Published in final edited form as:
*Nat Commun*. 2013 ; 4: . doi:10.1038/ncomms2877.

# Distinct bone marrow-derived and tissue resident macrophage-lineages proliferate at key stages during inflammation

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## Abstract

The general paradigm is that monocytes are recruited to sites of inflammation and terminally-differentiate into macrophages. There has been no demonstration of proliferation of peripherally-derived inflammatory macrophages under physiological conditions. Here we show that proliferation of both bone marrow-derived inflammatory and tissue resident macrophage lineage branches is a key feature of the inflammatory process with major implications for the mechanisms underlying recovery from inflammation. Both macrophage lineage branches are dependent on M-CSF during inflammation, and thus the potential for therapeutic interventions is marked. Furthermore, these observations are independent of Th2 immunity. These studies indicate that the proliferation of distinct macrophage populations provides a general mechanism for macrophage expansion at key stages during inflammation, and separate control mechanisms are implicated.

Monocytic cells play fundamental roles in both tissue homeostasis and immune regulation where they influence the initiation and resolution of inflammation. To support these activities, monocytes and macrophages (MØ) exhibit distinct effector functions and display a high degree of heterogeneity[1,2]. Human subsets of blood monocytes are distinguished by their expression of CD14 and CD16[3]. Similarly in mice, monocyte subsets are separated by expression of CCR2, Ly-6C, CD62L, $CX_3CR1$, CD43 and Ly-6B.2 (7/4 antigen)[4-6]. These monocyte subsets have been classified as ‘classical’ ‘intermediate’ and ‘non-classical’[7]. During inflammation, the generally accepted model is one where monocytes are recruited to inflammatory lesions whereupon they mediate various effector functions and differentiate

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**Author Contributions** P.R.T conceived and designed the project and wrote the manuscript; L.C.D. M.R., and P.R.T designed and conducted the majority of the experiments. S.J.J and J.E.A helped with design, execution and analysis of partial BM chimeras and Il-4Rα-deficient mouse experiments. C-T.L and M.J.S. helped with the analysis of specific peritonitis experiments. F.B provided IL-4Rα-deficient mice on a C57BL/6 genetic background. All authors contributed to the analysis and interpretation of the data. All authors contributed to the final version of the manuscript.

**Competing Financial Interests** The authors declare no competing financial interests.


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into MØ and dendritic cells (DC)[1,2,4,8-10]. It is, however, unclear how these various populations govern their numbers and activities to ensure both competent host defense and the restoration of normal tissue homeostasis. The bulk of recruited monocytes appear to be of the 'classical' subset recruited though a CCR2-mediated process[4,8]. In mice, these classical monocytes are also labeled with the Ly-6B.2 antigen (simplified henceforth to Ly-6B)[6,11,12], a marker, which appears to be retained for some time (>14 hours based on adoptive transfer experiments) on newly differentiated MØ within inflammatory lesions[11,12].

These monocytic populations are quite distinct from tissue resident (Res) MØ, which contribute to the initiation of inflammation, but also serve to maintain tissue homeostasis via surveillance of local microenvironment[2,13]. In several tissues, including serosal tissues such as the peritoneal cavity, Res MØ numbers are rapidly lost following the initiation of inflammation in a response referred to as the 'disappearance reaction'[14]. The disappearance reaction has been attributed to increased adherence to structural tissues, increased cell death or emigration to draining lymph nodes[14]. This disappearance is akin to the loss of Langerhans cells from the epidermis[15] and alveolar macrophages from the lung[16,17] during inflammation. However, as inflammation proceeds, cells displaying a characteristic Res MØ phenotype reappear in the presence of 'inflammation-associated' (Inf) MØ[6,18,19] through proliferation of residual mature Res MØ that survived the inflammatory process. These Res MØ appear capable of self-renewal with little input from the peripheral monocyte pool[13,18], indicating that they represent a distinct branch in the lineage. Indeed postnatal development of peritoneal Res MØ also appears to occur by proliferation[13,18]. These results are consistent with the development and expansion of Langerhans cells in the skin[20,21] and microglia in the brain[22,23]. Identification of this self-renewal mechanism adds a new layer of complexity to the classical model of monocyte to MØ differentiation and led us to re-evaluate the control of MØ turnover during inflammation.

In the peritoneal cavity, Res MØ are characterized by an F4/80$^{high}$CD11b$^{high}$ phenotype that distinguishes them not only from other tissue resident phagocytes[18,19,24], but also from Inf monocytes and MØ that broadly-express lower levels of F4/80[6,12,25]. We show that these subsets are not terminally differentiated and instead continue to actively proliferate at distinct stages during the resolution of an inflammatory response. As well as proliferation of Res MØ, this includes a distinct lineage of recruited bone marrow (BM)-derived MØ that proliferate within the inflammatory lesion. As a consequence, our data changes our understanding of monocyte/MØ biology by emphasizing that proliferative mechanisms, in addition to chemokine-directed trafficking, contribute to the turnover of monocytic cells at sites of inflammation.

## Results

### Characterization of Ly-6B positive inflammatory macrophages

Zymosan-induced peritonitis triggers a rapid, but transient elevation in neutrophil numbers and is followed by a more protracted elevation in eosinophil numbers (Fig. 1a, left). At the same time we observed a rapid drop in total monocyte/MØ numbers, caused by the loss of F4/80$^{high}$ Res MØ. This is followed by a rapid influx of peripheral Inf monocytes/MØ and a slower return of Res MØ, identified by their F4/80$^{high}$CD11b$^{high}$ phenotype[18] (Fig. 1a, right). The phenotype of the Inf MØ infiltrate is heterogeneous and Ly-6B$^{+}$ Inf MØ are clearly evident from day 2 of inflammation. The Ly-6B$^{+}$ Inf MØ are present throughout the resolution phase of inflammation and are still detectable at 14 days after zymosan administration (Fig.1b, c). Examination of MØ populations in a higher dose zymosan model ($2\times10^{7}$ particles i.p.) and an alternate thioglycollate peritonitis model showed similarities and differences (Fig. 1d). The higher dose zymosan model was characterized by lower

number of Res MØ (Fig.1d, middle) when compared to the low dose model (Fig.1d, left). This difference likely reflects the more substantial inflammatory burden, which may ultimately delay the resolution of inflammation. The thioglycollate model, which is characterized by substantial inflammation and a large accumulation of Inf MØ, showed little evidence of resolution at this time point (as indicated by the number of Res MØ) and notably no significant evidence of peritoneal Ly-6B expressing Inf MØ. Ly-6B expressing MØ were purified by flow-cytometric sorting and found to have a size and morphology consistent with MØ (Fig. 1e).

## Inflammatory macrophages are bone-marrow derived

We have classified Inf MØ as those 'associated with inflammation', but without definitively ascribing their origins to distinguish them from tissue resident cells (this article and [18]). Adoptive transfer experiments designed to track the persistence of resident peritoneal cells during an acute inflammatory episode indicated that Inf MØ, including the Ly-6B expressing cells, were not initially present in the peritoneal cavity[18]. We addressed the issue of the origins of Ly-6B$^+$ and Ly-6B$^-$ Inf MØ directly, with partial BM chimeras created by shielding the peritoneal cavity prior to irradiation and reconstitution with CD45 mismatched BM (CD45.2$^+$ donor cells into CD45.1$^+$ recipients)[26]. Six weeks later, the partial chimerism was confirmed by analysis of the myeloid cells in peripheral blood (Fig.2a). Zymosan peritonitis was induced in the partial chimeras, exactly as described[18], and then at day 3 the principle subsets of MØ under study were gated as shown (Fig.2b). Consistent with the direct evidence from previous adoptive transfer experiments in this model[18], the Res MØ remained of recipient origin, but both the Ly-6B$^+$ and Ly-6B$^-$ Inf MØ were chimeric (Fig. 2c). When quantified in individual chimeras, there was a clear correlation between blood monocyte chimerism and Inf MØ chimerism demonstrating that the Ly-6B$^+$ Inf MØ were BM derived (Fig.2d).

## The Ly-6B$^+$ subset ofinflammatory macrophages proliferate

Recent murine and human studies have highlighted the selective proliferative expansion of Res MØ, microglia and Langerhans cells during development, helminth infection and following recovery from acute inflammation[18,20-23,26]. Previously we showed minimal evidence for Inf MØ proliferation during inflammation[18]. To determine if a specific subset of Inf MØ were proliferating within an inflammatory lesion we adopted definitive measures of MØ proliferation[18] to identify cells in the S, G2 and M phases of cell cycle, and also those in active cell division (phospho-Histone H3 (pHH3) positive cells with 4N DNA content) (Fig. 3). We found that proliferating MØ were markedly enriched in the Ly-6B expressing subset (Fig. 3a, b). Similarly, Ly-6B$^+$ Inf MØ were also preferentially proliferating at this time in the high dose zymosan ($2\times10^7$ particles) challenge model (Supplementary Fig.S1a, b).

We compared zymosan peritonitis with thioglycollate broth-induced peritonitis. This comparison is interesting because thioglycollate broth recruits substantial numbers of MØ and is widely used to harvest MØ from mice. Thioglycollate-induced peritonitis is characterized by accumulation and retention of MØ[27], which is reflected in the lack of detectable proliferating cells at commonly used time points[26]. As already noted (Fig. 1), 'thioglycollate MØ' present at 72 hours post-injection were predominantly Inf, not Res, MØ and Ly-6B expressing MØ were absent. Since thioglycollate peritonitis at this time seemed to be slower to resolve (as evidenced by the lack of a restoring Res MØ population), we examined later time points to capture evidence of resolution of inflammation (Supplementary Fig.S1c, d). At later time points (>7 days after intraperitoneal thioglycollate broth), both Res and Ly-6B$^+$ Inf MØ were now evident and actively proliferating.

### Macrophage proliferation in inflammation depends on M-CSF

We have previously shown that Res MØ can self-renew by local proliferation[18]. Having demonstrated that a subset of BM-derived Inf MØ also proliferates during inflammation, we have identified that two distinct lineages of MØ are proliferating at specific stages within an inflammatory response. To identify the factors governing their turnover, we first examined the importance of M-CSF, a monocytic differentiation/survival factor, which is detectable at substantial levels in the peritoneal cavity of both naïve and inflammatory-challenged mice (Supplementary Fig. S2). First we tested whether elevated levels of M-CSF were sufficient to induce the expansion of Res MØ (Fig. 4). Naïve mice received 0.4 μg (i.p.) of recombinant M-CSF (Peprotec) or carrier alone (Fig.4a). M-CSF dosing caused only a mild increase in Res MØ proliferation above-that obtained by injection of carrier (Fig.4a). Next, to determine if M-CSF was required for proliferation of MØ during inflammation, mice were injected with $2\times10^6$ zymosan particles in conjunction with 0.5 mg of anti-M-CSF (clone 5A1) or IgG1 control antibody. Forty-eight hours later, when Res MØ proliferation was maximal, cells were recovered and analyzed by flow-cytometry. Blockade of M-CSF caused a total abrogation of Res MØ proliferation (Fig. 4b, c). Although Ki67 is a good indicator of proliferative potential and a measure of $G_1$ in these MØ ([18] and this study), it is a poor indicator of active cell division (Supplementary Fig. S3). However, the impact of M-CSF neutralization was evident across all measures of cell cycle.

When Inf MØ were examined 48 hours after induction of zymosan peritonitis M-CSF was also found to be important (Fig. 5). Here M-CSF neutralization led to a dramatic loss of the Ly-6B$^+$ Inf MØ, which was more marked than the impact of this treatment on the Inf MØ population as a whole (Fig. 5). Similar results were obtained in two distinct genetic backgrounds (129S6 and C57BL/6) (Fig. 5).

### IL-4Rα is not required for MØ proliferation

Lastly, given the recently identified role for IL-4 in the selective expansion of Res MØ in a Th2 dominated helminth infection model[26], we examined the role of IL-4:IL-4Rα in the proliferation of Res and Ly-6B$^+$ Inf MØ during acute inflammation. Mice were injected (i.p.) with 0.5 mg of anti-IL4 neutralizing antibody (clone 11B11) or control IgG at the same time as $2\times10^6$ zymosan particles and the proliferative state of Res MØ was analyzed 48 hours later. Data for cells in the definitive S, G2 or M phases of cell cycle (Fig.6a, left panel) and pHH3$^+$ mitotic phases (right panel) showed no differences in the proportions of Res MØ proliferating (Fig.6a). Similar results were obtained in two genetic backgrounds (129S6 and C57BL/6) (Fig.6a).

Examination of the Inf MØ present at this time demonstrated a partial reduction in the proliferation of these cells after neutralization of IL-4 in two independent experiments with different strains of mice (Fig.6b). However, examination of the proliferation of Res and Inf MØ subsets in wild type C57BL/6 and C57BL/6.*Il4ra*$^{-/-}$ backcrossed mice, 72 hours after induction of zymosan peritonitis resulted in a failure to observe any differences in either the number of cells present (Fig.6c, left panel) or the occurrence of proliferating cells in either of the Res or Inf MØ subsets (Fig.6c, middle and right panels).

## Discussion

Tissue MØ are traditionally viewed as terminally-differentiated populations, lacking in significant proliferative capacity. This view is however changing, and several murine studies have highlighted the selective proliferative expansion of Res MØ, microglia and Langerhans cells during development, helminth infection and following recovery from acute inflammation[18,20-23,26]. Comparable human studies also describe proliferation of

Langerhans cells[21] (and reviewed in [20]), MØ in glomerulonephritis[28,29] and atherosclerosis[30] and of a human monocyte subpopulation *in vitro* [31]. Treatment of human monocytes with M-CSF also induces genes involved in cell cycle[32]. While these studies provide a potential link between MØ proliferation and human pathology, a clear demonstration of the proliferation of monocyte-derived MØ in the physiological inflammatory environment *in vivo* has not been described.

We have now specifically identified a subset of proliferating BM derived MØ. Theese cells are a quite distinct lineage branch from that of Res MØ, which also proliferate as a mechanism of self-renewal in the tissue[18]. This separation in origins was confirmed by adoptive transfer with CD45 allotype mismatched cells[18]. However, the proliferative capacity of both subsets appears to be independently controlled. Ly-6B$^+$ Inf MØ retained a capacity to undergo proliferation at a stage of acute inflammation when Res MØ no longer exhibited significant levels of cell division[18]. The proliferation of Ly-6B$^+$ Inf MØ was confirmed with definitive quantification of mitosis. We next sought to define the signals that govern MØ proliferation. In this respect we show an absolute requirement for M-CSF. Not only is M-CSF fundamental for Res MØ proliferation during the proliferative burst[18] seen during the resolution of inflammation, but also basally during homeostasis, as determined with neutralizing antibody treatment of naïve mice (not shown). Indeed, mice deficient in M-CSF due to a naturally occurring mutation (the osteopetrotic (*op/op*) mice) display a marked deficiency in many macrophage populations[33,34]. This suggests little role for IL-34 in this model, which would be consistent with its tissue restricted function[35]. In spite of this, administration of additional exogenous M-CSF had only limited, albeit significant, impact on the proliferation of Res MØ indicating that whilst necessary for proliferation (as demonstrated with the M-CSF neutralization experiments), it was not sufficient to cause a substantial increase in Res MØ proliferation. In addition, Inf MØ numbers were reduced in all subsets following M-CSF neutralization, suggesting that M-CSF is necessary for the normal recruitment/differentiation/survival/proliferation of inflammatory MØ. This is particularly evident within Ly-6B$^+$ Inf MØ. Thus, M-CSF is essential for maintenance of two distinct lineage branches of proliferating MØ. When adoptively transferred into zymosan peritonitis, Ly-6B$^+$ Inf MØ persisted well with 75% of the transferred number of cells still present 90 hours later (Supplementary Fig. S4). These cells exhibited reduced Ly-6B expression indicating that Ly-6B$^+$ Inf MØ may become Ly-6B$^-$ Inf MØ (Supplementary Fig. S4).

Recently, IL-4 was identified as important for the expansion of Res MØ during helminth infection[26]. Whilst IL-4 driven MØ proliferation is consistent with a role during Th2 immunity, its role in other inflammatory settings has not been determined. Our data using both IL-4 neutralizing antibody and *Il4ra*$^{-/-}$ mice demonstrate that IL-4Rα is not essential for the proliferation of either Res or Inf MØ subsets in acute inflammation, although we cannot exclude that IL-4 has a more limited role as a modulator of Inf MØ proliferation. The lack of a requirement for IL-4Rα in this innate inflammatory model suggests that IL-4 driven proliferation is restricted to Th2 settings. Indeed, in helminth infection models, IL-4 dependent macrophage accumulation does not occur in the absence of Th2 cells[36], suggesting that innate sources of IL-4 are not sufficient to drive proliferation. Critically, these observations indicate that MØ proliferation is a general phenomenon that is not restricted to IL-4 and Th2-immunity.

Our findings indicate that both Res MØ and Inf MØ are capable of proliferating during key stages of the inflammatory response. While proliferating Inf MØ are found throughout the resolution phase of inflammation, Res MØ proliferation appears to be tightly governed and is restricted once normal tissue numbers are restored[18]. Res MØ and Ly-6B$^+$ Inf MØ are therefore likely to potentiate unique biological activities. Re-establishment of the tissue

resident population and continued presence of an inflammatory cell involved in pathogen or injury control are highly distinct functions and yet both require M-CSF. Considering the extensive development of M-CSF as therapeutic target[37] understanding these dynamics is essential, and illustrates the need to unravel the contribution of MØ origins to eventual function. It will be interesting to determine whether these findings within the peritoneal cavity apply to other tissues. In this context, Yona et al recently demonstrated that the relative autonomy of tissue resident macrophages, which we have reported in the serosal cavities[18,26] appears to be widespread[38]. The proliferation of Inf MØ in other contexts, whilst likely to occur, would need to be addressed in each context.

In summary, our data demonstrate that proliferation provides a general mechanism for the expansion of both peripherally derived (Inf) and Res MØ lineages during the resolution of inflammation. This raises critical new questions about the contribution of proliferation vs. recruitment to monocyte/MØ function and the different roles of MØ subsets. For example, does the failure of a particular subset to proliferate contribute to chronic inflammatory pathology, or does excessive proliferation promote unwanted tissue repair and fibrosis? The answers to these questions will open new avenues for understanding the optimal immune responses and intervention strategies required for the restoration of normal tissue homeostasis.

## Methods

### Mice

C57BL/6 female mice were obtained from Harlan Olac and 129S6/SvEv mice from our own breeding colonies and were used between 6-7 weeks of age (unless otherwise stated). C57BL/6.*Il4ra*$^{-/-}$ mice were generated by backcrossing *Il4ra*$^{-/-}$ [39] onto the C57BL/6 genetic background for at least 9 generations. Peritonitis was induced typically by i.p. injection of $2\times10^6$ (unless otherwise indicated) zymosan particles in 100 μl of PBS or with 1ml of 4% Brewer's thioglycollate medium (Sigma, B2551). For antibody modulation experiments, 0.5 mg of anti-M-CSF (clone 5A1), anti-IL-4 (clone 11B11) or control IgG were co-injected with the zymosan in a total volume of 250 μl of PBS. Exogenous M-CSF (Peprotec) was administered i.p. in 250 μl total volume in a solution of 0.05% BSA in PBS and control mice received this vehicle alone. Peritoneal cells were recovered at the indicated time points after sacrifice of the animal by lavage with 5ml of ice-cold 5 mM EDTA in PBS and absolute cell counts were determined using a hemacoytometer prior to further analysis. All animal work was conducted in line with Institutional and UK Home Office guidelines.

Partial BM chimeras were generated by shielding the peritoneal cavity and irradiation, as previously described[26].

### Antibodies and immunoassays

Antibodies used were: anti-Ly-6B.2-phycoerythrin (PE) or –A647 (clone 7/4 [6]; AbDSerotec); anti-Ly-6B.2-Peridinin-chlorophyll-protein (PerCP) (clone 7/4 labeled using the Phycolink PerCP-labeling kit from Europa Bioproducts); anti-Ly-6G-PE-Cy7 (clone 1A8; BD biosciences); anti-CD11b-APC or -APC-Cy7 or PerCP-Cy5.5 (clone M1/70; BD biosciences); anti-CD11b-FITC (clone 56C; homemade); anti-Tim4-PE (clone RMT4-54; eBiosciences); anti-F4/80-PE or -Alexa405 or -APC (clone CL:A3-1; AbDSerotec); anti-F4/80-PE-Cy7 (Biolegend) or -PE-Texas Red (PE-TxR) (clone BM8; Invitrogen); anti-Ki67-PE (clone B56) and IgG1κ-PE (clone MOPC-21; BD biosciences); anti-pHH3-A488 (clone D2C8 (Ser10); New England Biolabs); antiCD45.1-FITC (clone A20; Biolegend); anti-CD45.2-APC-Cy7 (clone 104; Biolegend); Streptavidin-PE-TxR and steptavidin-PerCP

was obtained from BD Biosciences. The supplier for 4′,6-diamidino-2-phenylindole (DAPI) was Invitrogen.

M-CSF in lavage fluid was measured using the mouse M-CSF ELISA kit (Peprotec) as directed by the manufacturer.

### Flow cytometry and flow-cytometric cell sorting

Flow cytometric staining was conducted using the standard protocols in our laboratory[6,12,18,25]. For cell purification, lavage from mice 7 days after induction of peritonitis with $2\times10^6$ zymosan particles were pooled, stained as indicated in Fig.1e and sorted on a MoFlo Legend (Beckman-Coulter) for single events (via pulse width) with the defined flow-cytometric phenotype and appropriate FSC/SSC profiles (see Fig.1 and [6,12,18]). The purity of the sorted populations was verified after sorting and morphology was investigated on cytospin preparations, which were stained and visualized using Microscopy Hemacolour cell stain (Merck). Analytical polychromatic flow-cytometry was conducted on a CyAn ADP analyzer (Beckman-Coulter) and analyzed using *Summit* software v4.3 (Beckman-Coulter) or on a Canto II (BD) and analyzed using FlowJo (TreeStar) and when appropriate was related to the absolute cell counts of the individual animals.

### Statistical analysis

Two-way ANOVAs, Student's *t* tests (paired or unpaired) and linear regression analysis were conducted using GraphPad Prism and the tests and results are indicated in the appropriate text. Statistical significance is also highlighted with the following notations: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

P.R.T is a Medical Research Council (MRC) (UK) Senior Non-Clinical Fellow (G0601617). L.C.D is an MRC DTG funded PhD student and Cardiff 125 for 125 scholar. Additional support was provided by a MRC Programme Grant to J.E.A. (G0600818). Animal work was approved by full local ethical review process and was conducted in accordance with Institutional and UK Home Office guidelines. We would like to thank the staff of our animal facilities for the care of the animals. We would also like to thank Dr. Chris Pepper and Mrs Janet Fisher for their help with cell purifications and Nicola Dierkes for her technical assistance.

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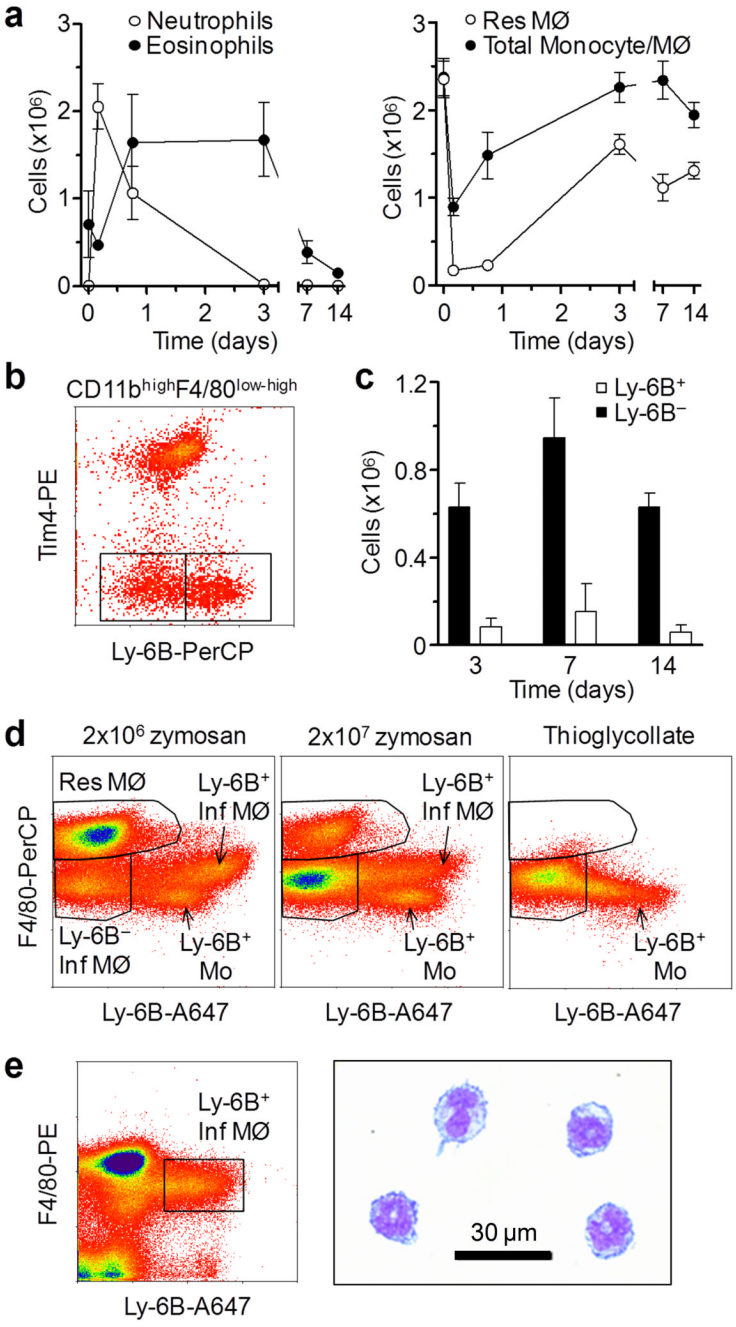


**Figure 1. Kinetics of acute zymosan induced peritonitis and appearance of Ly-6B expressing inflammatory macrophages**

**a)** Graphs showing the numbers of neutrophils and eosinophils (left panel) and MØ (both total monocyte/MØ and Res MØ) MØ (right panel) present at the indicated times after i.p. administration of $2\times10^6$ zymosan particles. Data represents mean±SEM of 3-5 individual mice per time point, taken from one of two similar experiments. **b)** Representative flow-cytometric profiles showing Ly-6B$^+$ Inf MØ, identified as previously described[6,18] by gating on CD11b$^{high}$F4/80$^{low\text{-}high}$ cells (excluding eosinophils), are clearly distinct from Tim4$^{high}$ Res MØ. Gates denote Ly-6B$^+$ and Ly-6B$^-$ Inf MØ populations. **c)** Approximately a fifth of cells classified as Inf MØ are Ly-6B$^+$ and are present for several weeks after induction of a

mild inflammatory reaction. Data is taken from the animals represented in (A) above and presented as mean±SEM. **d)** Representative flow-cytometric profiles (gated on F4/80$^{low\text{-}high}$CD11b$^{+}$ monocytes and MØ) taken from mice 72 hours after induction of peritonitis with zymosan (both $2\times10^{6}$ and $2\times10^{7}$ particles) and thioglycollate broth, as indicated. The higher dose of zymosan resulted in similar MØ populations, but with substantially reduced recoverable numbers of Res MØ when compared to the low dose, as expected[12,25]. In contrast, thioglycollate broth resulted in a near complete depletion of Res MØ and no evident Ly-6B$^{+}$ Inf MØ population at this time. The presence of both Ly-6B$^{+}$ monocytes and Ly-6B$^{+}$ Inf MØ populations are indicated. Flow-cytometric plots are representative of at least 3 mice per group from one of two similar experiments. **e)** The Ly-6B expressing MØ were gated as indicated (left panel) and purified (>90%) by flow-cytometric cell-sorting and analysed on cytospins stained with eosin and methylene blue. Cells were pooled from 6 week old female C57BL/6 mice 7 days after i.p. zymosan ($2\times10^{6}$ particles).



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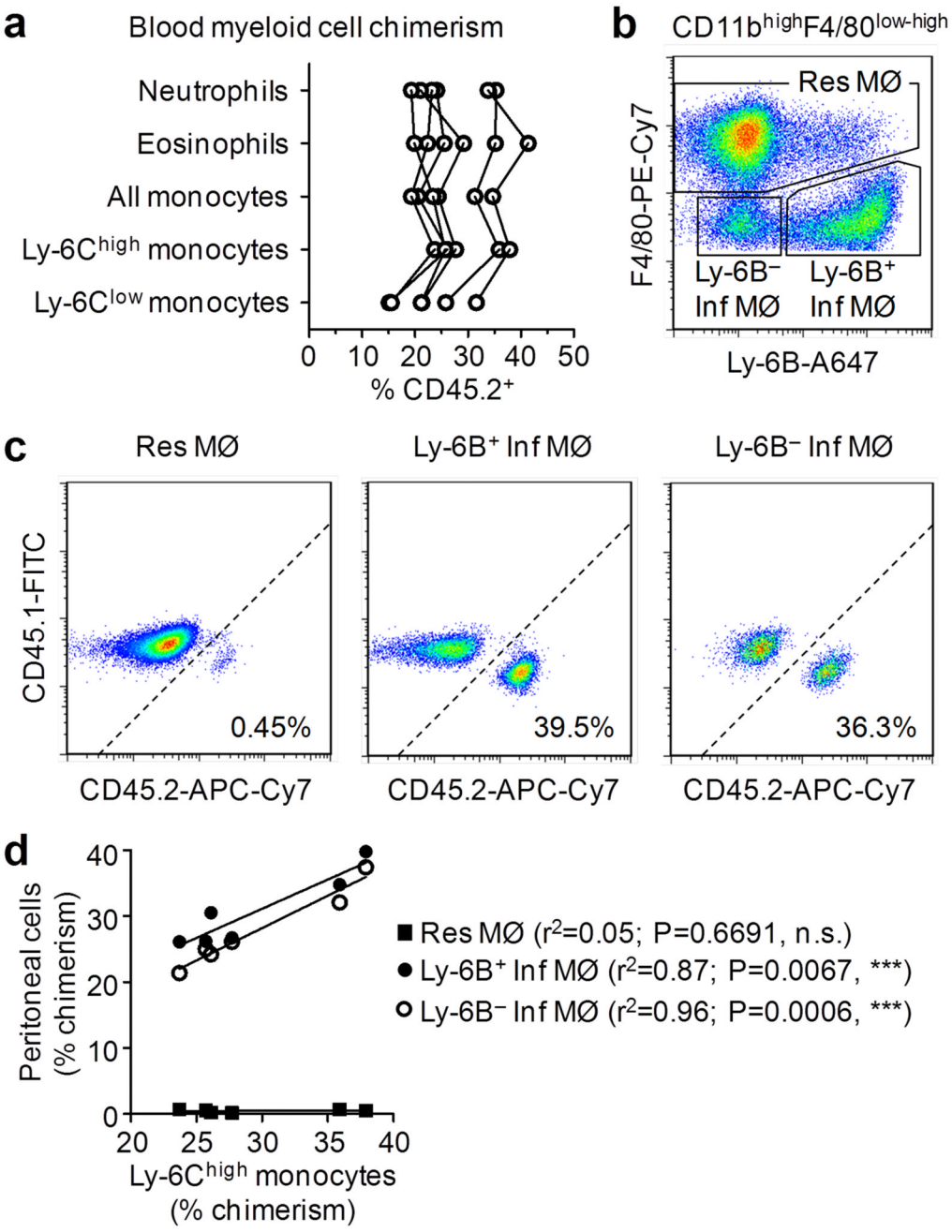


**Figure 2. Both Ly-6B$^+$ and Ly-6B$^-$inflammatory macrophages are derived from the bone marrow**

Partial BM chimeras were generated as previously described [26], after shielding the peritoneal cavity, and then were left to recover for 6 weeks. **a)** Analysis of blood myeloid cell chimerism one day before induction of peritonitis shows a stable chimerism in all experimental animals. Each line links the data from an individual mouse. **b)** Gating strategy for the identification of Res and Inf MØ (both Ly-6B$^+$ and Ly-6B$^-$) 72 hours after the induction of peritonitis by i.p. administration of $2\times10^6$ zymosan particles. The plot has been pre-gated on CD11b$^{high}$F4/80$^{low\text{-}high}$ cells as previously reported[18]. **c)** Representative flow-cytometric analysis of the indicated MØ subsets to determine the level of chimerism

between CD45.1 recipient cells and CD45.2 donor cells. **d)** Graphical representation of the chimerism in all of the experimental animals correlating the chimerism of the 3 peritoneal MØ subsets with that of Ly-6C$^{high}$ blood monocytes within the same animal. Data were analyzed by linear regression, and $r^2$ values are indicated alongside significance testing for a non-zero slope. Each symbol represents an individual irradiation chimera.



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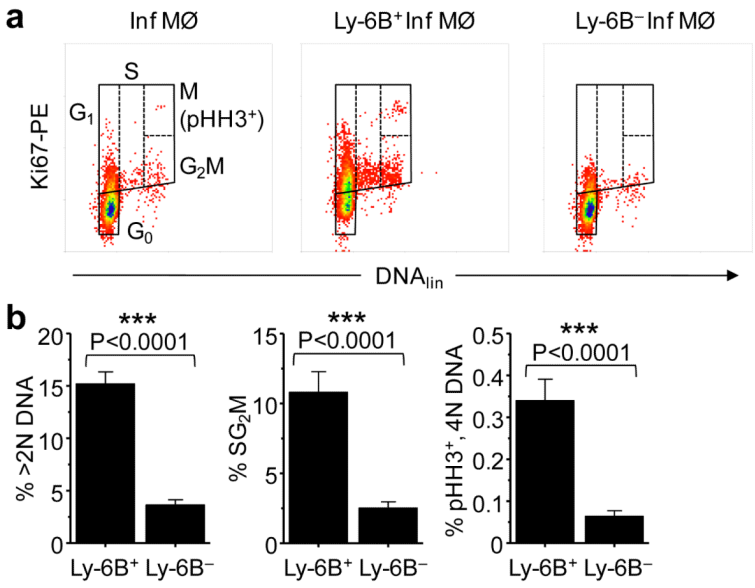


**Figure 3. Ly-6B expressing inflammatory macrophages actively divide*in situ***

**a)** The plots show total Inf MØ populations (left), Ly-6B$^+$ cells (middle) and their Ly-6B$^-$ counterparts (right) 72 hours after the induction of peritonitis with $2\times10^6$ zymosan particles. As previously reported there is only limited evidence of proliferation in the total Inf MØ population (left and [18]). However, when subdivided based on Ly-6B expression there was a clear enrichment of cells in the S,G2 and M stages of cell cycle amongst the Ly-6B expressing cells (middle) and notable lack of proliferating cells within the Ly-6B$^-$ population (right). Data are derived from individual mice and are representative of at least 3 experiments with 6-7 week old C57BL/6 female mice and typically 3-5 mice per experiment. **b)** The levels of proliferation within the two populations of Inf MØ in the same experiment shown above (A) was measured by the proportion of cells with >2N DNA content (left), >2N DNA content with Ki67 expression ('SG2/M', middle), phospho-Histone H3$^+$ phase of mitosis ('pHH3$^+$'; right). Data is expressed as mean±SEM of 5 mice per group (7 week old female C57BL/6) and is representative of at least 3 experiments. Data were analyzed by a paired *t*-test.

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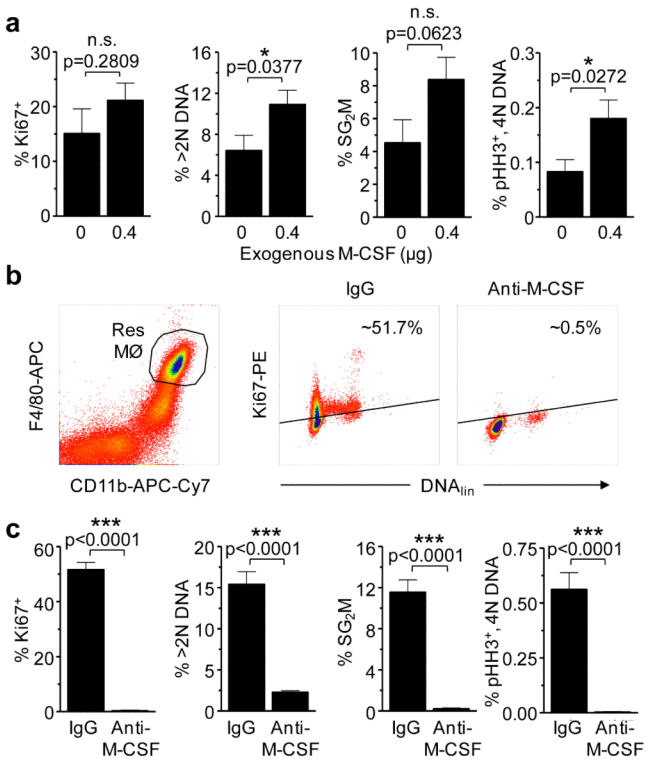


**Figure 4. M-CSF is required for tissue resident macrophage proliferation**

**a)** Data showing proliferation of Res MØ (gated by their F4/80$^{high}$CD11b$^{high}$ phenotype), 24 hours after i.p. administration of 0.4 μg of recombinant M-CSF (0.4) or carrier control (0). Data shown represents mean±SEM 10 mice per group (7 week old female C57BL/6) pooled from 2 identical experiments. Data were analyzed by a Student's *t*-test. **b)** Representative flow cytometric plots showing the identification of Res MØ in a similar way as previously described 48 hours after the i.p. injection of 2×10$^6$ zymosan particles in conjunction with 0.5 mg of either anti-M-CSF (clone 5A1) or and rat IgGisotype control (as indicated). Percentages shown are mean values from the analyses of groups of mice (shown below). **c)** Measurement of proliferation in Res MØ 48 hours after i.p. injection of 2×10$^6$ zymosan particles in conjunction with 0.5 mg of either anti-M-CSF (clone 5A1) or and rat IgGisotype control. Data shown represents mean±SEM 5 mice per group (7 week old female C57BL/6) from one of 2 similar experiments. Data were analyzed by a Student's *t*-test.

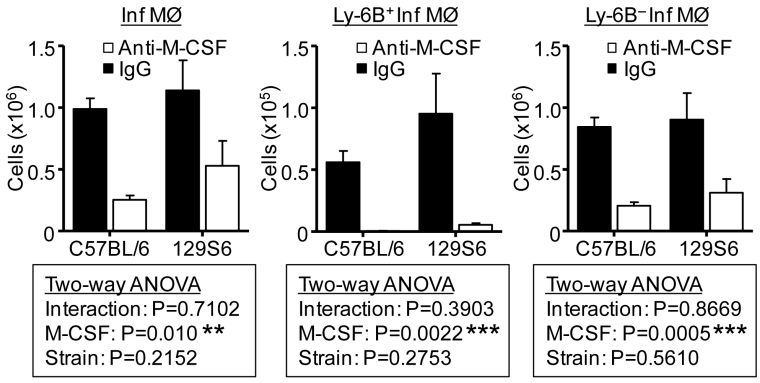


**Figure 5. Ly-6B expressing inflammatory macrophagesare M-CSF-dependent**

Inf MØ were quantified 48 hours after acute peritonitis induced with $2\times10^6$ zymosan particles in conjunction with 0.5 mg of either anti-M-CSF (clone 5A1) or and rat IgGisotype control. Neutralization of M-CSF results in a substantial drop in total Inf MØ numbers (left), which is caused by a near complete loss of Ly-6B$^+$ Inf MØ (middle) and substantial drop in Ly-6B$^-$ Inf MØ (right). Data shown represents mean±SEM of two independent experiments conducted with 7-8 week old female C57BL/6 mice (n=5 per group) and one with 9-12 week old female 129S6/SvEv (n=3-6 per group). Data was analyzed by Two-way ANOVA with the significance of the effects of both M-CSF and strain of mouse indicated.




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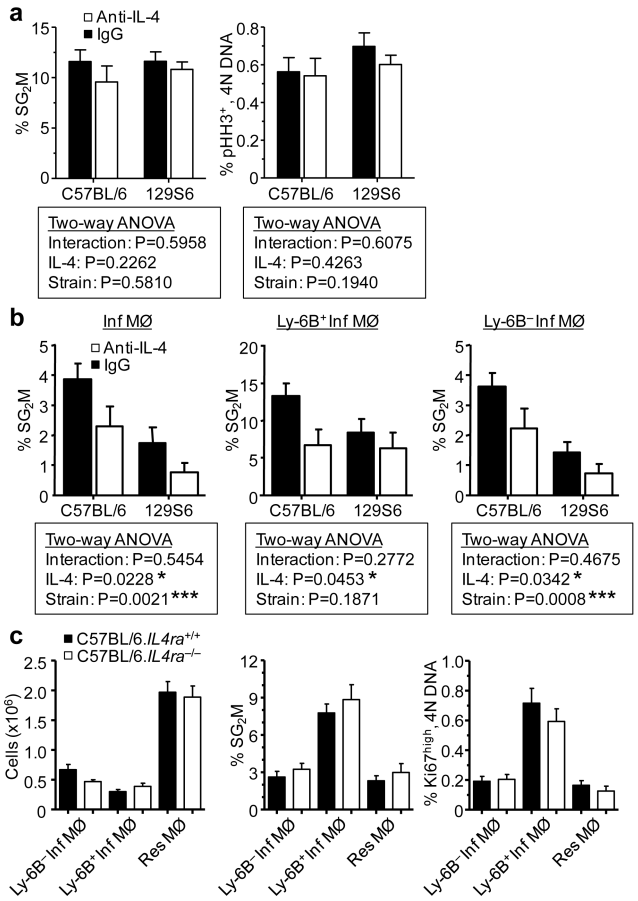


**Figure 6. Lack of an essential role for IL-4/IL-4Rα for macrophage proliferation during acute inflammation**

**a)** Measurement of proliferation in Res MØ 48 hours after i.p. injection of $2\times10^6$ zymosan particles in conjunction with 0.5 mg of either anti-IL-4 (clone 11B11) or and rat IgGisotype control. The left graph shows the proportion of Res MØ in the S, G2 and M phases of cell cycle whereas the right graph shows those cells in $pHH3^+$ definitive stages of mitosis. **b)** Analysis of proliferation of Inf MØ subsets in mice injected i.p. 48 hours earlier with $2\times10^6$ zymosan particles in conjunction with 0.5 mg of either anti-IL-4 (clone 11B11) or and rat IgGisotype control. Data shown for (A) and (B) represent mean±SEM from two-independent experiments (7 week old female C57BL/6 n=5 mice per group, and 6-9 week old female 129S6 mice n=3-6 mice per group). Data were analyzed by a Two-way ANOVA with the significance of the effects of IL-4 neutralization and mouse strain indicated. **c)** The number and proliferation of the Res and Inf MØ subsets were examined 72 hours after induction of peritonitis with $2\times10^6$ zymosan particles in wild type and IL-4Rα deficient mice on the C57BL/6 background. Data shown represents mean±SEM of 6 week old female mice (n=10 per group).