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Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis


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Although macrophages are widely recognized to have a profibrotic role in inflammation, we have used a highly tractable CCL4-induced model of reversible hepatic fibrosis to identify and characterize the macrophage phenotype responsible for tissue remodelling: the hitherto elusive restorative macrophage. This CD11b<sup>hi</sup> F4/80<sup>lo</sup> Ly-6C<sup>lo</sup> macrophage subset was most abundant in livers during maximal fibrosis resolution and represented the principle matrix metalloproteinase (MMP) -expressing subset. Depletion of this population in CD11b<sup>hi</sup> promoter–diphtheria toxin receptor (CD11b–DTR) transgenic mice caused a failure of scar remodelling. Adoptive transfer and in situ labeling experiments showed that these restorative macrophages derive from recruited Ly-6C<sup>hi</sup> monocytes, a common origin with profibrotic Ly-6C<sup>hi</sup> macrophages, indicative of a phenotypic switch in vivo conferring proresorptive properties. Microarray profiling of the Ly-6C<sup>lo</sup> subset, compared with Ly-6C<sup>hi</sup> macrophages, showed a phenotype outside the M1/M2 classification, with increased expression of MMPs, growth factors, and phagocytosis-related genes, including Mmp9, Mmp12, insulin-like growth factor 1 (Igf1), and Glycoprotein (transmembrane) nmb (Gpnmb). Confocal microscopy confirmed the postphagocytic nature of restorative macrophages. Furthermore, the restorative macrophage phenotype was recapitulated in vitro by the phagocytosis of cellular debris with associated activation of the ERK signaling cascade. Critically, induced phagocytic behavior in vivo, through administration of liposomes, increased restorative macrophage numbers and accelerated fibrosis resolution, offering a therapeutic strategy to this orphan pathological process.

Kupffer Cell | collagen | degradation | myofibroblast | proliferation

As the generic and common pathological endpoint to chronic injury, fibrosis has been estimated to contribute to 45% of all deaths in industrialized nations (1, 2). Currently, no direct antifibrotic therapeutic interventions exist. Long thought of as inexorably progressive, recent evidence, particularly in the liver (3) but also the kidney (4), lung (5), and heart (7), indicates that some reversibility exists, even in advanced disease. Therefore, a more detailed understanding of the specific mechanisms governing fibrosis regression will likely inform therapeutic approaches.

Macrophages have long been implicated in promoting tissue fibrosis (8–10). However, it has recently been shown that they also play a pivotal role in fibrosis regression (6, 11), in part through expression of matrix-degrading metalloproteinase enzymes (MMPs) (12). Macrophages are capable of distinct activation states and functions, which in vitro, can be broadly classified as M1 (classical) or M2 (alternative) (13, 14). It is generally postulated that M1 macrophages are proinflammatory, whereas M2 macrophages are responsible for immunomodulation and wound-healing responses (14). However, it is increasingly clear that this binary classification does not address the more complex heterogeneity in vivo, where macrophages adopt distinct phenotypes and even switch between phenotypes in response to the myriad of stimuli to which they are exposed (13). These in vivo macrophage phenotypes are impossible to recapitulate exactly in tissue culture models, emphasizing the importance of the characterization of macrophages on the basis of function (13).

Ly-6C is a cell surface glycoprotein that is widely used to identify functionally discrete murine circulating monocyte populations: Ly-6C<sup>hi</sup> monocytes (analogous to CD14<sup>hi</sup>CD16<sup>lo</sup> monocytes, a common origin of myo-macrophages, indicative of a phenotypic switch in vivo conferring proresorptive properties. Critically, induced phagocytic behavior in vivo, through administration of liposomes, increased restorative macrophage numbers and accelerated fibrosis resolution, offering a therapeutic strategy to this orphan pathological process.

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Ly-6C is a cell surface glycoprotein that is widely used to identify functionally discrete murine circulating monocyte populations: Ly-6C<sup>hi</sup> monocytes (analogous to CD14<sup>hi</sup>CD16<sup>lo</sup> human monocytes) are recruited early to inflammatory environments and thought to be proinflammatory, whereas Ly-6C<sup>lo</sup> monocytes (analogous to CD14<sup>lo</sup>CD16<sup>hi</sup> human monocytes) are a more patrolling cell type and can replenish resident tissue macrophages (15, 16). Differential Ly-6C expression in diseased tissues has identified functionally distinct macrophage populations (17–20). Indeed, an Ly-6C<sup>lo</sup> intrahepatic macrophage population, derived from recruitment of circulating Ly-6C<sup>lo</sup> monocytes, is critical for fibrogenesis (21). However, the nature, origin, and phenotype of the macrophage subset responsible for mediating fibrosis resolution have not been defined.

In this study, we have exploited differential Ly-6C expression in a tractable and reproducible model of reversible murine hepatic fibrosis to identify the specific macrophage population responsible for fibrosis resolution: the restorative macrophage. We have gone on to characterize this cell, and we have shown categorically that it is derived from recruited inflammatory monocytes after a phenotypic switch mediated by the ingestion of cellular debris and that it represents a newly identified phenotype distinct from the M1/M2 paradigm. Finally, we have established this mechanism to manipulate macrophage phenotype in vivo and accelerate fibrosis resolution.
Results

Experimental Liver Fibrosis Shows Distinct Phases of Fibrogenesis and Resolution. We established a model of liver fibrosis reversal from which macrophage populations could be isolated on a day-to-day basis. C57BL/6 mice were administered two times weekly i.p. carbon tetrachloride (CCl₄) for 4 wk followed by tissue harvests 24, 48, 72, 96, 168, and 256 h after the final CCl₄ injection (Fig. 1A). Comparison was made with age-matched uninjured (control) animals. Hepatic fibrosis and myofibroblast activation were assessed by immunohistochemistry and morphometric analysis of picrosirius red (PSR), collagen 1, collagen 3, and α-smooth muscle actin (α-SMA). Liver fibrosis and myofibroblast activation (α-SMA) peaked at 48–72 h, identifying 24 h as a time of active fibrogenesis, whereas maximal scar resolution and reduction in myofibroblast area occurred between 72 and 96 h and was followed by a more protracted regression of the residual fibrosis (96–256 h) (Fig. 1B and C). Scar resolution occurred after reduction in overall hepatic damage as assessed by serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Fig. 1D). Additionally, at the initiation of scar resolution, there was a significant reduction in hepatic levels of II-1β, Ccl2, Ccl3, and Cxcl2, suggesting an overall change in macrophage phenotype (Fig. 1E). As we have previously shown (3), loss of liver Timp-1 at a gene and protein level preceded fibrosis regression (Fig. S1 A and B).

Ly-6C<sup>lo</sup> Monocyte-Derived Macrophages Predominate During Maximal Fibrosis Resolution and Represent the Principle MMP-Expressing Subset. Having identified the time of early and maximal fibrosis resolution (72 h), we determined whether there were associated changes in specific hepatic macrophage subsets. Total hepatic macrophages were identified on flow cytometry as viable CD45<sup>+</sup> Ly-6G<sup>−</sup> NK1.1<sup>−</sup> CD3<sup>−</sup> B220<sup>−</sup> CD11B<sup>+</sup> F4/80<sup>+</sup> cells from the nonparenchymal cell fraction of digested livers (Fig. S2 A–E). Importantly, coinciding with maximal fibrosis resolution, total hepatic macrophage number peaked at 72 h (Fig. 2A), and macrophages closely associated with hepatic scars topographically (Fig. 2B).

Flow cytometric analysis of hepatic macrophages enabled identification of distinct subsets. F4/80<sup>hi</sup> CD11B<sup>bright</sup> intermediate macrophages predominated in the control (uninjured) liver and represent the resident Kupffer cell population (22) (Fig. 2C). The proportion of resident macrophages was reduced during active inflammation/fibrogenesis (24 h) and progressively increased during resolution (Fig. 2C and E). The CD11B<sup>hi</sup> F4/80<sup>int</sup> intermediate subset represents a recruited monocyte-derived macrophage population (22). Analysis of Ly-6C expression on this subset identified two clearly distinct hepatic recruited macrophage populations: Ly-6C<sup>hi</sup> and Ly-6C<sup>lo</sup> (Fig. 2D). Dynamic changes in these macrophage populations were seen during fibrogenesis and resolution (Fig. 2D and E). Whereas during fibrogenesis (24 h), Ly-6C<sup>lo</sup> (profibrotic) macrophages were the predominant subset (21) (Fig. 2E), at maximal scar resolution (72 h), when macrophage number peaked, there was a reduction in the Ly-6C<sup>lo</sup> population and a dramatic and significant increase in Ly-6C<sup>hi</sup> macrophages, which became the dominant population (Fig. 2E). Overall, these changes were also evident when absolute macrophage numbers were quantified (Fig. 2F). Therefore, Ly-6C<sup>lo</sup> monocyte-derived macrophages at the time of maximal scar resolution represented the most numerous macrophage population seen throughout the injury and recovery phases (4.13 ± 0.5-fold more than the total number of macrophages in the undamaged liver).

During late resolution (168 h), the relative proportions of macrophage subsets returned to control liver, although there remained an increase in the proportion of the Ly-6C<sup>lo</sup> subset (Fig. 2E). We have previously shown that macrophage MMP expression is critical for fibrosis regression (12). To identify the principle hepatic MMP-expressing macrophage subset, we used a pan-MMP substrate (MMPsense), which becomes fluorescent after cleavage by active MMPs in vivo (23), enabling us to identify a population of MMPsense-positive hepatic macrophages by flow cytometry (Fig.
2G). Subset analysis of these cells during both fibrogenesis (24 h) and maximal matrix degradation (72 h) showed that the predominant active MMP-expressing macrophage population at both time points was the Ly-6C<sup>lo</sup> macrophage (Fig. 2H). Therefore, Ly-6C<sup>lo</sup> monocyte-derived macrophages accumulate maximally during the most rapid phase of fibrosis resolution. Furthermore, they represent the principle MMP-expressing population during both fibrogenesis and fibrosis regression.

**Depletion of CD11B-Positive Macrophages Defines Ly-6C<sup>lo</sup> Cells as Being Critical for Scar Resolution.** To define the functional role of distinct macrophage subsets in mediating scar resolution, a well-described selective in vivo macrophage depletion strategy was used (11). CD11B promoter - diphtheria toxin receptor (CD11B-DTR) transgenic mice were given CCL<sub>4</sub> for 4 wk. To ensure maximal macrophage depletion throughout the rapid phase of scar resolution, i.e., diphtheria toxin (DT) (or PBS control) was administered 48, 72, and 96 h after the final CCL<sub>4</sub> injection followed by harvest at 120 h (Fig. 3A). In concordance with previous data (17), administration of DT was effective in depleting both populations of circulating monocytes (Ly-6C<sup>hi</sup> and Ly-6C<sup>lo</sup>) (Fig. S3A and B). The degree of depletion was more profound for the Ly-6C<sup>lo</sup> monocytes in keeping with them being a more mature cell type forming from differentiation of Ly-6C<sup>hi</sup> monocytes (15) and thus, taking longer to replenish after depletion (24).

We proceeded to analyze hepatic macrophage subsets in CD11B-DTR mice (Fig. 3 B and C). Importantly, administration of DT during maximal fibrosis resolution, when Ly-6C<sup>lo</sup> intrahepatic macrophages predominate, induced significant depletion of this subset until harvest, causing a 76.7 ± 3.16% reduction in relative number at 120 h (Fig. 3 B and C). No depletion of the smaller population of hepatic Ly-6C<sup>hi</sup> macrophages was seen, whereas there was a minor increase in the resident macrophage population (Fig. 3C). For comparison, we depleted macrophages during the inflammatory/fibrogenic phase when both Ly-6C<sup>hi</sup> and Ly-6C<sup>lo</sup> hepatic macrophages are present in large numbers. DT was administered to CD11B-DTR mice 8 h after the final CCL<sub>4</sub> with harvest at 24 h. Using this strategy, we observed a more general depletion of both Ly-6C<sup>hi</sup> and Ly-6C<sup>lo</sup> monocyte-derived macrophage subsets (Fig. S3D). Thus, timing depletion for when an individual population predominates is critical for selectivity.

What is also apparent from these data is that the Ly-6C<sup>lo</sup> hepatic macrophage subset is more susceptible to depletion after DT than the Ly-6C<sup>hi</sup> subset. This result is likely to reflect the higher level of CD11B expression in Ly-6C<sup>lo</sup> macrophages than the Ly-6C<sup>hi</sup> subset (Fig. S3D). Importantly, DT administration during fibrosis regression did not induce a change in the number of hepatic neutrophils or CD3-positive cells (Fig. S3 E and F). Furthermore, this depletion strategy caused persistent fibrosis, indicating a failure to remodel the hepatic scar (Fig. 3 D and E). No difference was detected in the a-SMA area after macrophage depletion, suggesting that the observed phenotype was a result of reduced matrix degradation rather than increased myofibroblast activation (Fig. 3 D and E). To confirm the specificity of these findings, we administered DT (or PBS control) to WT mice according to the same schedule (Fig. 3A). DT administration to WT mice had no effect on macrophage subsets or hepatic fibrosis (Fig. S3 G and H). To further show the specific effect of hepatic Ly-6C<sup>lo</sup> macrophages on fibrosis regression, we identified a statistically significant inverse correlation between the number of Ly-6C<sup>lo</sup> macrophages and the degree of fibrosis (Fig. 3F), indicating that the degree of depletion of this subset directly relates to the amount of residual scar. Critically, no significant correlations were seen between the number of Ly-6C<sup>hi</sup> resident macrophages and the degree of fibrosis (Fig. 3I).

These findings indicate that Ly-6C<sup>lo</sup> macrophages are critical for the resolution of hepatic fibrosis and the restoration of normal tissue architecture. Furthermore, given the temporal and numerical association of the Ly-6C<sup>lo</sup> subset with the time of maximal scar degradation (Fig. 2 D–F) and the fact that they are the principle MMP-expressing population (Fig. 2H), we postulated that these represent the elusive restorative macrophages.
CD11c+ dendritic cells (DCs) have been associated with resolution of liver injury (25, 26) and share a number of cell surface markers with macrophages (27). Given the dramatic increase in the number of Ly-6C<hi> macrophages at maximal resolution (72 h), only the Ly-6C<hi> monocytes remained elevated (Fig. S4A–C). To determine which of the circulating monocyte populations contributed to the formation of the restorative macrophages, adoptive transfer and in vivo labeling experiments were performed. For adoptive transfer, hepatic fibrosis was induced in C57BL/6 mice (CD45.2+); 4 h after the final CCI<sub>4</sub> injection, we injected 9 x 10<sup>5</sup> FACS-sorted bone marrow-derived CD45.1+ Ly-6C<hi> monocytes (Ly-6G<sup>L</sup>-CD115+ CD11B+ Ly-6C<hi> cells) (Fig. S5D) or vehicle control through the tail vein, with harvests at 24, 72, and 168 h (Fig. 4A). Adoptively transferred CD45.1+ Ly-6C<hi> monocytes could be detected in livers during active fibrogenesis (24 h) and early resolution (72 h) but not during late resolution (Fig. 4B). Even at 24 h, these monocytes had differentiated into Ly-6C<hi> macrophages (Fig. 4C–E). This population remained the predominant macrophage population formed from the adoptively transferred macrophages at 72 h (Fig. 4C–E). To determine the relative contribution of Ly-6C<hi> monocytes to the hepatic macrophage subsets, we used a well-validated in vivo labeling technique (29, 30). After chronic injury with 4 wk of CCI<sub>4</sub>, mice were given 200 µL fluorescent latex beads (which selectively label circulating Ly-6C<hi> monocytes) through the tail vein 4 h after the final CCI<sub>4</sub> injection. Animals were harvested at 24, 72, and 168 h (Fig. 4F). This technique caused selective labeling of circulating Ly-6C<hi> monocytes (Fig. S5E and F) as previously shown (29, 30). Latex-positive cells could not be identified in livers at 24 or 72 h, despite concurrent positive circulating Ly-6C<hi> monocytes (Fig. 4G). However, a population of intrahepatic latex-positive cells emerged during late resolution (168 h) (Fig. 4F), predominantly in the resident macrophage population (Fig. 4H and I).

Recent work has also shown a key role for local proliferation in the accumulation of macrophages during chronic inflammation (31). Given the dramatic increase in the number of Ly-6C<hi> macrophages at maximal resolution (29, 30). The fact that the number of Ly-6C<hi> macrophages rapidly declines (Fig. 2E and F) in the context of active proliferation emphasizes that this population undergoes a switch in phenotype in vivo.

These data show that restorative Ly-6C<hi> macrophages derive from circulating Ly-6C<hi> monocytes, a common origin to profibrotic macrophages, and that an in vivo phenotypic switch leads to fibrosis-modifying capabilities. Furthermore, Ly-6C<hi> monocytes make no contribution to the proresolusion population but contribute to repopulating the resident macrophage pool during late resolution.

Ly-6C<hi> Macrophages Show a Characteristic Gene Expression Profile Favoring Scar Resolution. Having identified that Ly-6C<hi> macrophages, derived from a phenotypic switch of Ly-6C<hi> monocytes, are critical for regression of hepatic fibrosis, we sought to define the mediators produced by this newly identified macrophage subset that confers its restorative properties. Affymetrix mouse gene microarrays were performed on FACS-sorted restorative 72-h Ly-6C<hi> macrophages and compared with the profibrotic 24-h Ly-6C<hi> macrophages given their common origin, distinct functional roles, and relative predominance at critical time points in regeneration.
the fibrogenesis resolution model. Specific microarray hits were confirmed by quantitative PCR.

A number of differentially regulated genes were identified, and the full list is presented in Tables S1 and S2. In keeping with the critical role of macrophage MMP expression in fibrosis resolution (12), the switch to a proresolution macrophage phenotype was associated with an up-regulation of MMPs (Fig. 5A). Furthermore, a number of proinflammatory cytokines and chemokines were down-regulated, and concurrently, genes associated with an antiinflammatory macrophage program [e.g., Chemokine (C-X3-C) receptor 1 (CX3CR1)] (32) or antifibrotic effects (e.g., Macrophage migration inhibitory factor (MIF) and CD74) (33) were increased (Fig. 5A). Expression of TGF-β, the archetypal profibrotic cytokine, was reduced in the restorative macrophage population along with Thrombospondin-1 (Tbbs1), a potent activator of latent TGF-β (34). We also identified additional proresolution mechanisms, such as a strong increase in expression of insulin-like growth factor 1 (Igf1), which has been implicated as being antifibrotic (35) (Fig. 5A). Thus, the switch to a restorative macrophage phenotype confers a number of proresolution features, highlighting the importance of a cellular mechanism for tissue fibrosis regression.

We performed pathway enrichment analysis on the differentially regulated genes from the two macrophage populations using the DAVID bioinformatics tool (36, 37). The proinflammatory macrophage population was enriched for pathways, including response to wounding, coagulation cascade, and chemotaxis (Fig. 5A), which are important for fibrogenesis (38, 39). Analysis of the restorative macrophages showed enrichment for pathways, such as lysosomes, endocytosis, scavenger receptors, and antigen presentation, which are implicated in phagocytosis (Fig. 5B). We also identified enrichment of pathways implicated in fatty acid metabolism and peroxisome proliferator activated receptor (PPAR) signaling (Fig. 5B). The enrichment of phagocytosis-related genes was confirmed individually, where a number of oposins, receptors, and genes involved in the recognition, binding, and clearance of apoptotic cells were up-regulated in the restorative macrophage population (Fig. 5B). Similarly, a number of PPAR-γ target genes was up-regulated in these proresolution macrophages (Fig. 5B). We also assessed the degree of expression of a number of previously described M1 and M2 macrophage markers to determine how hepatic inflammatory and restorative macrophages fit into the traditional paradigm (Fig. 5A and B). Although Ly-6C<sup>hi</sup> restorative macrophages show increased expression of some M2 genes, such as Macrophage Mannose receptor 1 (Mrc1), Arginase-1 (Arg1), and Retnla (Fizz-1), they also down-regulate other characteristic M2 genes, including Chi3l3 (YM-1), Il-1 receptor antagonist (Il1rn), Kdm6b (Jmjd3), Ccl24, Il-10, and TGF-β (14, 40). Simultaneously, these Ly-6C<sup>lo</sup> macrophages up-regulate traditional M1 genes, such as Cita (MHC class II transactivator), Cd16, Cd32, and Serpine1 (plasminogen activator inhibitor type 1) (14, 40, 41). Therefore, these hepatic macrophage populations do not fit into the M1/M2 classification and represent newly identified macrophage phenotypes (Fig. 5B).

We proceeded to confirm a number of the gene expression changes at a protein level using flow cytometry (Fig. 5C). Additionally, by administering MMPsense 24 h before harvest, we showed that the switch from inflammatory to restorative macrophage phenotype resulted in an increase in active MMP expression (Fig. 5C). Our microarray data also enabled us to identify the functionally distinct macrophage subsets in situ using immunohistochemistry for Chi3l3, MMP-12, and Glycoprotein (transmembrane) nmb (Gpmbm) (Fig. 5D). We confirmed the specificity of these markers in our CD11B-DTR depletion model, where the administration of DT causes a significant reduction in the number of MMP-12-positive cells histologically (Fig. 5C), whereas there was no significant difference in the number of Chi3l3-positive cells (Fig. 5D). We then went on to show the presence of similar MMP-12- and GPNMB-expressing cells associated with scars in cirrhotic human livers (Fig. 5D) and have identified them as a subpopulation of human CD68-positive macrophages (Fig. 5E and F).
These data indicate that the phenotypic switch to the restorative macrophage population results in a loss of proinflammatory gene expression, increased expression of matrix-degrading enzymes, and enrichment of phagocytosis-related genes. Furthermore, the identified macrophage phenotypes fall outside the M1/M2 paradigm, highlighting the limitations of this classification in an in vivo setting.

**Restorative Ly-6C hi Macrophages Are Postphagocytic.** Having identified up-regulation of phagocytosis-related pathways, we determined if restorative macrophages were postphagocytic. It is recognized that ingestion of cellular debris can influence macrophage phenotype (42). Furthermore, the switch to fibrosis resolution in our model followed a reduction in hepatocyte death as assessed by serum ALT and AST (Fig. 1D), indicating that the increase in the restorative Ly-6C hi population (Fig. 2 E and F) occurred after the clearance of cellular debris.

Flow cytometric and immunohistochemical analysis showed that, compared with the proinflammatory 24-h Ly-6C lo macrophages, restorative 72-h Ly-6C hi macrophages were larger [forward scatter area (FSC-A)], were more complex (side scatter area (SSC-A)), and showed features of being postingestion (Fig. 6 A and B). We FACS sorted these two macrophage subsets and TUNEL stained each to quantify the presence of intra- or extracellular apoptotic debris using confocal microscopy (Fig. 6C). No difference was seen in the percentage of each macrophage subset associated with TUNEL-positive debris (Fig. 6D). However, in the proinflammatory macrophages, apoptotic debris was predominantly bound to the cell surface, whereas in the restorative macrophage subset, the debris had been ingested (Fig. 6 C and E), confirming the postphagocytic phenotype of the Ly-6C hi macrophage population. These findings are consistent with the known ability of monocytes to bind apoptotic debris, but a delayed capacity to ingest until differentiation into a more mature macrophage subtype has occurred (43, 44).

**Macrophage Phagocytosis in Vitro Induces a Matrix-Degrading Phenotype Through ERK Signaling.** Having identified evidence of prior phagocytosis as a key feature of the restorative macrophage population, we sought to model this phenotype in vitro. Given that the predominant cellular debris in the CCl 4 model is hepatocyte-derived, we determined whether ingestion of hepatocyte debris might induce a similar change in macrophage phenotype. Primary bone marrow-derived macrophages (BMDMs), widely used to study macrophage biology in vitro (45), were cultured in the presence and absence of cell debris generated from strain-matched primary murine hepatocytes. Macrophage morphology changed significantly after coculture in keeping with ingestion of hepatocyte debris (Fig. 7A). Hepatocyte debris alone did not attach to the wells. After ingestion, BMDMs up-regulated Mmp12, Mmp9, and Igf1 and down-regulated Thbs1 and Chi3l3 (Fig. 7B). To confirm the active secretion of MMPs and determine if this effect was a general effect of phagocytosis on macrophages independent of the type of debris, we used the well-described model of phagocytosis of apoptotic thymocytes (46). Culture supernatants from BMDMs with apoptotic thymocytes for 12 h showed a robust increase in active MMP-9 and MMP-12 secretion detected by gelatin zymography and Western blotting, respectively (Fig. 7 C and D).

We then sought to determine which signaling pathways might link macrophage phagocytosis with the increase in matrix-degrading activity. MAPK signaling, specifically the ERK and p38 cascades, is activated in macrophages after phagocytosis, and it has been reported to regulate a number of macrophage responses (47, 48). Using immunohistochemistry, we could identify nuclear phos-
Having conclusively identified hepaticocyte–macrophages as postphagocytic. Comparison of macrophage phagocytosis in vitro induces a matrix-degrading phenotype in response to phagocytosis (Fig. S7C).

These data show that the matrix-degrading phenotype of the proresolution macropage can be modeled in vitro by the phagocytosis of cellular debris, and this phenotypic switch is, at least in part, mediated by phagocytosis-related MEK1/2 activation and ERK signaling in macrophages.

Induction of Phagocytic Behavior Using Liposomes Enhances the Restorative Macrophage Phenotype in Vivo and Accelerates Fibrosis Resolution. Having conclusively identified macrophage phagocytosis as a key determinant of the proresolution matrix-degrading phenotype, we wished to use this information to manipulate phag-ERK staining in macrophages at the 72-h time point (Fig. 7E), indicating activation of the ERK signaling pathway in scar-associated macrophages during maximal fibrosis resolution. To show a functional role for ERK signaling in the observed macrophage phenotype, we administered the specific ERK kinase [mitogen-activated protein kinase kinase 1 and 2 (MEK1/2)] inhibitor PD98059 (50 μM) or vehicle control to BMDMs at published doses (49, 50) for 1 h before and during feeding with hepaticocyte debris in vitro. Administration of PD98059 significantly inhibited macrophage up-regulation of Mmp9, Mmp12, and Igf1 in response to the ingestion of hepaticocyte debris (Fig. 7F). Furthermore, casein zymography on culture supernatants showed that ERK inhibition abrogated the increase in active Mmp9 and Mmp-12 secretion observed after phagocytosis (Fig. 7G), indicating a critical role for MEK1/2 activation in the increased matrix-degrading activity in macrophages in response to phagocytosis. MEK1/2 inhibition had no effect on the down-regulation of Thbs1 and Chi3l3 in response to phagocytosis (Fig. S7A), suggesting that cross-talk between signaling pathways is required for generating the complex overall phenotype of the restorative macrophage. We confirmed the role of MEK1/2 in macrophage Mmp12 up-regulation in response to phagocytosis using a second specific inhibitor UO126 (20 μM) (Fig. S7B). Administration of a p38 MAPK inhibitor (SB203580; 10 μM) at a published dose (51) had no effect on macrophage expression of Mmp9, Mmp12, or Igf1 in response to phagocytosis (Fig. S7C).

Fig. 6. Restorative Ly-6CH high macrophages are postphagocytic. Comparison of inflammatory (24 h Ly-6CH high) and restorative (72 h Ly-6CH high) macrophage subsets after 4 wk of CCl4. (A) Size [forward scatter area (FSC-A)] and complexity (side scatter area (SSC-A)) of macrophage subsets assessed by flow cytometry expressed relative to average MFI inflammatory macrophages (n = 13 from three independent experiments). (B) F4/80 immunohistochemistry shows larger scar-associated macrophages at 72 h. (Scale bar: 50 μm.) (C–E) TUNEL staining and confocal microscopy of FACS-sorted subsets. (C) Stained DAPI, TUNEL, F4/80, and merged image for macrophage subsets. (Scale bars: 10 μm.) Arrowheads, cell-surface debris; arrows, ingested debris. (D) Percentage of each subset associated with TUNEL-positive nuclei by cell counting (n = 3–4). (E) Percentage of TUNEL-associated macrophages with ingested or cell-surface debris (n = 3–4). Data shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. NS, nonsignificant. Representative images are shown.

Fig. 7. Macrophage phagocytosis in vitro induces a matrix-degrading phenotype through ERK signaling. (A and B) Coculture of BMDMs with hepaticocyte debris. (A) Changes in macrophage morphology on phase-contrast microscopy. Hepaticocyte debris alone was nonadherent. (B) Changes in macrophage gene expression after coculture expressed relative to mean expression of macrophages alone (n = 11–12 from two independent experiments). (C and D) Coculture of BMDMs with apoptotic thymocytes. (C) Gelatin zymography of culture supernatants showing active MMP-9 (representative zymogram from n = 4 from two independent experiments). (D) Western blot for MMP-12 on culture supernatants from n = 4 from two independent experiments. (E) Dual immunofluorescence for F4/80 and phospho-ERK in mouse liver 72 h after final CCl4 dose after 4 wk of injury. Arrows, nuclear pERK and F4/80 dual positive cells. (Scale bars: 10 μm.) (F and G) Culture of BMDMs ± MEK1/2 inhibitor (PD98059; 50 μM) ± hepaticocyte debris. (F) Changes in macrophage gene expression after coculture expressed relative to mean expression of macrophages alone (n = 6). (G) Casein zymography of culture supernatants equalized for protein content showing active MMP-9 and MMP-12 (representative zymogram from n = 3 shown). Data shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. Representative images are shown.
monocytes, a common origin to the pro-

Discussion

In a murine model of reversible hepatic fibrosis, we have used differential Ly-6C expression to identify and characterize the hitherto elusive restorative macrophage. This Ly-6C<sup>lo</sup> CD11B<sup>hi</sup> F4/80<sup>-</sup> macrophage population accumulates in the liver, and it is the main MMP-expressing macrophage subset during maximal fibrosis resolution, is necessary for degradation of tissue scar, is derived from infiltrating Ly-6C<sup>hi</sup> inflammatory monocytes, has a distinct pattern of gene expression, including matrix degradation and phagocytic and growth factors, and is characterized by evidence of prior phagocytosis of dying cells. The restorative phenotype can be recapitulated in vitro by phagocytosis-induced ERK signaling and can be induced in vivo by the administration of liposomes, which accelerates scar resolution.

Although evidence for a central role for macrophages in inflammation and tissue fibrogenesis has been described across organ systems (8), data have recently emerged to suggest a central role for macrophages in vivo. Furthermore, recent studies have shown that macrophage administration can alter macrophage phenotype in vivo, in part by induction of ERK signaling after infection (53, 54). We proceeded to feed BMDMs with liposomes in vitro, which induced a change in macrophage phenotype (Fig. S4) analogous to the change that we observed in vivo (Fig. S4A and B) and similar to the tissue culture models after the ingestion of cellular debris (Fig. 7B). Thus, ingestion of liposomes models the phagocytosis of cellular debris and the generation of restorative macrophages. We went on to administer liposomes (or vehicle control) to chronically injured mice during maximal fibrosis resolution for 48, 72, and 96 h after the final CCl<sub>4</sub> injection, with harvest at 120 h (Fig. 8D). In keeping with an induction of phagocytic behavior, liposome administration caused a reduction in proinflammatory Ly-6C<sup>hi</sup> macrophages and an increase in restorative Ly-6C<sup>lo</sup> hepatic macrophages during fibrosis resolution (Fig. 8C). Liposomes, when fluorescently labeled, were rarely detected in Ly-6C<sup>lo</sup> macrophages but frequently seen in Ly-6C<sup>hi</sup> macrophages, indicative of a postphagocytic phenotype (Fig. 8D).

Critically, this manipulation accelerated the regression of liver fibrosis (Fig. 8E and F), indicating that, by inducing phagocytosis, macrophages could be switched to a phenotype promoting fibrosis resolution in vivo.
local proliferation to tissue macrophage expansion during inflammation is critically dependent on the nature of injury and organ involved. Investigators should consider this information in future studies on macrophage dynamics.

A major difficulty in studying macrophage heterogeneity in vivo is the lack of defined specific markers for functionally distinct populations, necessitating the use of flow cytometry on freshly isolated tissue to recognize subsets. Thus, existing strategies for macrophage depletion are unable to specifically select for functionally distinct subsets. In this work, we used the widely used CD11B-DTR system (11, 17). This transgenic strategy shows selectivity for CD11B<sup>+</sup> F4/80<sup>+</sup> monocytes and monocyte-derived macrophages compared with CD11B<sup>+</sup> F4/80<sup>+</sup> resident tissue macrophages. We have gone on to show that specific depletion of subsets of CD11B<sup>+</sup> F4/80<sup>+</sup> cells is critically dependent on timing. Furthermore, our data indicate an increased susceptibility of Ly-6C<sup>+</sup> macrophages to depletion with DT, which is likely to be a result of higher levels of CD11B expression in this population. We have discovered a number of genes that are differentially expressed by the functionally distinct populations. These findings could inform transgenic studies, where individual macrophage subsets could be specifically depleted or labeled in vivo. Using these data, we could identify distinct macrophages in situ using immunohistochemistry, enabling more easy translation to studying human tissue fibrosis. Our findings are based on the highly tractable and predictable CCL2 model of reversible hepatic fibrosis. To study macrophage dynamics and phenotype on a day-by-day basis, we deliberately focused on an early fibrosis, which resolves rapidly and completely. A future goal is the more detailed analysis of hepatic macrophage subsets in cirrhotic human liver to identify analogous populations to those populations described in this study; until this analysis is undertaken, extrapolation of our findings to human models must be guarded.

The data presented also show that the switch to a proresolution macrophage population confers a number of potential antifibrotic properties. Principally, there is a change from expression of proinflammatory cytokines, chemokines, and profibrotic genes, such as thrombospondin-1 (34), to a profile incorporating genes responsible for scar degradation, such as Mmp12 and Mmp9, genes critical for the clearance of cellular debris, and a number of potential antifibrotic pathways, such as Igf1 (35) and CD74/MIF (33). Further studies expose the limitations of categorizing macrophage populations from an in vivo setting into the widely used but restrictive M1/M2 paradigm. Moving forward, we suggest that a more functional classification of macrophage subsets should be used to better represent their biology.

Phagocytosis can elicit significant effects on macrophage phenotype and function (19, 42). By showing that the proresolution macrophage phenotype can be promoted by ingestion of debris and that the increase in matrix-degrading activity is mediated by phagocytosis-induced ERK signaling, we have identified a potential therapeutic approach to manipulate these cells in situ. Crucially, our data showing that the in vivo phenotypic switch can be induced through phagocytosis of administered liposomes with a beneficial effect on fibrosis resolution identify a possible translational strategy for the treatment of tissue fibrosis. An attractive alternative therapeutic strategy would be the use of macrophages modified in vitro by feeding with liposomes to generate pro-resolution features as a cell therapy to induce fibrosis regression. This use would require modification of the macrophages to ensure adequate trafficking to the fibrotic liver after peripheral injection, but it remains an intriguing area for additional study.

In conclusion, we have identified and characterized a specific macrophage phenotype responsible for the resolution of tissue fibrosis. In addition to the value in studying macrophage biology, this study has important implications for fibrosis research and the future development of antifibrotic therapies aimed at targeting macrophages in vivo.

**Materials and Methods**

**Mice.** C57BL/6 mice (CD45.2<sup>+</sup>) were purchased from Harlan. CD11B-DTR mice, originally obtained from R. Lang, Children’s Hospital Research Foundation, Cincinnati, OH and as previously described (11), were maintained as heterozygotes on C57BL/6 Outbred background. CD45.1<sup>+</sup> C57BL/6 mice (33) were provided by S. M. Anderton, University of Edinburgh. CD11C-DOG mice (28) were provided by A. S. MacDonald, University of Edinburgh. Mice were bred under specific pathogen-free conditions at the University of Edinburgh. All experiments had local ethical approval and were conducted under UK Home Office Legislation.

**Liver Fibrosis Models.** Adult male mice at least 6 wk of age were used. Hepatic fibrosis was induced by two times per week i.p. CCl<sub>4</sub> (0.4 μl/g; Sigma) diluted 1:3 in olive oil (Sigma) for 4 wk (nine injections). Animals were culled at stated time points after the final CCl<sub>4</sub> injection. For depletion studies, DT (10 ngg in PBS; List Biological Laboratories) or PBS control was administered to fibrotic CD11B-DTR or WT mice i.v. through the tail vein at the stated time points. DC depletion in fibrotic CD11C-DOG mice was performed by administration of DT (12 ngg) or PBS control i.p. at the stated time points. Animals were culled at stated time points using (i) 9 x 10<sup>5</sup> FACS-sorted CD45.1<sup>+</sup> Ly-6C<sup>+</sup> monocytes from bone marrow in RPMI 1640 or vehicle control; (ii) 250 μl fluorescent latex beads (0.5 μm; Fluoresbrite polychromatic red microspheres; 2.5% solids (wt/ vol) diluted 1:25 in PBS for injection; Polysciences Inc) or vehicle control; and (iii) 250 μl liposomes (60) (provided by N.v.R.), CM-DiI-labeled (Intravital) liposomes (labeled according to the manufacturer’s protocol), or PBS control.

**Flow Cytometry and FACS Sorting.** Flow cytometry (using BD LSR Fortessa II) and FACS sorting (using BD FACSaria II) were performed on hepatic nonparenchymal cells containing the total hepatic leukocyte population (SI Materials and Methods). FACS sorting routinely yielded cell purity levels of over 95%.

**Detection of in Vivo MMP Activity.** To detect in vivo MMP activity, 2 nmol MMPsense 680 (Perkin-Elmer) (or vehicle control) was administered to animals through the tail vein 24 h before harvest according to the manufacturer’s protocol. Hepatic macrophages were identified using flow cytometry followed by identification of MMPsense-positive macrophages with excitation laser at 635 nm (23).

**Microarray Analysis.** Fifty nanograms RNA from FACS-sorted cells was processed using the Ovation Pico WTA system (NuGen) according to the manufacturer’s protocol (n = 3 per group). Processed RNAs were hybridized at Affymetrix GeneChip Mouse Gene 1.0 ST Arrays. RNA/microarray processing was carried out by ARK Genomics (Roslin Institute). Data analysis was performed as described (SI Materials and Methods). Fold change > 2 with adjusted P < 0.05 was considered significant for individual gene changes. Gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was done with the DAVID tool (36, 37) on gene lists that were significantly differentially expressed. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-3177.

**In Vitro Phagocytosis Assay.** BMDMs, primary murine hepatocyte debris, and apoptotic thymocytes were prepared as described (SI Materials and Methods); 2 x 10<sup>6</sup> BMDMs were seeded per well in 12-well plates followed by the addition of 5 x 10<sup>5</sup> washed dead hepatocytes (or control medium) and 1 x 10<sup>6</sup> washed dead thymocytes or liposomes at 1:10 dilution by volume (or PBS control) cultured for 16 h or 12 h for thymocytes) at 37 °C 5% (vol/vol) CO<sub>2</sub> in DMEM/F12 Glutamax (Gibco) medium with 10% FCS. Where stated, inhibitors PD98059 (50 μM; Cayman Chemical), UO126 (20 μM; New England Biolabs), SB203580 (10 μM; Cayman Chemical), or DMSO control were added to the plated BMDMs for 1 h before and maintained throughout the 16-h incubation with hepatocyte debris. Supernatants were then harvested and stored at -80 °C, noningested hepatocyte or liposomes were removed by vigorous washing three times with PBS, and residual adherent macrophages were used for additional analysis. In control wells containing hepatocyte debris alone, no adherent cells were detected.

**Statistical Analysis.** All data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism software. Statistical evaluation of data was performed using one-way ANOVA with post hoc Tukey test. Statistical evaluation of two groups was performed using Student t test or Mann–Whitney test if data were not normally distributed. A value of P < 0.05 was considered statistically significant.
Additional Methods. Additional methods are shown in SI Materials and Methods.

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