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ADAM17 limits the expression of CSF1R on murine hematopoietic progenitors

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Abstract

All-lymphoid progenitors (ALPs) yield few myeloid cells *in vivo*, but readily generate such cells *in vitro*. The basis for this difference remains unknown. We hypothesized that ALPs limit responsiveness to *in vivo* concentrations of myeloid-promoting cytokines by reducing expression of the corresponding receptors, potentially through post-transcriptional mechanisms. Consistent with such a mechanism, ALPs express higher levels of *Csf1r* transcripts than their upstream precursors, yet show limited cell surface protein expression of CSF1R. ALPs and other hematopoietic progenitors deficient in ADAM17, a metalloprotease that can cleave CSF1R, display elevated cell surface CSF1R expression. *Adam17*$^{-/-}$ ALPs, however, fail to yield myeloid cells upon transplantation into irradiated recipients. Moreover, *Adam17*$^{-/-}$ ALPs yield fewer macrophages *in vitro* than control ALPs at high concentrations of M-CSF. Mice with hematopoietic-specific deletion of *Adam17* have grossly normal numbers of myeloid and lymphoid progenitors and mature cells *in vivo*. These data demonstrate that ADAM17 limits CSF1R protein expression on hematopoietic progenitors, but that compensatory mechanisms prevent elevated CSF1R levels from altering lymphoid progenitor potential.

Introduction

Hematopoietic stem cells traverse through a series of developmental intermediates, termed progenitors, en route to lineage commitment and maturation [1]. As differentiation progresses, these progenitors lose their ability to undergo self-renewing divisions. At specific developmental branchpoints, progenitors also lose their ability to generate specific

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subsets of mature blood lineages. At each of these branches, these progenitors are considered to be committed to the remaining blood lineages that they can still generate.

Complicating the definition and analysis of lineage commitment, *in vivo* assays can yield different results than *in vitro* experiments [2]. For example, common- or all-lymphoid progenitors (CLPs or ALPs) that yield primarily lymphocytes and dendritic cells *in vivo* can readily generate macrophages and neutrophils *in vitro* with high efficiencies [2–10]. These data demonstrate that CLPs and ALPs have not epigenetically silenced their myeloid programs [11], yet myeloid cells are infrequently generated from these progenitors under physiological conditions [4]. Thus, there is substantial disagreement on whether CLPs and ALPs should be considered lymphoid-committed.

As highlighted by this disagreement, the mechanisms by which lymphoid progenitors limit myeloid output *in vivo* remain incompletely understood. One possibility is that lymphoid progenitors home to distinct "niches" *in vivo* in which local concentrations of myeloid cytokines are low [12, 13]. Another non-mutually exclusive possibility is that ALPs reduce the expression of myeloid cytokine receptors such that they are unresponsive to the *in vivo* concentrations of such factors. Yet by providing excess amounts of myeloid lineage-promoting cytokines *in vitro*, lymphoid progenitors can still generate macrophages and neutrophils. Indeed, ectopic expression of certain cytokine receptors allows for robust myeloid cell production by lymphoid progenitors [14]. Endogenous cytokine receptor expression can be regulated both by transcriptional and post-transcriptional mechanisms. For example, the metalloprotease ADAM17 can cleave CSF1R, an essential and instructive cytokine receptor for M-CSF which mediates macrophage commitment and homeostasis [15–19].

ADAM17 belongs to a family of metalloproteases with broad target specificities and essential roles in many biological processes. ADAM17 is best known for its role in cell-intrinsic processing of TNFα to its secreted form, and is often referred to as TNFα-converting enzyme (TACE) [20, 21]. However, many studies have identified other ADAM17 targets in addition to TNFα, including CSF1R [18]. Although the role of ADAM17 in mature myeloid cells and responses to bacterial endotoxin challenge has been studied [22–26], to our knowledge there have been no reports describing its function during hematopoietic development. Given similar roles in other cell types, we hypothesized that ADAM17 limits CSF1R expression on lymphoid progenitors, thereby preventing macrophage and granulocyte production *in vivo*. Here we demonstrate that ALPs express *Csf1r* transcripts, and that ADAM17 does indeed limit cell surface expression of CSF1R on ALPs and other hematopoietic progenitors. Yet despite its role in limiting CSF1R on the surface of ALPs, ADAM17 is not required for preventing myeloid cell production by lymphoid progenitors *in vivo*.

Materials and Methods

Mice

Adam17$^{fl/f}$ [22], Vav1-iCre [27], C57BL/6, and B6.SJL mice were originally purchased from The Jackson Laboratory and subsequently housed and maintained in our animal care

facility. The genotype of ADAM17 knockout mice in all experiments was ADAM17$^{fl/fl}$ Vav1-iCre^{+} while wild type mice were ADAM17$^{fl/+}$ Vav1-iCre^{-} or ADAM17$^{fl/fl}$ Vav1-iCre^{-}. All studies were carried out according to the Institutional Animal Care and Use Committee at Washington University.

Microarray and quantitative real-time PCR

Csf1r expression levels were analyzed in LMPP and ALP from previously published microarrays [5]. For quantitative real-time PCR analysis, cells were double-sorted into TRIzol reagent (Invitrogen) using a BD FACSAria (BD Biosciences). SuperScript III First Strand Kit (Invitrogen) was used to generate cDNA using random hexamers per the manufacturer's instructions. SybrGreen PCR master mix (Applied Biosystems) was used for real-time PCR assays per the manufacturer's instructions. The ABI 7000 Sequence Detection System (Applied Biosystems) was used to quantify expression. Primer sequences were: *Csf1r* 5'-ACACGCACGGGCCACCATGAA-3': and 5'-GCATGGACCGTGAGGATGAGGC-3' and *Gapdh*, 5-GGCAAATTCAACGGCACAGT-3′ and 5-GATGGTGATGGGCTTCCC-3′.

M-CSF ELISA assay

Epiphyses were removed and dissected femurs from wild type mice were flushed with 1 ml PBS. Cells were pelleted, and M-CSF levels were quantified from bone marrow supernatant or blood serum using a mouse M-CSF ELISA kit as per manufacturer's instructions (Sigma). Bone marrow M-CSF concentrations were calculated by dividing the total amount of M-CSF in the bone marrow supernatant by the marrow volume of a mouse femur, estimated to be 9.4μl. This estimate is based upon the approximations that a mouse femur is a normal cylinder, the cross-sectional marrow radius *r* is 0.46mm [28], the length *l* is 15mm [28], and that in turn the volume can be calculated as $\pi r^2 l$.

Flow cytometry and cell sorting

Staining buffer consisted of 2% adult bovine serum (Hyclone)/PBS with 1mM EDTA. Dead cells were gated out using propidium iodide (Sigma-Aldrich). Cells were acquired and sorted on the FACSAria (BD Biosciences) or analyzed on a LSRII (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). For a list of antibodies used in the experiments, see Supplemental Table 1

In vitro differentiation assay

500 ALPs were double-sorted into culture media that consisted of 10% Defined FBS (Hyclone) in DME-F12 + 10 mM Hepes (SAFC Biosciences) and supplemented with NEAA (Lonza), Sodium pyruvate (Lonza), penicillin/streptomycin (Sigma-Aldrich), Glutamax (Invitrogen), and 50μM 2-mercaptoethanol (Invitrogen). M-CSF (Peprotech) was added at the indicated concentration. Cells were cultured for 4 days before staining and flow cytometric analysis on a BD LSRII. To quantify total numbers of cells, software acquisition and recording was initiated before the sample was loaded and continued until the sample was completely consumed and no additional live events were observed. Viability was quantified by the percentage of cells incorporating propidium iodide.

In vivo differentiation assay

5000 ADAM17 wild type or knockout ALPs (CD45.2$^+$) were double-sorted into PBS and then injected into each 800 cGy-irradiated B6.SJL (CD45.1$^+$) recipient mice via retro-orbital injection. Ten days post-injection, bone marrow and spleens were harvested and mechanically dissociated in staining buffer. Cells were stained and analyzed as described in Results.

Results

Cytokine signaling in hematopoietic progenitors can play an instructive role in directing fate decisions [14, 17, 19, 29]. Thus, we hypothesized that lymphoid progenitors display reduced expression of myeloid-promoting cytokine receptors relative to their uncommitted multipotent precursors. To test this hypothesis, we examined global gene expression profiles of ALPs, which generate only lymphocytes *in vivo* [5], and lymphoid-primed multipotent progenitors (LMPPs), which can generate macrophages *in vivo* through CSF1R-expressing progeny [5, 30–32]. Unexpectedly, the expression of CSF1R, which promotes monocyte and macrophage development [15], was increased in ALPs relative to LMPPs (Fig. 1A). This increase was also observed through quantitative RT-PCR analysis (Fig. 1B). Thus, ALPs express more *Csf1r* transcripts than do LMPPs, but less than do common myeloid progenitors (CMPs) (Fig. 1B).

Protein levels of CSF1R can be regulated through several post-transcriptional mechanisms [33]. Therefore, increased transcript levels may not strictly correlate with increased cell surface protein levels or responsiveness to cytokines. To test cell surface expression of CSF1R in LMPPs and ALPs, we performed flow cytometric analysis. CSF1R surface expression was low on ALPs, similar to that seen in LMPPs and markedly less than that observed in CMPs (Fig. 1C, gating strategies shown in Supplemental Figure 1). Thus, ALPs use post-transcriptional mechanisms to limit CSF1R expression.

We hypothesized that the diminished levels of surface CSF1R on ALPs would render these cells insensitive to physiological concentrations of M-CSF. To quantify the endogenous levels of M-CSF, we performed ELISA analyses. These data demonstrated that the bone marrow M-CSF concentration is ~2 ng/ml (Fig. 1D). Interestingly, M-CSF was undetectable in the serum of these same animals (Fig. 1D). These data demonstrate that M-CSF levels are locally restricted. Local concentrations of M-CSF could potentially also vary greatly within distinct marrow regions from the average value of ~2 ng/ml. Thus, to determine if the low levels of cell surface CSF1R expression on ALPs would allow for macrophage development, we cultured purified ALPs in the presence of a broad range of M-CSF concentrations. ALPs readily generated macrophages at high concentrations (10ng/ml) of M-CSF, but this output fell sharply at lower doses (Fig. 1E). At all doses, CMPs generated more macrophages than did ALPs, potentially due to higher levels of surface CSF1R expression (Fig. 1E). The proportion of macrophages generated was comparable between ALPs and CMPs, with some small differences at the lower concentrations of M-CSF (Fig. 1E). Lowering the concentration of M-CSF led to a progressive decline in viability, although CMPs were less sensitive to death than were ALPs (Fig. 1E). These data demonstrate that ALPs express

relatively little cell surface CSF1R despite transcription, and robustly respond to M-CSF only at high doses.

Previous studies have shown that the metalloprotease ADAM17 can cleave CSF1R protein in activated macrophages [18]. We thus hypothesized that a similar mechanism limits CSF1R expression in ALPs. To test this hypothesis, we generated *Adam17*$^{fl/fl}$ × *Vav1-iCre* mice, which selectively lack ADAM17 in the hematopoietic compartment [24]. ALPs and other progenitors were then assessed for cell surface CSF1R levels. Indeed, ALPs, and also granulocyte macrophage progenitors (GMPs), CMPs, and LMPPs all showed elevated expression of surface CSF1R (Fig. 2, gating strategies shown in Supplemental Figure 1). These data demonstrate that hematopoietic-intrinsic ADAM17 limits the expression of CSF1R *in vivo*.

We next sought to determine the functional consequences of ADAM17-deficiency on ALP behavior *in vivo*. ALPs were purified from *Adam17*$^{fl/fl}$ × *Vav1-Cre* or control littermates and transferred into sublethally-irradiated recipients. For both wild type and ADAM17-deficient ALPs, mean splenic donor chimerism was identical at 1.9% (data not shown), and B cells and dendritic cells were readily generated from ALPs of both genotypes (Fig. 3A). However, we observed no evidence of monocyte production by ADAM17-deficient ALPs (Fig.3A). At this same timepoint, CMPs readily generate monocytes [31, 34]. We cannot exclude the possibility that ALPs generate mature cells such as monocytes with much different kinetics than do CMPs. However this possibility seems unlikely, as both of these progenitors yield mature dendritic cells with similar kinetics [34–36]. As monocytes are commonly identified using CSF1R expression as a marker, we were concerned that this strategy would not be faithful for ADAM17-deficient monocytes. To address this, we examined wild type and ADAM17-deficient splenocytes. Although ADAM17-deficient monocytes did indeed express elevated levels of CSF1R and required slightly different gates for quantification, they could still be readily identified (Fig. 3B). A similar proportion of wild type and ADAM17-deficient monocytes expressed Ly6C (data not shown), a marker of inflammatory monocytes, thus further validating the gating strategy. Therefore, in this adoptive transfer system, elevated levels of CSF1R are not sufficient to confer *in vivo* myeloid potential to ALPs.

Because irradiation can alter *in vivo* concentrations of cytokines and homing properties of progenitors [37–39], we next sought to determine if ADAM17-deficiency led to any changes in the numbers of progenitors or mature cells under steady-state conditions. Despite elevated surface levels of CSF1R, no significant defects were observed in the numbers of lymphoid or myeloid progenitors in ADAM17-deficient animals aside from a modest reduction in CDPs (Fig. 3C, gating strategies shown in Supplemental Figure 1). Similarly, no defects were observed in the numbers of mature dendritic cells, monocytes, or lymphocytes in the spleen (Fig. 3D) or bone marrow (Fig. 3E), except for a slight increase in splenic *Adam17*$^{-/-}$ T cell numbers (gating strategies shown in Supplemental Figure 2).

To explore the basis for the lack of dramatic *in vivo* effects of ADAM17-deficiency, we compared the *in vitro* responsiveness of wild type and *Adam17*$^{-/-}$ ALPs to M-CSF. Unexpectedly, ADAM17-deficient ALPs yielded fewer macrophages *in vitro* than did wild

type ALPs at the highest doses of M-CSF (Fig. 4), despite expressing higher levels of CSF1R (Fig. 2). At lower M-CSF doses, the numbers and frequencies of macrophages generated were similar between wild type and ADAM17-deficient ALPs (Fig. 4). Overall viability was also similar between genotypes at all doses of M-CSF (Fig. 4, bottom panel). We cannot exclude the possibility that a distinct proteolytic target of ADAM17 somehow prevents monocyte and macrophage development. However, given that M-CSF is the only cytokine included in these *in vitro* cultures, our data suggest that excessive M-CSF signaling may either prevent the differentiation of macrophages from lymphoid progenitors or selectively kill those which have already formed. Thus, negative feedback signaling through CSF1R may prevent major changes from occurring *in vivo* in ADAM17-deficient animals. This may also help explain the modest reduction in ADAM17-deficient CDPs (Fig. 3C), which already express high levels of CSF1R even in ADAM17-sufficient animals [40, 41]. Together, these data demonstrate that although ADAM17 limits CSF1R expression in progenitors, it is not necessary for lymphoid lineage commitment or myeloid cell homeostasis under steady-state conditions.

Discussion

Fate decisions during hematopoietic differentiation are regulated by complex interactions between cell-extrinsic and cell-intrinsic cues. At certain intermediates, progenitor cell differentiation *in vivo* is often more restricted than their epigenetic profiles or *in vitro* potentials might predict [2, 11]. In these cases, it is likely that extrinsic factors dictate cellular outcomes *in vivo*, either through instructive actions or by selectively permitting the survival or proliferation of specific downstream lineages. By tuning their sensitivities to these extrinsic factors, progenitors could regulate outcomes *in vivo*.

We hypothesized that one way in which lymphoid progenitors regulate their sensitivities to myeloid cytokines is by ADAM17-mediated cleavage of CSF1R. Indeed, CSF1R surface levels were significantly higher in *Adam17*$^{-/-}$ ALPs and other progenitors relative to their *Adam17*$^{+/+}$ counterparts. Thus it seemed reasonable to expect that cells expressing elevated CSF1R levels would increase macrophage or monocyte production *in vivo*. Contrary to our hypothesis, however, we observed no differences in *Adam17*$^{-/-}$ ALP output *in vivo* compared to controls, and there were no major differences in mature cell subsets in the spleen or bone marrow. This may in part be attributable to negative feedback inhibition of CSF1R signaling, as *Adam17*$^{-/-}$ ALPs generated relatively few macrophages at the highest doses of M-CSF *in vitro* compared to *Adam17*$^{+/+}$ ALPs.

The physiological importance of elevated CSF1R transcription in ALPs relative to LMPPs is unclear, as is the latent myeloid potential of these cells. Lymphoid progenitors can alter their lineage output when exposed to pathogen-associated molecular patterns, such as Toll-like receptor ligands [42]. By maintaining a reservoir of *Csf1r* transcripts, it is possible that ALPs can rapidly contribute to emergency myelopoesis upon systemic infection. Yet under steady-state conditions, this monocyte and macrophage potential is not utilized.

The full mechanisms by which lymphoid progenitors restrict myeloid output *in vivo* thus remain unresolved. Specific lymphoid niches and consequent restriction of access to M-CSF

are possible explanations as to why ALPs generate so few myeloid cells *in vivo* [13, 43–45]. Our data are consistent with this mechanism, and justify further studies on specialized niches and cytokine gradients *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1) Lymphoid progenitors yield macrophages *in vitro* robustly only at high doses of M-CSF.

2) Lymphoid and other progenitors limit CSF1R through post-transcriptional mechanisms.

3) *Adam17*$^{-/-}$ lymphoid and other progenitors have elevated CSF1R cell surface expression.

4) *Adam17*$^{-/-}$ lymphoid progenitors yield few macrophages *in vitro* at high doses of M-CSF.

5) ADAM17-deficiency does not alter lymphoid potential or myeloid homeostasis *in vivo*.

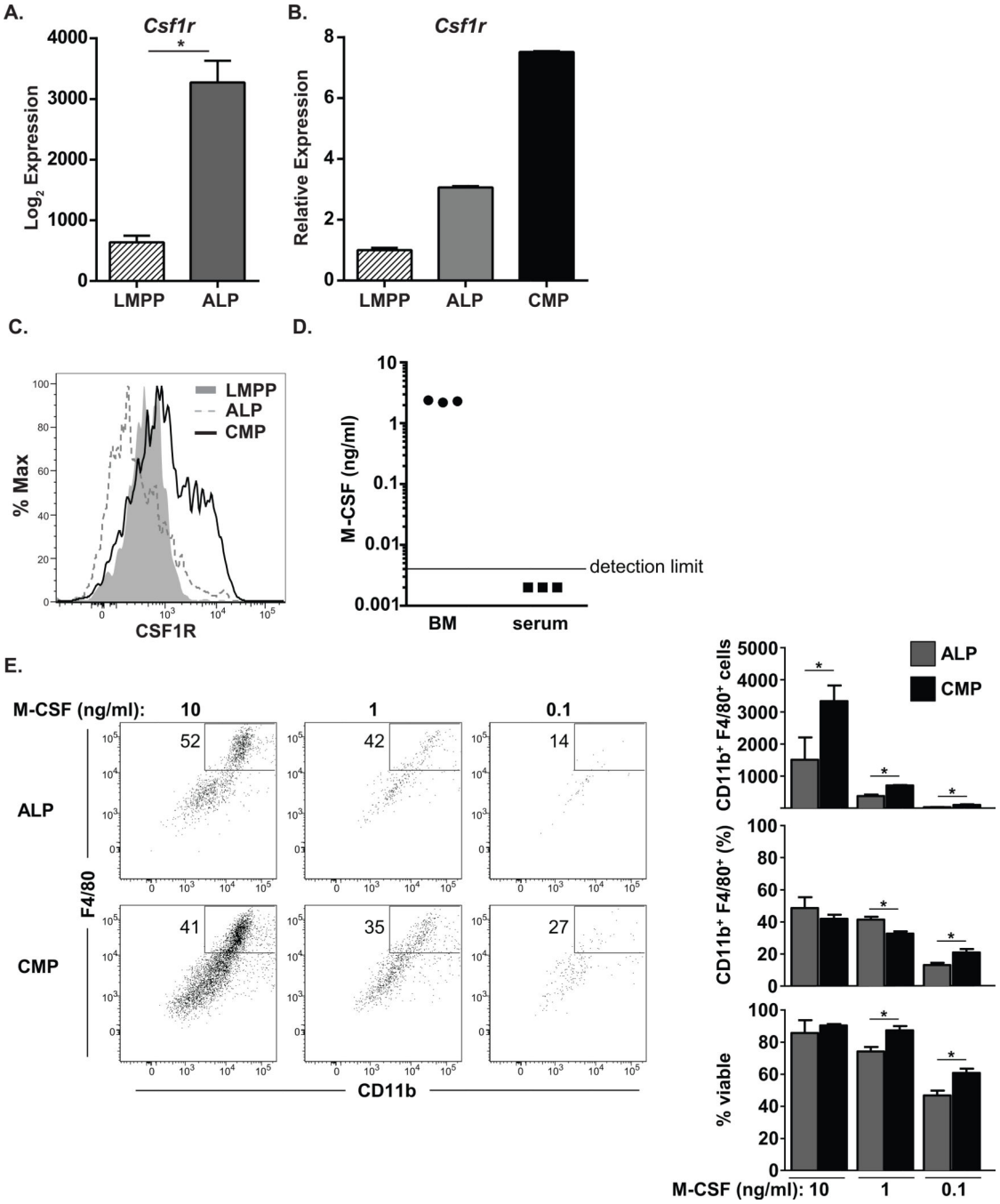

Figure 1. ALPs express low levels of CSF1R and differentiate into monocytes *in vitro* with exogenous M-CSF administration

(A) Microarray data obtained from Inlay et al. [5] showing log2 expression values for *Csf1r*. Signals from probeset 1419872_at are shown. Three biologically distinct samples were analyzed for each population. (B) Quantitative real-time PCR analysis of *Csf1r* for LMPPs, ALPs, and CMPs. *Csf1r* expression was normalized to *Gapdh* expression. Data are representative of 2 independent experiments. (C) Flow cytometry plots show the surface levels of CSF1R expressed on LMPPs, ALPs, and CMPs. These data are representative of 3 independent experiments. (D) ELISA analysis of bone marrow (BM) and serum M-CSF

levels. Femurs were flushed with 1 ml of PBS, levels of M-CSF were quantified in the supernatant, and BM concentrations were estimated assuming a femur volume of 9.4μl (see Materials and Methods for details). Data are cumulative from two independent experiments. (E) Flow cytometric analysis of ALP output. 500 ALPs or CMPs were double-sorted and cultured in the presence of M-CSF for 4 days. Flow cytometric plots in the left panel depict representative data from ALP and CMP cultures. Values within the plots depict the percentage of $F4/80^{+}CD11b^{+}$ macrophages generated. Column graphs in the right panel show cumulative data for the absolute number or frequency of macrophages or the frequency of viable cells. Data are inclusive of 4 independent experiments and represent mean values ± SEM. * $p \leq 0.05$, using students' 2-tailed unpaired t-test.

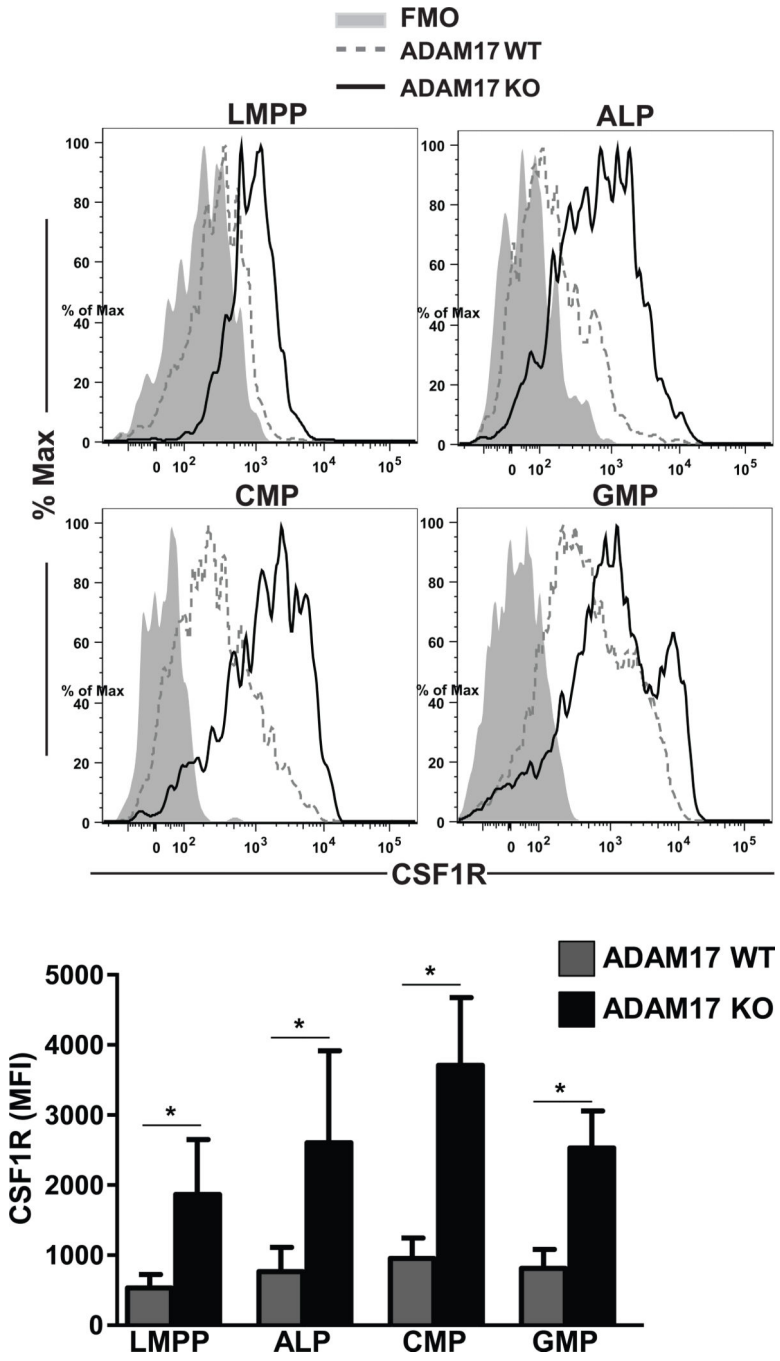

Figure 2. ADAM17 regulates the surface expression of CSF1R on hematopoietic progenitor cells

Flow cytometric analysis of CSF1R expression on wild type and ADAM17-deficient progenitors. Histograms are representative plots from three independent experiments. Bottom panels show an average of CSF1R mean fluorescence intensities (MFI) ± SEM for the same three experiments. * $p \leq 0.05$, using students' 2-tailed unpaired t-test.

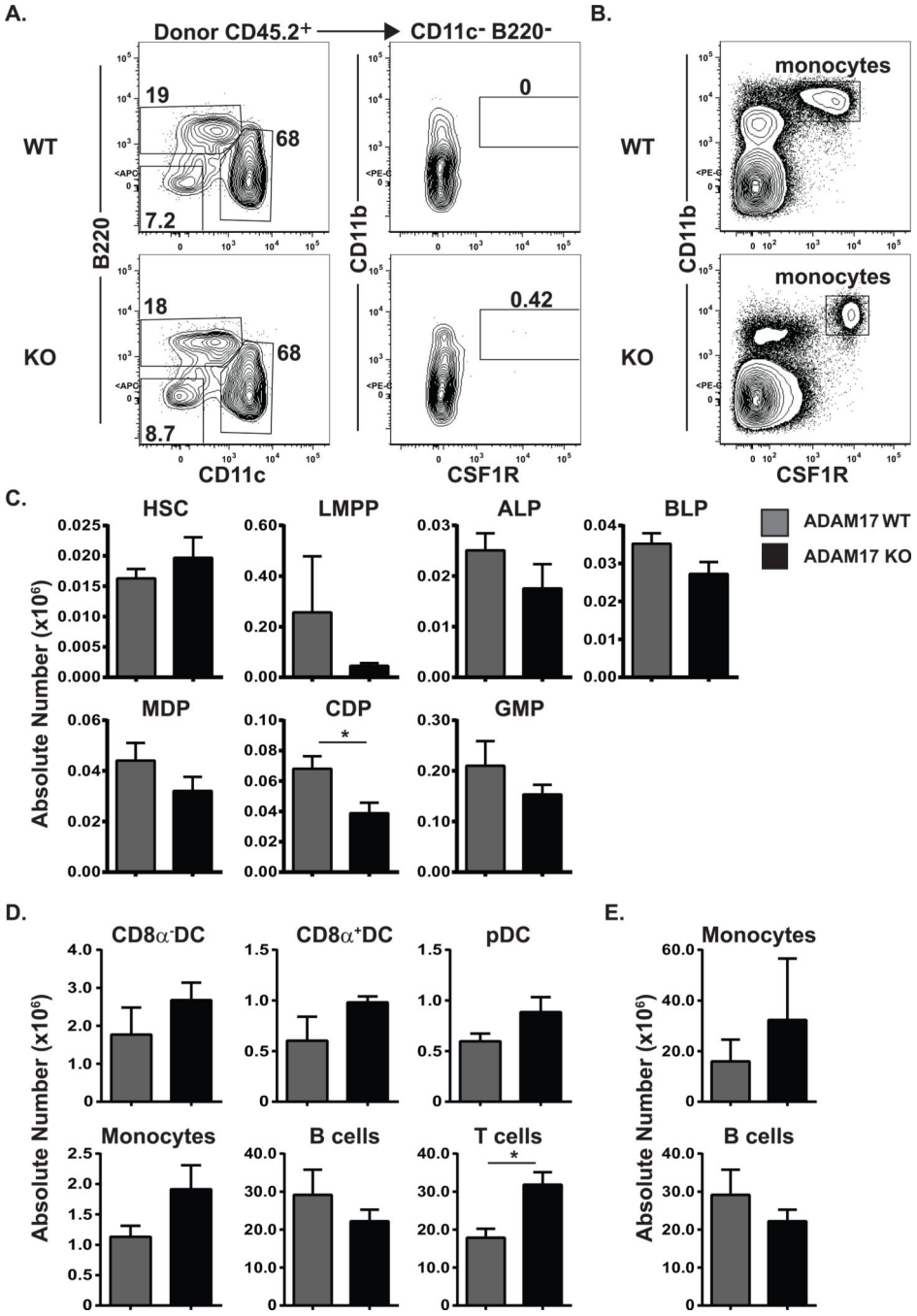

Figure 3. ADAM17 deficient ALPs do not produce monocytes *in vivo*

(A) Flow cytometric analysis of ALP output *in vivo.* 5000 ALPs were double-sorted from ADAM17 wild type or knockout mice. Cells were injected into sublethally irradiated congenic recipients. Ten days post-injection, spleens were harvested and analyzed for donor-derived ($CD45.2^+CD45.1^-$) monocytes ($B220^-CD11c^-$ $CD11b^+CSF1R^+$), B cells ($B220^+$), and dendritic cells ($CD11c^+$). (B) Flow cytometric analysis of CSF1R expression on splenic monocytes (pregated on $B220^-$ $Ly6G^-$ $CD11c^-$ cells) from ADAM17 wild type or knockout mice. (C–E) Flow cytometric analysis of hematopoietic stem and progenitor cells (C) or

mature progeny (D, E) in the bone marrow (C, E) or spleens (D) of ADAM17 wild type or knockout mice. Bar graphs show the mean absolute number cells in each tissue ± SEM. Data are cumulative from three independent experiments. * $p \leq 0.05$, using students' 2-tailed unpaired t-test.

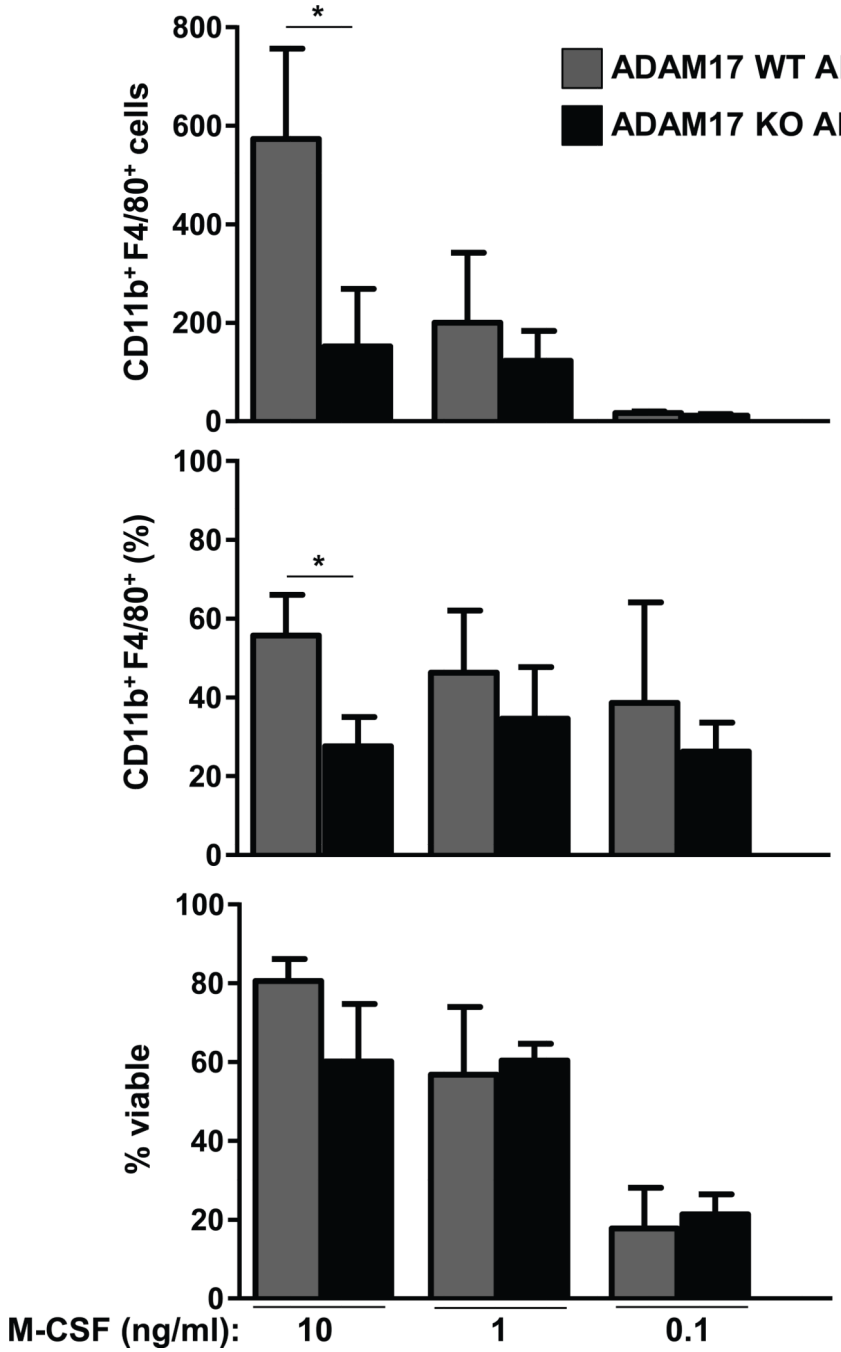

Figure 4. ADAM17-deficient ALPs yield few macrophages at high M-CSF doses

Flow cytometric analysis of wild type and *Adam17*$^{-/-}$ ALPs *in vitro*. 500 ALPs from wild type or ADAM17-deficient donors were cultured in the presence of varying concentrations of M-CSF for 4 days. CD11b^{+} F4/80^{+} macrophages and propidium iodide^{+} dead cells were quantified by flow cytometry. Three ADAM17-deficient animals and wild type siblings were analyzed. Bar graphs show mean values ± SEM. *$p<0.05$, using students 2-tailed t-test.