The Monocyte to Macrophage Transition in the Murine Sterile Wound

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Abstract

The origin of wound repair macrophages is incompletely defined and was examined here in sterile wounds using the subcutaneous polyvinyl alcohol sponge implantation model in mice. Phenotypic analysis identified F4/80<sup>+</sup>Ly6G<sup>−</sup>CD64<sup>+</sup>-MerTK<sup>+</sup> monocytes and F4/80<sup>+</sup>Ly6C<sup>low</sup>CD64<sup>+</sup>MerTK<sup>−</sup> macrophages in the wound. Circulating monocytes were the precursors of inflammatory Ly6C<sup>hi</sup> wound monocytes. Ly6C<sup>low</sup>MerTK<sup>−</sup> macrophages appeared later, expressed CD206, CD11c, and MHC class II, produced cytokines consistent with repair function, and lacked a gene expression profile compatible with mesenchymal transition or fibroblastic transdifferentiation. Data also demonstrated that Ly6C<sup>hi</sup> wound cells were precursors of Ly6C<sup>low</sup> macrophages, although monocytes did not undergo rapid maturation but rather persisted in the wound as Ly6C<sup>low</sup>MerTK<sup>−</sup> cells. MerTK-deficient mice were examined to determine whether MerTK-dependent signals from apoptotic cells regulated the maturation of wound macrophages. MerTK-deficient mice had day 14 cell compositions that resembled more immature wounds, with a smaller proportion of F4/80<sup>+</sup> cells and higher frequencies of Ly6G<sup>+</sup> neutrophils and Ly6C<sup>hi</sup> monocytes. The cytokine profile and number of apoptotic cells in day 14 wounds of MerTK-deficient mice was unaffected despite the alterations in cell composition. Overall, these studies identified a differentiation pathway in response to sterile inflammation in which monocytes recruited from the circulation acquire proinflammatory function, persist in the wound, and mature into repair macrophages.

Introduction

Tissue injury induces an inflammatory response that results in the recruitment of polymorphonuclear leukocytes and monocytes to the site of damage. Circulating monocytes are recruited to wounded tissues as macrophage precursors [1–9]. The experimental depletion of wound monocyte/macrophase populations has revealed their essentiality to the repair process [10–13]. The order and timing by which infiltrating blood monocytes acquire macrophase traits in the sterile wound remains incompletely defined. This monocyte-to-macrophage transition was investigated using cells isolated from subcutaneously implanted polyvinyl alcohol (PVA) sponges in mice.

A recent publication challenged the traditional view that monocytes which extravasate into tissues obligatorily differentiate into macrophages or dendritic cells (DCs) [14]. In that report, specific populations of blood monocytes were shown to migrate into normal skin, lungs, and lymph nodes, where they retained monocyte markers without acquiring the molecular signature of macrophages or DCs. Data to be presented indicate that monocytes migrating into a site of sterile inflammation, here an experimental wound, can either persist as monocytes with a pro-inflammatory phenotype or differentiate in situ to macrophages capable of producing mediators associated with repair.

To examine the transition from monocyte to macrophase in the wound, F4/80<sup>+</sup> cells were monitored for the acquisition of a CD64<sup>+</sup>Mer tyrosine kinase (MerTK)<sup>+</sup> macrophage signature phenotype, as recently defined by the Immunological Genome Project [15]. Co-expression of CD64 and MerTK on F4/80<sup>+</sup> cells allows for the distinction of macrophages from CD64<sup>+</sup>MerTK<sub>low/-neg</sub> monocytes [14]. Results demonstrated that monocytes arriving in the early wound remained MerTK<sub>low/-neg</sub> until day 3 after wounding, and rapidly transitioned to early pro-inflammatory macrophages through acquisition of CD14 expression and TNF-α production. MerTK expression on F4/80<sup>+</sup> wound cells increased over time and was accompanied by loss of Ly6C expression. Ly6C<sup>low/-neg</sup>MerTK<sup>+</sup> wound macrophages were capable of releasing pro-repair mediators, including VEGF and TGF-β, and evidence to be presented suggests that this population arose from the maturation of Ly6C<sup>hi</sup> pro-inflammatory monocyte/macrophase precursors.

Reports by others have demonstrated the upregulation of fibroblast and myofibroblast markers, including procollagens and α-smooth muscle actin, in inflammatory myeloid cells, suggesting...
that wound macrophages may undergo transdifferentiation during the repair process [16–18]. This potential fate of wound macrophages was assessed by examining genes associated with fibroblastic and/or mesenchymal transition.

MerTK is a member of the Tyro3/Axl/Mer (TAM) receptor tyrosine kinase family, whose functions include the phagocytosis and clearance of apoptotic cells by macrophages [19–24], and the subsequent dampening of inflammatory responses [25–29]. Previous work from this laboratory revealed that wound macrophages can induce apoptosis in neutrophils, which are recruited in large numbers to early wounds, and ingest the apoptotic debris [30–32]. Based on the known functions of MerTK, its role in mediating the transition from an inflammatory to a reparative wound monocyte/macrophage phenotype was also examined.

The work described here demonstrates that MerTK+ wound macrophages arise from the maturation of inflammatory monocytes recruited from the circulation. Monocytes entered the wound rapidly after injury, where they persisted before acquiring a repair macrophage phenotype. MerTK deficiency slightly affected the monocyte-to-macrophage transition by altering the cell composition, although not the cytokine environment, of wounds. Overall, these studies identified two functionally and phenotypically distinct myeloid cell subsets in the wound, and demonstrate that these populations are related along a maturation pathway.

Materials and Methods

Ethics Statement

All animal studies were carried out according to the Guide for the Care and Use of Animals of the National Institutes of Health and were approved by the Rhode Island Hospital Institutional Animal Care and Use Committee (Protocol number 0146-12). Surgical procedures were performed under isofluorane anesthesia and all necessary steps to minimize suffering were taken.

Mice

All mice were housed in pathogen-free facilities at Rhode Island Hospital. C57BL/6J (B6; CD45.2), B6. SJL-Ptprc aPepcb/BoyJ (CD45.1), B6;129-Mertk tm1Gr1/J (MerTK control) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Heterozygous CX3CR1-gfp/+ (CX3CR1-GFP) mice were generated by breeding homozygous male B6.129P-Cx3cr1tm1Litt/J mice (CX3CR1−/−) with female C57BL/6J mice (The Jackson Laboratory). Male mice were used for experiments at 8–10 weeks of age.

Polyvinyl Alcohol Sponge Insertion and Wound Cell Isolation

All surgery in mice was performed under anesthesia by isofluorane inhalation. Backs were shaved and cleaned with iodopovidon solution and 70% EtOH. Six 1 cm×1 cm×0.6 cm polyvinyl sponges (Ivalon) were placed subcutaneously through a midline dorsal incision under sterile conditions, and the incision closed with surgical clips. At indicated time points, mice were euthanized by CO2 asphyxiation and blood was obtained by cardiac puncture using heparinized syringes. Leukocyte-containing buffy coats were isolated following centrifugation of whole blood in Wintrobe tubes. Red blood cells were lysed, and blood leukocyte yield and viability was determined by trypan blue exclusion using a hemocytometer.

Identification of Monocytes/Macrophages by Flow Cytometry

Markers used to identify monocyte/macrophage subsets included: F4/80-PerCP Cy5.5, or APC (clone BM8; Bioskience & Biologend), Ly6C-FTTC, PE or APC-Cy7 (clone AL-24; BD Biosciences), CCR2-PE, or APC (Clone 475301; RD Systems), MHC class II-PE (clone M5/114.15.2; BD Biosciences), CD11c-APC (clone HL3; BD Biosciences), Cd206-APC (C-type Mannose Receptor 1, clone MR5D3; AbD Serotec), CD14-PE (clone Sa2-8; eBioscience), MerTK-APC (clone 108928; R&D Systems) and CD64-PE (clone X54-5/7.1.1; BD Biosciences). Flow Cytometry III was blocked with 2.4G2 antibody (BD Biosciences). Cells were surface-stained for 30 minutes on ice in staining buffer (1x DPBS/1% FCS). Where indicated, cells were fixed for 30 minutes, treated with 1x permeabilization wash (Cytofix/Cytoperm Kit; BD Biosciences) and intracellularly stained with TNF-α-PE (clone MP6-XT22; BD Biosciences).

Intracellular Staining of in vivo Phagocytosed Neutrophils

To measure phagocytosis of neutrophils by flow cytometry, wound cells were stained with Ly6G-FTTC and F4/80-APC to identify macrophages and with Ly6G-V450 (clone 1A8; BD Biosciences) to exclude neutrophils. Cells were fixed and permeabilized as described above, then intracellularly stained with Ly6G antibodies to ensure specificity of intracellular Ly6G staining.

Flow Cytometric Analysis of Caspase-3/7 Activity

Measurement of activated caspase-3/7 was performed using the Vybrant® FAM Caspase-3 and -7 Assay Kit for flow cytometry according to manufacturers instructions (Invitrogen). Samples were acquired on a FACSCalibur using CellQuest software or on a FACSArray using FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (Tree Star Inc.). Analysis gates were determined using isotype controls or appropriate negative controls where indicated.

Cell Sorting and Macrophage Culture

Macrophages were enriched from isolated wound cells by negative selection. Specifically, cells were stained with Ly6G-PE (clone 1A8; BD Biosciences), CD2-PE (clone RM2-5; BD Biosciences), TER119/Erythroid Cells-PE (clone Ter-119; BD Biosciences), and/or Siglec-F (MerTK−/− experiments; clone E50-2440; BD Biosciences) for ≤1 hour, washed, and incubated with anti-PE magnetic beads (Miltenyi Biotec) to deplete non-monocyte/macrophage populations. Enriched monocytes/macrophages were assessed for viability by trypan blue exclusion and surface stained with Ly6G-FTTC and F4/80-APC as described above. Eosinophils were identified as F4/80+SSCbre (Ly6Chi and Ly6C++) populations were sorted to greater than 90% purity under sterile conditions on a FACSArray (BD Biosciences).
Sorted cells were assessed for viability by trypan blue exclusion, resuspended in complete medium (DMEM/5% FCS/Penicillin-Streptomycin) and cultured at 37°C/5% CO₂.

Cytokine Measurement
Unfractionated wound cells, column-enriched monocytes/macrophages or FACS-sorted wound monocyte/macrophage populations were cultured in complete medium (DMEM/5% FCS/Penicillin-Streptomycin) or serum-free X-Vivo media (for the detection of TGF-β, Lonza) and cell supernatants removed after 24 h or 72 h for cytokine analysis. 72 h cell supernatants were tested by sandwich ELISA for TNF-α (BD Biosciences OptEIA mono/mono ELISA Set II), and TGF-β (DuoSet, R&D Systems). 24 h cell supernatants were tested by sandwich ELISA for IL-1β (BD Biosciences OptEIA mono/mono ELISA Set II) and VEGF (DuoSet, R&D Systems). Culture times were selected for optimal detection of cytokines in supernatants within the ELISA standard curves.

In vivo Microparticle Labeling of Monocyte Subsets
Selective labeling of circulating monocyte subsets was performed with methods adapted from those described in Tacke et al [33]. To label Ly6C<sup>hi</sup> monocytes, 250 μL of liposome-encapsulated clodronate (Dr. Nico van Rooijen; ClodoronateLiposome.s.com) was administered i.v. to B6 mice to deplete circulating monocytes. 18 h after clodronate injection, mice received an i.v. injection of Fluoresbrite Polychromatic Red Microbeads (PE-MP; 0.2 μg per mouse, Polysciences, Inc.). PVA sponges were implanted 1 day after bead injection, harvested 1 or 7 days later and processed for cell surface staining. The presence of PE-microbeads in blood and wound monocyte/macrophage subsets was assessed by FACS.
To label Ly6C<sup>low</sup> monocytes, PE-MP were administered i.v. without prior treatment with liposome-encapsulated clodronate. B6 mice received sponges 1 day after bead injection. Blood and sponge cells were harvested 1 or 7 days later and prepared for flow cytometry analysis as previously described.

PVA Sponge Adoptive Transfer
CD45.1 congenic mice received sponges for 1 day. Sponges were then removed from CD45.1 congenic (donor) mice under sterile conditions and transferred to unwounded B6 (recipient) mice. Sponges were harvested from recipient mice at 1, 3 or 7 days post-transfer. Cells were isolated from sponges and surface stained as previously described. CD45.1<sup>+</sup> and CD45.2<sup>+</sup> wound monocyte/macrophage subsets were analyzed by FACS at indicated time points.

Gene Expression Analysis of Macrophage Subsets
Macrophages from the day 14 wound were enriched by negative selection on a magnetic column (Miltenyi Biotec) using the following depletion cocktail: Ly6G-PE (clone 1A8, BD Biosciences), CD2-PE (clone RM2–5; BD Biosciences), Siglec-F-PE (clone E50–2440; BD Biosciences) and TER119/Erythroid Cells-PE (clone E50–2440; BD Biosciences), CD2-PE (clone RM2–5; BD Biosciences), Siglec-F-PE and F4/80-APC for 30 minutes on ice. Sytox Blue (Invitrogen) was used for dead cell exclusion. Ly6G<sup>hi</sup>F4/80<sup>+</sup>Ly6G Siglec-F<sup>−</sup> and Ly6G<sup>low/int</sup>F4/80<sup>+</sup>Ly6G Siglec-F<sup>−</sup> subsets were sorted to ≥90% purity under sterile conditions using a FACSaria.
RNA was isolated from sorted cells using the RNeasy Micro Kit (Qiagen) and cDNA was synthesized with the High Capacity RNA-to-cDNA Kit (Life Technologies). Gene expression analysis was performed by real-time quantitative PCR on a Viia 7 system (Life Technologies) using TaqMan Assays (Life Technologies) and TaqMan Gene Expression Master Mix (Life Technologies). TaqMan Assay IDs for individual genes are shown in Table S1. Hprt and 18 s served as endogenous controls. Both reference genes produced similar results after normalization. Results using Hprt are shown here. Analysis was carried out using the 2<sup>–ΔΔCt</sup> method.

Statistical Analysis
Statistical analysis was performed using ANOVA or Mann-Whitney tests. Statistical significance was defined as a p value of <0.05.

Results
Time Dependent Accumulation of Monocyte/Macrophage Subsets in the Sterile Wound
Differential analysis of cellular infiltrates in the sterile PVA sponge has demonstrated that monocytes are recruited to the wound within one day post-implantation, and that the frequency of monocytes/macrophages in the wound increases with time [34]. Distinct subsets of wound monocytes/macrophages could be distinguished by expression of the markers F4/80 and Ly6C. The day 1 F4/80<sup>+</sup> wound cell population was predominantly Ly6C<sup>hi</sup> (Figure 1D). The frequency, although not the absolute number, of this subset declined concurrent with the appearance of Ly6C<sup>low</sup> cells by day 3 (Table S2). The proportion of Ly6C<sup>hi</sup> cells increased at day 7 before falling slightly at day 14 (Figure 1A). By day 14, distinct F4/80<sup>+</sup>Ly6C<sup>hi</sup> (hereafter Ly6C<sup>hi</sup>) and F4/80<sup>+</sup>Ly6C<sup>low</sup> (hereafter Ly6C<sup>low</sup>) subsets were apparent in the wound (Figure 1A).
Subsets were further examined for expression of CD64 and MerTK. These molecules, when co-expressed, have been identified as macrophost core markers [14, 15]. Ly6C<sup>hi</sup> wound monocytes expressed CD64 at 76 ± 7%, 54 ± 15%, 66 ± 16%, and 80 ± 5% days after injury. The frequency of MerTK expression by these cells at all examined time points was ≤2% (Figure 2B). This phenotype was similar to that of circulating Ly6C<sup>hi</sup> monocytes, which were CD64<sup>+</sup>/MerTK<sup>+</sup> or CD64<sup>+</sup>MerTK<sup>−</sup> (Figure 1C). In contrast, wound Ly6C<sup>low</sup> cells were more likely to co-express CD64 and MerTK (9 ± 3% of cells 3 days after injury, 57 ± 9% by post-wound day 7, and 59 ± 2% on day 14; Figure 1B). qPCR analysis similarly revealed a higher relative expression of MerTK in Ly6C<sup>low</sup> macrophages when compared to Ly6C<sup>hi</sup> monocytes/macrophages at day 14 after wounding (Figure 1D). Expression of TAM family members Axl and Tyro3 on wound monocyte/macrophage subsets could not be detected by flow cytometry (data not shown). Further cell surface marker analysis revealed that the day 1 and day 14 Ly6C<sup>hi</sup> subsets were CD11c<sup>low/int</sup>MHC class II<sup>low</sup>/CD206<sup>+</sup>. The expression of CD11c and MHC class II was higher on day 14 wound Ly6C<sup>hi</sup> cells when compared to their day 1 counterparts. The day 14 Ly6C<sup>hi</sup> subset was MHC class II<sup>+</sup>, CD11c<sup>−</sup> and CD206<sup>+</sup> (Figure 2). A similar pattern of surface marker expression to that seen at day 14 was detected on monocyte/macrophage subsets isolated from day 7 wounds. MHC class II, CD11c and CD206 were more highly expressed on day 7 Ly6C<sup>low</sup> macrophages than on Ly6C<sup>hi</sup> monocytes, although the difference between the two subsets was not as pronounced as that observed.
Cell cycle analysis revealed no monocyte/macrophage proliferation in the day 1 or day 14 wound (data not shown).

Ly6C<sup>low</sup> Wound Macrophages Arise from Ly6C<sup>hi</sup> Monocytes

Four independent approaches were taken to examine the origin of monocytes/macrophages in the wound. These included the tracking of in vivo-labeled monocytes migrating to the wound,

![Figure 1. Phenotype of wound monocyte/macrophage subsets.](image)

Figure 1. Phenotype of wound monocyte/macrophage subsets. C57BL/6J mice were wounded for 1, 3, 7 or 14 days. Wound cells were isolated and examined by flow cytometry. FSC and SSC were used to gate viable cells. (A) Wound monocytes/macrophages were defined as F4/80<sup>+</sup>SSC<sub>int</sub>, F4/80<sup>+</sup>SSC<sup>hi</sup> eosinophils were excluded from analysis. Gated F4/80<sup>+</sup> wound monocytes/macrophages were additionally examined for expression of Ly6C. (B) Expression of CD64 and MerTK was examined on gated Ly6C<sup>hi</sup> and Ly6C<sup>low</sup> monocyte/macrophage subsets. (C) Blood cells were isolated from naive C57BL/6J mice and examined by flow cytometry. An identical gating strategy to that described for wound cells was employed to examine MerTK and CD64 expression on blood F4/80<sup>+</sup>Ly6C<sup>hi</sup> and Ly6C<sup>low</sup> monocytes. Numbers accompanying gates are the mean ± SD, n = 3 mice per group, and are representative of 2–3 independent experiments. (D) Mer<sub>k</sub> gene expression was determined in day 14 Ly6C<sup>hi</sup> and Ly6C<sup>low</sup> wound monocyte/macrophage subsets by qPCR. Mer<sub>k</sub> expression was normalized to Hprt and data are presented as ΔCt Expression. Data shown are the mean ± SD, n = 3 mice per group.

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![Figure 2. Surface marker expression on day 1 and day 14 wound macrophage subsets.](image)

Figure 2. Surface marker expression on day 1 and day 14 wound macrophage subsets. C57BL/6J mice were wounded for 1 or 14 days, and the surface expression of MHC class II, CD11c and CD206 on wound monocyte/macrophage subsets was determined by flow cytometry. Wound monocyte/macrophage subsets were defined as F4/80<sup>+</sup>Ly6C<sup>hi</sup> (Ly6C<sup>hi</sup>) and F4/80<sup>+</sup>Ly6C<sup>low</sup> (Ly6C<sup>low</sup>). Mean fluorescence intensities (MFI; geometric mean) were calculated and are presented as the mean ± SD, n = 4 mice per group. Data are representative of at least 3 independent experiments.

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blood monocyte subset analysis before and after wounding, examination of chemokine receptor expression on blood and wound monocyte/macrophage subsets, and the adoptive transfer of wound sponges between CD45 congenic mice.

The monocyteic origin of wound Ly6C^hi cells was first verified using the in vivo labeling techniques described by Tacke et al [33]. The i.v. injection of fluorescent microparticles (MP) to animals previously depleted of monocytes by the administration of liposome-encapsulated clodronate results in the selective labeling of F4/80^hi Ly6C^hi blood monocytes. In contrast, the MP are taken up almost exclusively by F4/80^hi Ly6C^low monocytes when injected into naive mice. MP uptake has been demonstrated to not affect the recruitment of monocytes from the blood, nor to induce phenotypic markers of activation or the accumulation of proinflammatory cytokines in plasma. Further, no overt adverse effects of clodronate-mediated depletion were reported [33]. Indeed, this method of assessing monocyte trafficking presents advantages over adoptive transfer methods by eliminating the need for ex vivo cell manipulations that may alter the behavior of transferred cells, and provides greater sensitivity for quantification of migration [33].

Figure 3A is a schematic demonstrating the order and timing of liposome-encapsulated clodronate and MP administration prior to wounding. In the blood, MP administered after liposome-encapsulated clodronate treatment were observed predominantly in the F4/80^hi Ly6C^hi subset at 1 and 7 days post-wounding, while approximately 1% of blood monocytes were MP^Ly6C^low at day 7 (Figure 3B). As expected, clodronate treatment resulted in a reduction of the Ly6C^low circulating monocyte population at days 1 and 7 after wounding (Figure 3B). However, this did not affect the pattern of wound monocyte/macrophage accumulation, as the distribution of Ly6C^hi and Ly6C^low wound monocytes/macrophages was similar with or without clodronate treatment (Figures 3B and 3D). MP-containing cells were detected in the day 1 and day 7 wound following selective labeling of Ly6C^hi blood monocytes. The majority of MP-labeled F4/80^hi wound monocytes at both time points were Ly6C^hi. A small proportion of the labeled monocytes at day 7 were Ly6C^low, possibly due to maturation of Ly6C^hi cells in the wound or the migration of the small fraction of MP^Ly6C^low blood monocytes observed at this time point [1,3,35].

A similar tracking approach was adopted to examine whether circulating Ly6C^low monocytes were recruited to the wound (schematic shown in Figure 3C). After intravenous injection of labeled MP into naive animals, F4/80^MP^+ monocytes were detected in the blood after wounding, and nearly 100% of these cells were Ly6C^hi (Figure 3D). Examination of wound cells at 1 and 7 days after sponge insertion failed to detect labeled Ly6C^low monocytes/macrophages among infiltrating F4/80^+ cells (Figure 3D). Approximately 0.3% of wound monocytes were MP^Ly6C^hi at day 1, perhaps due to the migration of MP-labeled Ly6C^hi monocytes from the blood (0.1% MP^Ly6C^low, Figure 3D).

It was further noted that Ly6C^hi but not Ly6C^low monocytes were transiently diminished in the circulation at 1 day after wounding, suggesting preferential trafficking of this subset to the wound (Figure 4A). The Ly6C^low monocyte count in the circulation remained constant over the time points examined (Figure 4A).

The presence of CD45 congenic mice expressing GFP under the control of the CX3CR1 promoter after wounding (Figure 4B). In contrast, wound monocytes/macrophages harvested 1 or 14 days after wounding were CX3CR1^GFP^hi (Figure 4B). Ly6C^hi and Ly6C^low subsets at day 14 expressed similar levels of CX3CR1 (Figure 4C). Expression of CX3CR1 was moderate on day 14 wound cell subsets when compared to blood CX3CR1^hi monocytes, but higher than that seen on day 1 Ly6C^hi wound monocytes/macrophages (Figure 4C). Furthermore, in contrast to the inverse relationship between CX3CR1 and CCR2 expression on blood monocytes, day 1 and day 14 F4/80^hi wound cells were found to co-express these chemokine receptors, regardless of Ly6C status (Figure 4C).

To examine the potential for Ly6C^hi wound monocytes to mature into Ly6C^low cells in situ, sponge adoptive transfer experiments were performed using mice expressing the congenic markers CD45.1 or CD45.2. Wound cells from congenic strains had comparable expression of F4/80 and Ly6C. Sponges were removed from CD45.1 donor mice 1 day after wounding when >70% of F4/80^+ cells were Ly6C^hi and ≤10% were Ly6C^low (Figure 1A) and transferred to CD45.2^+ recipients. Sponges were harvested at 1, 3, or 7 days post-transfer, and the phenotype of donor CD45.1^+ cells was analyzed by flow cytometry (Figure 5). The frequency of donor-derived CD45.1^+ cells decreased over time due to the accumulation of CD45.2^+ recipient cells in the wound. However, a similar number of F4/80^CD45.1^+ donor-derived cells were recovered at days 1 and 7 after transfer (Table S3). The presence of CD45.1^+ donor-derived cells in the sponge did not affect the recruitment or phenotype of CD45.2^+ recipient cells in the wound upon sponge transfer. Examination of donor-derived CD45.1^+F4/80^+ cells revealed a decrease in the proportion and total number of Ly6C^hi cells over time (Figure 5 and Table S3). At days 1 and 3 post-transfer, 35 ± 15% and 32 ± 12% of donor monocytes/macrophages, respectively, were Ly6C^hi. At day 7, only 2 ± 0.5% of donor-derived F4/80^+ wound cells were Ly6C^hi. In contrast, the frequency and total number of Ly6C^low donor-derived F4/80^+ cells increased with time (Figure 5 and Table S3). At day 1, 29 ± 2% of the F4/80^+ population was Ly6C^low. This proportion increased to 41 ± 10% at day 3 and 67 ± 11% at day 7. The recovery of a similar number of CD45.1^+F4/80^+ cells at 1 and 7 days post-transfer suggests that these results are not an artifact of selective survival of donor Ly6C^low cells, but rather due to in situ conversion of Ly6C^hi monocytes.

Early Wound Monocytes Acquire Function in the Wound and are Distinct from Late Macrophages

Ly6C^hi cells in blood and wound were compared to determine the acquisition of inflammatory traits upon entry to the wound. FACS analysis of intracellular TNF-α revealed that 20% of day 1 Ly6C^hi wound monocytes spontaneously expressed this cytokine (Figure 6A). This was in contrast to Ly6C^hi blood monocytes from naive and day 1-wounded mice, where less than 5% of the population expressed TNF-α. CD14 was also upregulated upon entry to the wound. Circulating monocytes in naive and day 1-wounded mice expressed low levels of CD14, while nearly 100% of Ly6C^hi monocytes in the day 1 wound were CD14^hi (Figure 6B). Additionally, analysis of forward scatter (FSC) and side scatter (SSC) properties revealed that Ly6C^hi monocytes in the day 1 wound had a similar FSC profile but higher SSC than circulating monocytes, suggesting that the cells gained greater internal complexity upon entry to the wound (Figure 6C and D).

The functional transition from early wound inflammatory monocytes to repair macrophages was examined by ex vivo cytokine production profiles (Figure 7). Day 1 and day 14 Ly6C^hi monocytes express a variety of cytokines including TNF-α, IL-6, and IL-1β. In contrast, day 14 Ly6C^low cells expressed much lower levels of these cytokines, indicating an acquired regulatory function.
and day 14 Ly6C low wound monocytes/macrophages were FACS sorted and cultured alongside unfractionated wound cells. Culture supernatants were tested by ELISA for production of proinflammatory and repair-associated cytokines. The highest levels of both IL-1β and TNF-α were detected in culture supernatants of Ly6Chi cells enriched from day 1 wounds (Figure 7A and B). These cells produced 3.5-fold more IL-1β than unfractionated cells from day 1 wounds, 7-fold more than day 14 total cells, and 2- and 5-fold more than Ly6Chi and Ly6Clow cells recovered from day 14 wounds, respectively (Figure 7A). TNF-α production by day 14 unfractionated wound cells was below the level of detection of the assay (Figure 7B).

The highest concentrations of TGF-β and VEGF were detected in day 14 wound cell cultures. A comparison of TGF-β production by day 14 wound monocyte/macrophage subsets revealed that Ly6C low cells preferentially produced this cytokine, making 6-fold more than Ly6C hi cells at the same time point (Figure 7C). Production of TGF-β by day 14 wound Ly6C low cells was also higher than that by day 1 unfractionated and Ly6C hi cells. Similarly, day 14 Ly6C low cells produced over 5-fold more VEGF than their Ly6C hi counterparts, and more than day 1 total and Ly6Chi cells (Figure 7D). For both TGF-β and VEGF, the highest concentrations were detected in day 14 unfractionated cell cultures, indicating that monocytes/macrophages are not the exclusive source of these cytokines (Figure 7C and D).

Comparative gene expression analysis revealed relatively higher levels of Il1b, Tnf, Tgfb and Vegf cytokine message in the Ly6C hi subset relative to the Ly6C low subset. The difference between populations was most pronounced for Il1b expression, which was 36-fold higher in Ly6C hi relative to Ly6C low F4/80+ cells. The Ly6C hi subset had an approximately 2-fold higher expression level of Tnf, Vegfa and Tgfb1 in comparison to Ly6C low cells (Figure S1).
Gene Expression Analysis of Day 14 Wound Monocytes/Macrophages

Day 14 wound Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{low} monocytes/macrophages were assessed for the differential expression of genes related to fibroblastic and/or mesenchymal transition. Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{low} monocytes/macrophages were sorted from the day 14 wound, and relative expression of matrix metalloproteinase 9 (\textit{Mmp9}), vimentin (\textit{Vim}), tissue inhibitor of metalloproteinase 1 (\textit{Timp1}), type I collagen (\textit{Col1a1}), type III collagen (\textit{Col3a1}), and \(\alpha\)-smooth muscle actin (\textit{Acta2}) was determined by qPCR (Figure 8). These genes were selected based on reports examining mesenchymal transition in myeloid cells [16–18]. Ly6C\textsuperscript{low} wound macrophages had approximately 6-fold higher levels of \textit{Mmp9} expression relative to Ly6C\textsuperscript{hi} cells. In contrast, \textit{Acta2} was expressed at lower levels in Ly6C\textsuperscript{low} macrophages. \textit{Vim}, \textit{Timp1}, \textit{Col1a1} and \textit{Col3a1} relative expression was approximately equal between both monocyte/macrophage subsets (Figure 8).

MerTK Regulates the Cellularity but not the Cytokine Environment in 14 Day-old Wounds

The role of MerTK in shaping the monocyte/macrophage response after PVA sponge implantation was investigated using mice that are deficient in MerTK signaling (MerTK\textsuperscript{\textminus/\textminus}). The day 14 wound was chosen for analysis because the highest level of MerTK expression was detected on Ly6C\textsuperscript{low} wound macrophages at this time point (Figure 1C). MerTK expression on wound macrophage subsets at day 14 post-sponge implantation was comparable between B6129SF2/J control and B6 mice, and undetectable by flow cytometry analysis on cells isolated from the wounds of MerTK\textsuperscript{\textminus/\textminus} mice (Figure 1B and data not shown).
MerTK deficiency did not alter the total number of wound cells recovered 14 days after injury (control, 5.1 ± 2.7x10⁶ vs. MerTK⁻⁄⁻, 7.3 ± 2.9x10⁶, n = 6, p > 0.05). However, day 14 wounds from MerTK⁻⁄⁻ mice contained a greater frequency of Ly6G⁺ neutrophils and a lower proportion of F4/80⁺ cells than controls (Figure 9A). The F4/80⁺ population isolated from wounds in MerTK⁻⁄⁻ mice contained a higher frequency of Ly6C hi cells when compared to controls (Figure 9B). Overall, the composition of cellular infiltrates in day 14 wounds from MerTK⁻⁄⁻ mice resembled that seen in less mature wounds in normal mice.

MerTK has been reported to function in the phagocytic clearance of apoptotic debris. However, the fraction of wound cells expressing activated caspase-3/7 in day 14 wounds was similar in MerTK⁻⁄⁻ and control mice (26% ± 5 and 37% ± 8, respectively, n = 6 mice; Figure 9C). Moreover, when day 14 wound macrophages from B6 mice were stained for intracellular Ly6G as a marker for phagocytosed neutrophils, Ly6C hi MerTK⁻ and Ly6C lo MerTK⁺ wound monocyte/macrophage populations were comparable in their expression of this marker by both frequency (Figure 9D) and total number (Figure 9E).

Because MerTK activation has also been shown to modulate cytokine production by macrophages [26,28,36], TNF-α, VEGF and TGF-β concentrations were measured in fluids and enriched macrophage culture supernatants from day 14 wounds of control and MerTK⁻⁄⁻ mice. No differences were detected between groups (Table 1).

Discussion

Studies presented here were performed to examine the monocyte-to-macrophage transition in sterile wounds.
were conducted using the subcutaneously implanted PVA sponge wound model. This model recapitulates the inflammatory, angiogenic, and fibrotic responses seen in all soft tissue wounds for at least two weeks following sponge implantation. Thereafter the continued fibrotic response to the sponge models for a sterile foreign body reaction. The PVA sponge wound model offers the singular advantage of allowing for the full recovery of viable infiltrating cells by simple mechanical disruption, without requiring the use of enzymatic digestion of the wound material. The biological response to the sponge does not depend upon an irritative effect of the implanted material, because an identical sequence of inflammation and fibrosis follows the subcutaneous insertion of sterile stainless steel mesh Hunt-Schilling chambers [37]. Importantly, the phenotype of macrophages isolated from the sponge wound has been shown to be the same as that of cells isolated from murine skin wounds [38].

Previous work with the PVA sponge model described the sequential accumulation of Gr-1high and Gr-1low F4/80+ cells in the healing wound [34]. The addition of new phenotyping tools allowed for the identification of a subset of proinflammatory F4/80+Ly6Chigh monocytes and repair-like F4/80+Ly6Clow macrophages. Analysis of CD64 and MerTK expression on F4/80+ cells demonstrated that Ly6C<sub>low</sub>F4/80<sub>high</sub>CD64<sup>+</sup>MerTK<sup>+</sup> macrophages do not appear in the sterile wound immediately following injury. The early response was dominated by Ly6C<sub>high</sub>F4/80<sub>high</sub>CD64<sup>-</sup>MerTK<sup>-</sup> cells that phenotypically resembled circulating monocytes but expressed CD14 and had a proinflammatory signature including the ability to produce TNF-α and IL-1β. This observation was consistent with the report that alveolar monocytes expressed TNF-α and CD14 upon entry to the lung following CCL2-mediated inflammation, but otherwise resembled their circulating precursors [39].

A Ly6C<sub>low</sub>F4/80<sub>low</sub>CD64<sup>+</sup>MerTK<sup>+</sup> population appeared in the wound by 3 days post-injury and persisted through day 14. The proportion of Ly6C<sub>low</sub> macrophages co-expressing CD64 and MerTK increased over time. This subset also expressed CD206 along with CD11c and MHC class II, and produced cytokines consistent with repair function (VEGF and TGF-β) [9,34].

Figure 6. Changes in monocyte phenotype upon entry to the wound. Gated F4/80+Ly6Chigh blood monocytes from naive and day 1-wounded C57BL/6J mice were compared to wound monocytes from day 1-wounded mice by flow cytometry analysis. (A) Proportion of TNF-α<sup>+</sup> cells and representative FACS plots, (B) proportion of CD14<sup>+</sup> cells and representative histograms, (C) FSC median fluorescence intensity (MFI) and (D) SSC MFI with representative histograms. Data shown are the mean ± SD, n = 3 mice per group for (A) and (B); n = 2–3 mice per group for (C) and (D). Data are representative of 2–3 independent experiments.

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The cytokine profile of the Ly6C\textsuperscript{low} monocyte/macrophage subset suggested that these cells promote angiogenesis and fibrosis in the wound. Studies by others have proposed that macrophages exhibit functional and phenotypic plasticity with the ability to undergo transdifferentiation to fibroblast- or myofibroblast-like cells, which are essential to the fibrotic response. Multiple studies have described the upregulation of \( \alpha \)-smooth muscle actin, a myofibroblast marker, on cells of myeloid origin during granulation tissue formation or in response to TGF-\( \beta \) [16–18]. Others found that Mac-1-expressing macrophages upregulated procollagen expression during \textit{Schistosoma} infection, suggesting transdifferentiation into fibroblasts [40].

It is also possible that co-expression of myeloid and mesenchymal markers may result from the differentiation of a common precursor rather than lineage reprogramming. In this regard, fibrocytes are bone marrow-derived cells that co-express myeloid and hematopoietic markers, participate in fibrotic and angiogenic processes, and act as fibroblast markers and myofibroblast precursors [41]. Fibrocytes are characterized by their expression of the mesenchymal marker vimentin, and their ability to produce extracellular matrix proteins including collagens, as well as matrix remodeling enzymes, in particular MMP-9 [41–45]. It was hypothesized that the fate of the Ly6C\textsuperscript{low} wound macrophages described here may involve the acquisition of fibroblast or mesenchymal traits. However, qPCR analysis of collagens, \( \alpha \)-smooth muscle actin, and vimentin did not reveal a subset-specific pattern of expression, at least at the singular time point examined here. Gene expression analysis did indicate a several-fold higher level of \textit{Mmp9} in the Ly6C\textsuperscript{low} cells relative to the Ly6C\textsuperscript{hi} subset, while no difference was detected in \textit{Timp1}. Despite this difference, the results do not support a fibrocyte origin or transdifferentiation program for the day 14 Ly6C\textsuperscript{low} wound macrophage. Ongoing studies will examine whether transdifferentiation is evident at other time points or by morphological analysis. While it is possible that transdifferentiation may result in loss of macrophage markers, evidence provided by Mooney et al. (2010) demonstrated that csf1r-EGFP cells recruited to a peritoneal foreign body implant acquired \( \alpha \)-smooth muscle actin while retaining macrophage morphology and F4/80 expression, the marker used here for macrophage identification [16]. Experiments were performed 14 days after

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure7}
\caption{Cytokine production by wound monocyte/macrophage subsets. F4/80\textsuperscript{hi}Ly6C\textsuperscript{hi} monocytes/macrophages were sorted from day 1 wound cells. F4/80\textsuperscript{hi}Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{low} monocytes/macrophages were sorted from day 14 wound cells. Sorted populations were cultured as described in Materials & Methods and supernatants were tested by ELISA for (A) IL-1\( \beta \), (B) TNF-\( \alpha \), (C) TGF-\( \beta \) and (D) VEGF production. Data shown are the mean \( \pm \) SD; \( n = 3 \) mice per experiment; data represent 2–3 independent experiments. \# = below the level of detection. doi:10.1371/journal.pone.0086660.g007}
\end{figure}
wounding because others have reported evidence for macrophage transdifferentiation at this time point in foreign body reactions in mice [16]. The findings reported here, however, do not rule out that mesenchymal transition may occur in wounds at a later time point.

The sequential nature of monocyte and macrophage accumulation in the wound led to the hypothesis that wound inflammatory monocytes derive from circulating monocytes that differentiate within the wound to become repair macrophages. Multiple approaches aimed at determining the origin and fate of Ly6C<sup>hi</sup> wound monocytes/macrophages indicated that Ly6C<sup>hi</sup> monocytes preferentially migrated from the circulation to the wound, and that this population changed in situ to Ly6C<sup>lo</sup> macrophages. These studies demonstrated that recruited inflammatory monocytes are precursors to repair macrophages in this model of sterile wound healing, which is consistent with the recruitment of F4/80<sup>lo</sup>-Ly6C<sup>lo</sup> proinflammatory monocytes and subsequent conversion to anti-inflammatory F4/80<sup>hi</sup>Ly6C<sup>hi</sup> monocytes/macrophages that occurs during skeletal muscle repair [5]. The selective migration of Ly6C<sup>hi</sup> monocytes to the PVA sponge is in contrast to a model of recruitment of Ly6C<sup>hi</sup> inflammatory wound monocytes and Ly6C<sup>lo</sup> macrophages. The proportion of Ly6C<sup>lo</sup> macrophages expressing MerTK increased over time, reaching 59 ± 2% by day 14 after wounding, and the level of MerTK expression (as measured by MPI) was higher on Ly6C<sup>lo</sup> than on Ly6C<sup>hi</sup> cells at all time points examined (data not shown). Of note, the MerTK<sup>hi</sup>F4/80<sup>lo</sup>Ly6C<sup>lo</sup> population detected at day 14 after injury resembled a recently described human anti-inflammatory macrophage population that preferentially expresses MerTK and CD206 and possesses the ability to clear early apoptotic cells [20].

Figure 8. Comparative gene expression analysis of day 14 Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> macrophages. F4/80<sup>−</sup>Ly6C<sup>hi</sup> and F4/80<sup>−</sup>Ly6C<sup>lo</sup> macrophages were FACS-sorted from the day 14 wound. Expression of matrix metalloproteinase 9 (Mmp9), vimentin (Vim), tissue inhibitor of metalloproteinase 1 (Timp1), collagen 1 (Col1a1), collagen 3 (Col3a1) and α-smooth muscle actin (Acta2) was examined by qPCR. Target gene expression was normalized to Hprt. ΔΔCt Expression is presented as fold difference in relative gene expression of Ly6C<sup>lo</sup> relative to Ly6C<sup>hi</sup> cells as described in Materials & Methods. Equal gene expression between subsets is indicated by a grey dashed line placed at a fold change of 1. Data are means ± SD, n = 3 mice per group.

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The kinetics and subset-specific MerTK expression on wound monocyte/macrophage populations raised the question of whether this kinase is involved in development of the repair macrophage phenotype. The reported functions of MerTK include phagocytosis of apoptotic cells and dampening production of proinflammatory mediators [19,20–24,26,29,46]. Accordingly, mice that lack MerTK are prone to LPS-induced endotoxic shock and the development of autoimmune disorders [21,25,27,28]. Differential analysis has shown that neutrophils dominate the early wound, and that these cells persist even as monocytes/macrophages accumulate [32]. Work from this laboratory previously demonstrated that soluble products released from murine wound neutrophils dampen LPS-induced macrophage inflammatory responses in vitro [32]. Furthermore, using the PVA sponge model in rats, wound macrophages were found to both induce apoptosis and ingest wound neutrophils [30,31]. It was hypothesized that MerTK may mediate some of these processes in the sterile wound and, as a result, promote the transition from inflammatory monocyte to repair macrophage.

The composition of inflammatory infiltrates from the wounds of MerTK<sup>−−</sup> mice was more immature than that from controls. Day 14 wounds from MerTK<sup>−−</sup> animals contained a higher proportion of neutrophils and F4/80<sup>−</sup>Ly6C<sup>hi</sup> monocytes. This alteration in the wound cellularity, however, did not impact the cytokine and growth factor composition of wound fluids or monocyte/macrophage culture supernatants.

Contrary to what was expected given the known role of MerTK as a phagocytic receptor for apoptotic cells, there was no difference between MerTK-deficient and control mice in the number of wound cells expressing activated caspase-3/7, or in the frequency of wound macrophages that had ingested neutrophils. In further support of MerTK-independent phagocytosis, both Ly6C<sup>hi</sup> MerTK<sup>−−</sup> and Ly6C<sup>lo</sup> MerTK<sup>−−</sup> wound monocytes/macrophages showed evidence of neutrophil ingestion in vivo.

While studies here did not reveal a difference in the wound environment beyond small changes in leukocyte populations after PVA sponge implantation in MerTK<sup>−−</sup> mice, a recent report found that MerTK-deficiency during myocardial infarction (MI) resulted in accumulation of apoptotic cardiomyocytes and subsequent adverse outcome [24]. Similar to what is reported here, this study found that MerTK was expressed predominantly on Ly6C<sup>tm</sup> macrophages in the myocardium after MI. Using bone marrow-derived macrophages, MerTK was found to promote the ingestion of apoptotic cardiomyocytes in vitro; it was not established, however, whether macrophages directly isolated from the healing myocardium were similarly MerTK-dependent for cardiomyocyte phagocytosis. Furthermore, as indicated from studies here, Wan et al. did not link MerTK-deficiency to reduced clearance of neutrophils. This suggests that MerTK may function in vivo in the clearance of parenchymal, but not inflammatory cells. As the PVA sponge wound model lacks a site-specific parenchymal cell type [9], this observation may explain the lack of overt phenotype seen in MerTK<sup>−−</sup> mice. These data support the existence of alternative pathways for the clearance of apoptotic cells and modulation of cytokine production in this model of sterile injury, as has been reported elsewhere [47,48].
Taken together, these studies more thoroughly define time-dependent accumulation of monocyte and macrophage subsets in the healing wound. The data revealed the persistence of Ly6Chi monocytes in an inflamed sterile environment. While these cells expressed low to intermediate levels of CD11c and MHC class II, they were not TNF/iNOS-producing DCs (Tip-DCs), given that inflammatory cells do not express iNOS protein in the sterile wounds of mice [34,49]. The finding of monocyte persistence in the wound is congruent with recent work by Jakubzick et al. [14], which reported that blood monocytes entering multiple tissue and lymph node sites under steady state conditions retained their monocyte status without acquiring macrophage or dendritic cell properties. The authors also proposed that Ly6C expression could provide a molecular signature for the identification of tissue monocytes. In the experiments presented here, however, it was noted that F4/80+Ly6Clow cells were heterogeneous for the expression of the macrophage-specific marker MerTK. This raises the question of

![Figure 9. Cellular responses to sterile injury in MerTK+- mice.](image)

**Table 1.** Day 14 wound cytokines in MerTK+- and control mice.

| Sample | TNF-α (pg) | VEGF (pg) | TGF-β (pg) |
|--------|------------|-----------|------------|
| Wound fluid | Control | 86±40 | 608±124 | 1029±143 |
| MerTK+- | 102±26 | 709±218 | 852±403 |
| Mono/Mac | Control | 690±40 | 1650±370 | 820±190 |
| MerTK+- | 701±35 | 1730±410 | 870±210 |

Mice were wounded for 14 days. Cytokine and growth factor concentrations were measured in wound fluids and monocyte/macrophage (Mono/Mac) culture supernatants. Data shown are means ± SD, n = 4-6 mice per group. Wound fluid cytokine concentrations are pg/mL; macrophage-secreted cytokines are pg/10^6 cells.

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Taken together, these studies more thoroughly define time-dependent accumulation of monocyte and macrophage subsets in the healing wound. The data revealed the persistence of Ly6C^hi_ monocytes in an inflamed sterile environment. While these cells expressed low to intermediate levels of CD11c and MHC class II, they were not TNF/iNOS-producing DCs (Tip-DCs), given that inflammatory cells do not express iNOS protein in the sterile wounds of mice [34,49]. The finding of monocyte persistence in the wound is congruent with recent work by Jakubzick et al. [14], which reported that blood monocytes entering multiple tissue and lymph node sites under steady state conditions retained their monocyte status without acquiring macrophage or dendritic cell properties. The authors also proposed that Ly6C expression could provide a molecular signature for the identification of tissue monocytes. In the experiments presented here, however, it was noted that F4/80^+Ly6C^low_ cells were heterogeneous for the expression of the macrophage-specific marker MerTK. This raises the question of...
whether Ly6C<sup>hi</sup>-MerTK cells in the sterile wound are true monocytes or macrophages, or represent a transitional phenotype. Studies described here indicate that monocytes recruited to a sterile wound can undergo eventual, but not immediate, transition to macrophages with a reparative phenotype, which neither show proliferative nor markers associated with transdifferentiation. The factors regulating the acquisition of inflammatory traits by monocytes upon entry to the sterile wound, as well as those that promote their development into repair macrophages, are unclear and are the subject of ongoing investigation.

Supporting information

**Figure S1** Relative expression of cytokine genes in monocyte/macrophage subsets. Expression of IL-1β (Il1b), TNF-α (Tnf), TGF-β (Tgfb1) and VEGF (Vegf) was determined from FACS-sorted day 14 Ly6C<sup>hi</sup> and Ly6C<sup>low</sup> wound monocytes/macrophages by qPCR. Data are shown as the ratio of gene expression in Ly6Chi cells relative to Ly6C low cells. A dashed grey line placed at a fold change of 1 is indicative of equal expression between subsets. Data shown are the mean ± SD, n = 3 mice per group.

**Table S1** TaqMan Assays Used in qPCR Experiments. (DOCX)

**Table S2** Number of wound monocyte/macrophage subsets in the wound. (DOCX)

**Table S3** Donor-derived cells recovered after sponge adoptive transfer. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: MJC JMD OVH JEA. Performed the experiments: MJC JMD OVH WLH. Analyzed the data: MJC JMD OVH. Wrote the paper: MJC JEA.

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