 2009; **114**: 1289–1298.
70 Uchida K, Beck DC, Yamamoto T, Berclaz PY, Abe S, Staudt MK *et al.* GM-CSF autoantibodies and neutrophil dysfunction in pulmonary alveolar proteinosis. *N Engl J Med* 2007; **356**: 567–579.
71 Hopper KE. Kinetics of macrophage recruitment and turnover in peritoneal inflammatory exudates induced by Salmonella or thioglycollate broth. *J Leukoc Biol* 1986; **39**: 435–446.
72 Metcalf D, Robb L, Dunn AR, Mifsud S, Di Rago L. Role of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in the development of an acute neutrophil inflammatory response in mice. *Blood* 1996; **88**: 3755–3764.
73 Hamilton JA. Colony stimulating factors, cytokines and monocyte-macrophages—some controversies. *Immunol Today* 1993; **14**: 18–24.
74 Hamilton JA. Rheumatoid arthritis: opposing actions of haemopoietic growth factors and slow-acting anti-rheumatic drugs. *Lancet* 1993; **342**: 536–539.
75 Cook AD, Turner AL, Braine EL, Pobjoy J, Lenzo J, Hamilton JA. Bone marrow cell-derived granulocyte-macrophage colony stimulating factor regulates systemic and local myeloid populations in inflammatory arthritis. *Arthritis Rheum* 2011 (in press).
76 Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ *et al.* A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 2003; **101**: 1155–1163.
77 Drevets DA, Schawang JE, Mandava VK, Dillon MJ, Leenen PJ. Severe Listeria monocytogenes infection induces development of monocytes with distinct phenotypic and functional features. *J Immunol* 2010; **185**: 2432–2441.
78 Vlahos R, Bozinovski S, Chan SP, Ivanov S, Linden A, Hamilton JA *et al.* Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. *Am J Respir Crit Care Med* 2010; **182**: 34–40.