Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair

Jeremy S. Duffield, Stuart J. Forbes, Christothea M. Constandinou, Spike Clay, Marina Partolina, Srilatha Vuthoori, Shengji Wu, Richard Lang, and John P. Iredale

Macrophages perform both injury-inducing and repair-promoting tasks in different models of inflammation, leading to a model of macrophage function in which distinct patterns of activation have been proposed. We investigated macrophage function mechanistically in a reversible model of liver injury in which the injury and recovery phases are distinct. Carbon tetrachloride–induced liver fibrosis revealed scar-associated macrophages that persisted throughout recovery. A transgenic mouse (CD11b-DTR) was generated in which macrophages could be selectively depleted. Macrophage depletion when liver fibrosis was advanced resulted in reduced scarring and fewer myofibroblasts. Macrophage depletion during recovery, by contrast, led to a failure of matrix degradation. These data provide the first clear evidence that functionally distinct subpopulations of macrophages exist in the same tissue and that these macrophages play critical roles in both the injury and recovery phases of inflammatory scarring.

Introduction

Macrophages feature prominently at diverse sites of inflammation and have been linked to the process of inflammation and repair, demonstrating a repertoire of activities that include regulating inflammatory cells, tissue debridement, cell killing, recruiting and activating myofibroblasts, and regulating spontaneous recovery of fibrosis in physiologic or aberrant wound healing (1–6). In response to injury, cells of mesenchymal origin transdifferentiate into myofibroblasts, migrate, and generate the matrix components of scar tissue (7, 8). Myofibroblasts also have the capacity to express MMPs and tissue inhibitors of metalloproteinase (TIMPs), through which they regulate degradation of matrix components (9). The precise role of macrophages at the site of injury is unclear (10, 11), but they are known from in vitro experiments to generate a diverse array of agents that regulate the degradation of matrix (7, 10, 12, 13).

Recent cell surface phenotyping studies suggest that distinct macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. For example, the hemoglobin scavenger receptor CD163 is expressed by a subpopulation of macrophage populations exist. 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ularly perisinusoidal fibrosis. These data define 2 distinct activities of macrophages with opposite but complementary functions.

Results

Macrophages are associated with liver fibrosis during injury and repair. In order to determine the role of macrophages in liver injury and repair, we induced fibrosis (4 weeks of CCl4), cirrhosis (12 weeks of CCl4), and spontaneous recovery following both fibrosis and cirrhosis in rats as previously described (7, 24). Liver sections were stained for markers of collagen, macrophages, and activated HSCs (Figure 1). At all stages of progressive fibrosis and cirrhosis, SAMs were found in significant numbers. SAMs were found within and apposed to the bands. In particular, the perisinusoidal fibrosis was rich in SAMs (Figure 1B). SAMs were also found to be associated with resolving areas of fibrosis in spontaneous recovery in both the fibrotic and cirrhotic ure 1B). SAMs were also found to be associated with resolving areas during spontaneous recovery from 4 weeks of fibrosis and counted 

As expected, the F4/80-positive peritoneal population was unaffected in wild-type mice receiving 2 doses of DT (Figure 2D) but was eliminated in CD11b-DTR mice (Figure 2E). Importantly, in CD11b-DTR mice with complete peritoneal macrophage elimination, the CD3+ peritoneal cavity T cells were unaffected (Figure 2E). The relative increase in CD3+ and double-negative cells in the DT-treated CD11b-DTR mice is due to the plotting of equal numbers of events. B220+ and CD3+ populations in the spleen were unaffected in either wild-type (Figure 2F) or CD11b-DTR mice (Figure 2G).

Because the endogenous CD11b gene is expressed in both macrophages and granulocytes, we determined which of the cell...
types recruited to the peritoneal cavity by injections of sterile Brewer’s thioglycollate (BTG) could be killed by subsequent injections of DT. In CD11b-DTR homozygotes, BTG was injected 6 hours prior to injection of DT at 25 ng/g mouse weight and cells harvested for analysis 24 hours later. A morphological analysis showed that cells of monocyte/macrophage, eosinophil, neutrophil, basophil, and lymphocyte morphology were present in control mice (Figure 2H and Table 1) and that cells of monocyte/macrophage morphology only were greatly reduced in those treated with DT (Figure 2I and Table 1). It was particularly striking that most BTG-elicited, DT-treated peritoneal cells were neutrophils (Figure 2I).

Importantly, this showed that granulocytes were not sensitive to DT. Furthermore, blood leukocytes from DTR mice 24 hours following a single i.v. injection of DT revealed a selective depletion of F4/80-positive monocytes from 6.5% ± 3.3% to 1.5% ± 1.3%. It was particularly striking that neutrophils was apparent in mice treated with DT.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monocytes/macrophage</th>
<th>Basophils</th>
<th>Polymorphs</th>
<th>Lymphocytes</th>
<th>Mast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTG/DTmut</td>
<td>33 ± 1.9 (54%)</td>
<td>1.1 ± 0.5 (1.8%)</td>
<td>2.6 ± 0.4 (4.3%)</td>
<td>1.8 ± 0.5 (3.0%)</td>
<td>1.0 ± 0.8 (2.0%)</td>
</tr>
<tr>
<td>BTG/DT</td>
<td>8.1 ± 0.9 (14%)</td>
<td>2.0 ± 0.7 (3.4%)</td>
<td>1.5 ± 0.2 (2.5%)</td>
<td>2.3 ± 0.4 (3.9%)</td>
<td>0.9 ± 0.3 (1.5%)</td>
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*A single i.p. injection of DT at 25 ng/g mouse weight 6 hours after i.p. injection of 4 ml of 4% BTG in PBS.*
SAMS promote matrix deposition and support HSCs during injury. Livers depleted of SAMS during CCl₄ injury were assessed for matrix components. Picrosirius red staining highlights polymeric and monomeric collagen matrix (29). Liver sections were assessed for the area of sirius red (Figure 4, A and E). Macrophage depletion markedly reduced sirius red. Sections were stained for collagen III, a representative fibrillar collagen secreted in excess in liver fibrosis (Figure 4, C and G). Macrophage depletion also resulted in substantial reduction in the amount of this abnormal collagen. Another component of mature scar tissue that may promote matrix cross-linking is elastin (30). Depletion of macrophages leads to marked reduction of elastin at 12 weeks compared with that in controls (Figure 4, D and H). Thus, each representative component of the scar matrix examined was substantially reduced during progressive liver injury in the absence of SAMS, indicating that SAMS either directly or indirectly promote the expression of matrix proteins and/or regulate the maintenance of abnormal extracellular matrix.

Because the collagens and elastin are produced primarily by myofibroblast HSCs (9, 31), we wondered whether the profound effect of SAMS on the pattern of fibrosis was via regulation of the HSC population. The number of α-SMA–positive cells in the scars was assessed (Figure 4, B and F). Macrophage depletion by administration of DT either i.p. or i.v. led to a marked reduction in the number of HSCs. This indicates that SAMS support the activated HSC (myofibroblast) population, and, at least in part via this mechanism, promote fibrosis. We have previously determined that the population of HSCs is determined by the balance of proliferation and apoptosis (32). Therefore, BrdU uptake in scar cells was assessed in all livers (Table 2). During SAM depletion, the number of proliferating scar-associated cells was markedly reduced, suggesting that SAMS directly induce HSC proliferation. The number of scar-associated apoptotic cells was also decreased following depletion of SAMS (Table 2). Therefore, SAMS may regulate both proliferation and apoptosis of myofibroblast HSCs.

SAMS promote the resolution of fibrosis during recovery. Livers depleted of SAMS during recovery were assessed for matrix components. Sirius red material decreased by 85% in control (PBS-treated) mice after recovery compared with control mice at peak fibrosis (PBS and CCl₄) (Figure 5, A and E). However, those mice with depletion of SAMS induced by i.p. or i.v. administration of DT during recovery had persistence of sirius red material, increasing from 1% of the biopsy to more than 3% of the biopsy. While the overall thickness of the bands of fibrosis had reduced during recovery in spite of SAM depletion, the most prominent feature was a persistence of perisinusoidal fibrosis in the absence of SAMS (Figure 5C). The amount of perisinusoidal fibrosis was assessed using an established scoring system (33) and was found to persist at a comparable level to peak fibrosis in the tissue from recovering livers as a result of depletion of SAMS (Table 2). The increase in perisinusoidal fibrosis score from 1.0 to 1.9 as a result of SAM depletion compares with a score of 2.5 at peak fibrosis in the control diseased mice. Thus, there is very little attenuation of perisinusoidal fibrosis in the absence of SAMS. Therefore, in stark contrast to the function of SAMS during CCl₄ injury, SAMS during recovery promote degradation and resorption of matrix components. To be sure that this resorption included complex scar-associated matrix proteins, both collagen III (Figure 5, C and G) and elastin (Figure 5, D and H) were assessed. There was 50% more collagen III in livers from mice depleted of SAMS during recovery (predominantly due to a failure to resorb the perisinusoidal fibrosis). Elastin, a durable component of scar tissue, declined in response to recovery. However, following depletion of SAMS, there was no evidence of a reduction in elastin content.

Table 2
The effect of macrophage depletion on proliferation, apoptosis, and perisinusoidal fibrosis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>i.p. DT</th>
<th>i.v. DT</th>
<th>Control</th>
<th>i.p. DT</th>
<th>i.v. DT</th>
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<tbody>
<tr>
<td>Scar-associated cells per 50 hpf</td>
<td></td>
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<tr>
<td>BrdU</td>
<td>142 ± 47</td>
<td>47 ± 5</td>
<td>57 ± 3</td>
<td>8.8 ± 6.7</td>
<td>16.7 ± 3</td>
<td>9.7 ± 2.9</td>
</tr>
<tr>
<td>TUNEL</td>
<td>43 ± 12</td>
<td>9 ± 3</td>
<td>12 ± 5</td>
<td>3.4 ± 2.3</td>
<td>4.5 ± 2.5</td>
<td>3.7 ± 2.5</td>
</tr>
<tr>
<td>Perisinusoidal fibrosis score</td>
<td>2.5 ± 0.9</td>
<td>1.0 ± 1.2</td>
<td>1.8 ± 0.8</td>
<td>1.9 ± 0.7</td>
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*A: Mean ± SEM; P < 0.01 by ANOVA. **Mean ± 95% confidence interval; P < 0.05 by Wilcoxon rank sum test.*
Thus, SAMs during recovery mediate opposite effects on matrix components and fibrosis compared with SAMs during injury. Both macrophages and activated HSCs can liberate a range of matrix-degrading MMP enzymes and regulate their activity via expression of TIMPs. Moreover, matrix degradation in spontaneous recovery from fibrosis is associated with a loss of activated HSCs (33), which are known to be quantitatively the major source of matrix (9). We therefore set out to determine whether the persistence of scar matrix during recovery in macrophage-depleted mice was due to persistence of myofibroblast-like activated HSCs. The area of \( \alpha \)-SMA cells during recovery was assessed in the presence of SAMs or following SAM depletion. Although there was a trend toward persistence of \( \alpha \)-SMA cells during recovery was assessed in the presence of SAMs or following SAM depletion. Although there was a trend toward persistence of \( \alpha \)-SMA cells (Figure 5, B and F), this was not significant. Furthermore, when proliferation and apoptosis were assessed in the scars, no clear difference was seen (Table 2).

**SAMs derive from both circulating monocytes and resident cells of the liver.** In the liver there are 2 potential sources of SAMs. One derives from the circulating pool of BM-derived monocytes, and the other from the population of resident liver macrophages, the Kupffer cells. Because we had determined that 2 functional subsets of macrophages exist in liver injury, it was important to determine what role the Kupffer cells played in the disease process. To date, no phenotypic marker has been described that distinguishes Kupffer cells from monocyte-derived macrophages. We therefore took advantage of the observation that Kupffer cells in healthy livers of mice chimeric for BM cells are not replaced by BM-derived cells after 8 weeks of chimerism (data not shown). Thus, the population of Kupffer cells can be distinguished from monocyte macrophages in BM chimeric mice. In female mice with male BM, SAMs in livers following 12 weeks of liver injury were assessed for the presence of the Y chromosome using established scoring methods (Figure 6) (34). In 5-\( \mu \)m tissue sections, 28.3% ± 4.2% of SAMs contained the Y chromosome, indicating that 45.7% ± 6.8% of SAMs were derived from the BM-derived monocyte pool (see Methods for details). These data indicate that 2 populations of macrophages exist: 1 derived from monocytes, the other derived from a nonhematogenous source. It is likely that the second population derives from resident Kupffer macrophages.

**SAMs during disease are phenotypically distinct from macrophages during recovery.** We have demonstrated that SAMs during disease are...
functionally distinct from SAMs during recovery. It was important
to determine whether a phenotypic marker could be elucidated.

Analysis of tissue sections stained with macrophage markers
CD11b, CD68 (data not shown), and F4/80 (Figure 3) unveiled no
differences in macrophage expression during disease and recovery.

In vitro studies of BM macrophages have shown that macrophages
can be polarized into distinct cell types that are distinguished by
expression of TNF-α, IL-10, and TGF-β1 (11). We used a quantita-
tive RT-PCR method to determine the amounts of \textit{tnf}α and \textit{il-10}
transcripts during injury and repair (Table 3). Previous studies
have shown that RNA levels correlate closely with protein levels
for these cytokines (35). During injury, \textit{tnf}α transcript was 14-fold
more abundant than during recovery (Table 3). During injury, \textit{il-10}
transcript was also expressed more abundantly than during recov-
ery, though the differences were less marked. Furthermore, \textit{tnf}α transcript was 360-fold more abundant than \textit{il-10}. These data sug-
gest that SAMs during injury express high levels of TNF-α and lit-
tle IL-10, but that SAMs during recovery express little TNF-α and
little IL-10. Although total \textit{tgf}β1 transcript was not significantly
altered between disease and recovery (Table 3), \textit{tgf}β1 transcript
levels have been shown not to correlate well with protein prod-
uct (36), and TGF-β1 expression is not limited to macrophages
or immune cells. Liver sections were therefore immunolabeled for
TGF-β1 expression using a well-validated antibody (37). In scars of
active disease we found abundant expression of TGF-β1 in cells of
the scar (morphologically the source cells were inflammatory cells
and activated HSCs/myofibroblasts), extracellular matrix, and
cells with the morphology of hepatocytes immediately adjacent
to the scars (Figure 7A). Sections labeled with control rabbit IgG
revealed no specific binding (data not shown). In contrast TGF-β1
was absent from the scars during recovery (Figure 7B). When SAMs
were depleted during injury, the scars also did not express TGF-β1,
though expression could still be seen in cells with the morphology of hepatocytes (data not shown). Furthermore, when SAMs were depleted during recovery, TGF-β1 expression was still not seen in scars. Taken together, these observations indicate that SAMs during injury not only express TGF-β1, but also likely induce expression in other cells of the scar, including HSCs, whereas the SAMs present after the onset of recovery do not express this cytokine.

Discussion

To assess macrophage function at distinct phases of disease and recovery, we developed a novel system for depletion of macrophages during disease. The role of the inflammatory tissue macrophage has remained a subject of debate, at least in part because macrophages are associated with both tissue injury and repair (10). In addition, when macrophages have been depleted in a series of injury models, the results have been conflicting (38, 39). Macrophage have been implicated either during injury or during repair and resolution has dramatically different effects on the overall fibrotic response. Specifically, in progressive inflammatory injury, macrophage depletion results in amelioration of fibrosis. By contrast, depletion during recovery results in a failure of resolution with persistence of cellular and matrix components of the fibrotic response. These data represent the first definitive demonstration that macrophages play distinct roles in injury and repair and highlight the critical observation that a given cell may be both pathogenic and beneficial.

Our experiments suggest that over a period of a few days, 2 functionally distinct types of macrophages exist in the injured liver. During the injury phase (a time when chemokines, complement components, and proinflammatory cytokines are abundant) injury–associated macrophages promote myofibroblast proliferation and apoptosis. On balance, macrophages have the effect of increasing myofibroblast numbers. As a result, fibrogenesis predominates and matrix is deposited. The phenotype of macrophages associated with the injury phase is closest to that of the aa-Ms described by others (11). However, it is present in response to injury normally associated with classically activated macrophages, as it is present at a stage when proinflammatory cytokines are released.

In contrast, during recovery from injury, a population of macrophages predominates that does not support HSC survival and promotes matrix degradation and, in that regard, resembles classically activated macrophages. This macrophage population, however, is present during resolution of injury and at a time when proinflammatory mediator levels are decreasing, an observation reinforced by our studies of TNF-α in this model. Moreover, these 2 functional phenotypes are separated chronologically in the liver by only a few days, suggesting that they may represent a single population. Therefore, we suggest that the yin and yang of the classically activated macrophage/aa-M model is inadequate to reflect the complex roles of these cells in vivo during liver injury.

In our study, the SAMs during injury are associated with high expression of TGF-β1 in the scars and high levels of liver tnfα transcript. In contrast, during recovery, SAMs are not associated with TGF-β1 generation in the scars, and the liver tnfα transcript is attenuated. We have identified 2 sources of SAMs. One is monocyte-derived, and the other is derived from a nonhematogenous source. It is likely that this other source of SAMs is the resident liver macrophages, the Kupffer cells. It is tempting to speculate as to whether one functional population of macrophages derives from monocytes and the other from Kupffer cells. Further experiments (beyond the scope of the current study) will be required to determine whether the functional distinctions are clearly related to macrophage origin.

Our study indicates that SAMs during disease are associated with high levels of proliferation and apoptosis in the scars, but that these

Figure 6

Y chromosome–positive SAMs after 12 weeks of liver injury in female BALB/c mice chimeric for male BM (magnification, ×600). F4/80-labeled macrophages (red) are located in the region of a scar. Y chromosome–FITC hybridization (green) can be seen in nuclei labeled with DAPI (blue). Note macrophages with nuclei containing Y-chromosomal DNA (arrowheads) and also macrophages without Y-chromosomal DNA (arrows).

Figure 7

TGF-β expression in the fibrotic bands (magnification, ×400). (A) After 12 weeks of disease in the mouse model, fibrotic bands (denoted by arrowheads) contain numerous cells showing immunofluorescence for TGF-β. In addition, cells with the morphology of hepatocytes also express cytoplasmic TGF-β (arrow). (B) Following 7 days of recovery from injury, the fibrotic bands show no evidence of TGF-β expression. In addition, no hepatocytes show TGF-β expression.
parameters are markedly decreased by depletion of SAMs. Thus, the SAMs may induce both proliferation and apoptosis and, as such, maintain a population of HSC myofibroblasts. Macrophages are known to induce cell cycle–dependent cell death in some settings (42), and so there is reason to consider this mechanism of cell number regulation in the injured liver. Furthermore, macrophages can liberate both potent myofibroblast growth factors, such as PDGF, and proapoptotic factors, such as TNF-α. Indeed, SAMs during disease generate TNF-α. Our data reveal that after 7 days of recovery, apoptosis and proliferation in the scars is reduced and the population of HSC myofibroblasts is much reduced. Thus, SAMs during recovery do not share these characteristics with SAMs during disease. In a previous study, we have shown that the loss of HSCs during recovery in rats is due to a wave of apoptosis occurring predominantly in the first 3 days (7). We do not see this wave of apoptosis in the current study, in which we focused on 7 days of recovery in order to demonstrate changes in matrix components. It is likely that the wave of apoptosis occurred at an earlier time point.

Previous studies using other methods to modify or deplete macrophages have pointed to a role for macrophages in mediating the fibrotic response (4, 39). Those studies have been inconsistent, however, which may reflect nonspecific depletion of other cell types or the existence of distinct functional populations of macrophages in the different models of inflammation and scarring that were studied. Our new conditional ablation system is nontoxic, is cell type–specific, is effective in many tissues, and offers the possibility of time point–selective deletion of cells during a range of inflammatory processes.

We suggest that our description of the roles of macrophages in functional terms should provide the basis for a series of future functional studies in vivo to define more accurately the nature, specific attributes, and dynamics of the macrophage population(s) present in inflammation and repair. We further suggest that a description of macrophage populations based on functional studies in vivo should form the basis of a rational and clinically relevant classification of the cell.

Methods

Materials

All reagents were from Sigma-Aldrich unless stated otherwise. DT and mutant DT (DTmut), which lacks intracellular enzyme activity, were from List Biological Laboratories Inc. Animals were housed in a standard sterile animal facility with free access to chow and water. All manipulations and procedures were undertaken with approved license from the Animal Scientific Procedures Division of the Home Office (London, UK).

Transgenic construct

The CD11b promoter from coordinates –1,704 to +83 (28) was used to drive expression of the human hbetaGF cDNA (27). Splicing and polyadenylation signals were provided by a region of the human growth hormone gene that had previously worked effectively with the CD11b promoter (28). The fusion protein between hbetaGF and GFP was generated by continuing the open reading frame of hbetaGF at residue 1. The transgene fragment was excised using the HindIII and NotI restriction endonucleases and purified using standard procedures (35). Transgene expression was detected by hypotonic lysis on ice, followed by further PBS washes. A total of 2 × 10^8 cells were preincubated with FACS buffer containing Fc block (BD Biosciences — Pharmingen). Fifty microliters of FACS buffer containing FITC– or PE-conjugated anti-B220, anti-CD3 (BD Biosciences — Pharmingen), or anti-F4/80 (Serotec) was then added and incubated at 4°C for 1 hour. Cells were washed 3 times in FACS buffer and fixed in 2% formaldehyde in PBS prior to analysis.

FACS analysis

Mouse peritoneal and spleen cells were isolated using conventional methods (35). Blood leukocytes were purified from heparinized blood by centrifugation followed by aspiration ofuffy coat. The latter was washed 3 times in PBS to remove platelets. Remaining erythrocytes were removed by hypotonic lysis on ice, followed by further PBS washes. A total of 2 × 10^6 cells were preincubated with FACS buffer containing Fc block (BD Biosciences — Pharmingen). Fifty microliters of FACS buffer containing FITC– or PE-conjugated anti-B220, anti-CD3 (BD Biosciences — Pharmingen), or anti-F4/80 (Serotec) was then added and incubated at 4°C for 1 hour. Cells were washed 3 times in FACS buffer and fixed in 2% formaldehyde in PBS prior to analysis.

Disease models

Progressive fibrosis demonstrating complete spontaneous recovery. CCl₄ liver fibrosis was induced in Sprague-Dawley rats by twice weekly i.p. injections of CCl₄ in sterile olive oil (7). After 4 weeks, cohorts of animals were allowed to spontaneously recover for periods of up to 28 days (7).

Advanced fibrosis and micronodular cirrhosis. CCl₄ liver fibrosis was induced and analyzed after 6 weeks (fibrosis) and 12 weeks (advanced micronodular cirrhosis). Cohorts were allowed to recover for 28 days (9, 24). The more advanced cirrhosis demonstrated a reduced capacity for spontaneous resolution. Nevertheless, there was considerable remodeling during the recovery period.

Mouse model of hepatic fibrosis and spontaneous recovery from fibrosis in DTR mice. Twenty-four adult DTR mice (FVB/N) were injected with 0.25 μl/g of CCI₄ i.p. twice weekly for 12 weeks. During the 12th week, half the mice (disease group) were given either (a) DT (10 ng/g i.v. by tail vein) immediately following the first injection of CCI₄, that week, DT (i.v.) 24 hours later, and DT (i.v.) a further 48 hours later to coincide with the next injection of CCI₄ (b) DT (25 ng/g i.p.) over a similar schedule; or (c) 100 μl PBS (i.v.) as control to coincide with the DT injections. During the 13th week, the remaining mice (recovery group) did not receive any further CCI₄ injections. However, 72 hours following the final CCI₄ injection, mice were given either (a) DT (10 ng/g i.v.), DT (i.v.) 24 hours later, and DT (i.v.) a further 48 hours later; (b) DT (25 ng/g i.p.) over a similar schedule; or (c) 100 μl PBS (i.v.) to coincide with the DT injections. Livers were harvested 24 hours following the final dose of DT or PBS.

Mouse BM transplant model to trace the lineage of the SAMs

In order to trace the lineage of the SAMs, we performed sex-mismatched BM transplants from male donor mice into female recipients, which allowed us to detect male BM-derived SAMs through in situ hybridization for the Y chromosome combined with immunostaining for F4/80 antigen. Six-week-old female recipient BALB/c mice (n = 7) underwent whole-body, lethally irradiation (8 Gray) in divided doses. This was followed by a tail vein injection with whole BM from 3 male BALB/c donors. Femurs from the male donors were removed and marrow was flushed with PBS using a needle syringe. The product was filtered twice through a 70-micron tube filter and centrifuged to obtain red blood cells. A total of 2 × 10⁶ cells were preincubated with FACS buffer containing Fc block (BD Biosciences — Pharmingen). Fifty microliters of FACS buffer containing FITC– or PE-conjugated anti-B220, anti-CD3 (BD Biosciences — Pharmingen), or anti-F4/80 (Serotec) was then added and incubated at 4°C for 1 hour. Cells were washed 3 times in FACS buffer and fixed in 2% formaldehyde in PBS prior to analysis.

Immunostaining

Rat SAMs were detected in 3-μm paraffin-embedded sections using ED-1 antibody (Serotec) as previously described (45). α-SMA was detected by incubation with 1A4 antibody (DAKO) as previously described (7). Mouse SAMs were detected in 3-μm methyl Carnoys–fixed paraffin-embedded sections using anti-F4/80 antibody (46), except for the tissue analysis requiring in situ hybridization, where 5-μm formalin-fixed sections were digested with trypsin.
at 37°C for 20 minutes prior to the immunodetection. Collagen III was detected by incubating sections with anti-collagen III (Southern Biotechnologies) as described (47). Elastin was detected by anti-elastin antibody (Cedar Lane Labs), followed by secondary stages of immunostaining as previously described (7). TGF-β1 was detected in formalin-fixed sections using anti-mouse TGF-β1 antibodies, followed by donkey anti-rabbit Cy3-conjugated secondary antibody (Jackson Laboratories). Immunolabeled sections were mounted in Vectorshield with DAPI/Citifluor prior to fluorescent microscopy.


**Real time RT-PCR**

RNA was purified from diseased livers using the RNeasy kit (Qiagen). In brief, tissue was homogenized in a buffer containing guanidine isothiocyanate and then passed over a silica-based gel that selectively binds RNA. Purified RNA was eluted following washing of the gel. Following DNase treatment, cDNA was generated from 1 ng of whole RNA using iScript cDNA synthesis kit (BioRad Laboratories), which relies on a combination oligo (dT) and random hexamers for annealing and an RNase H+ reverse transcriptase for accurate synthesis. cDNA was analyzed for content using a SYBR green–based, quantitative fluorescent PCR methodology (Qiagen). Fluorescence was detected with the iCycler system (BioRad Laboratories). The following primer pairs were used for detection of specific cDNAs: GADDH, 5′-CTGAGAAACCTGCAAGTATG-3′, 5′-AAGATGGGAGTTGCTGTGG-3′; TGF-β1, 5′-GGATCTCTGTCCTCACTAGAC-3′, 5′-GATGAGAGGGAGGCCATT-3′; IL-10, 5′-CAGCCTTGACAAAGAGAG-3′, 5′-GAGAGTGCGTGGACGTTATTG-3′; TGF-β, 5′-CTCACCTGCAAGACCAT-3′, 5′-CTTAGGTTTGGACAGGATCTGG-3′. Each assay was performed in duplicate with 25 μl reaction volume with 2X SYBR green mastermix, 1 μl of cDNA, and primers at 300 nM. The following PCR conditions were used: 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for a final 10 minutes. Each PCR reaction was also tested to assure a single product of the predicted size. Quantification of each PCR product was expressed relative to GAPDH.

**Assessment of tissue sections**

Rat SAMs were scored as number per high-power field (hpf). An ED-1–positive cell was included if it was within or apposed to the scar. Rat α-SMA–positive cells were counted as previously described (7). Mouse SAMs were assessed morphometrically using Fovea Pro 3.5 software (Reindeer Graphics) to quantify the area of tissue occupied by positive staining (47). At least 10 serial images were taken of the tissue section (*n* = 100), covering the whole tissue section. Collagen III immunostaining was assessed morphometrically for percentage area of liver occupied by collagen III matrix. Picrosirius red–stained sections were assessed morphometrically using Fovea Pro 3.5 software to determine the origins of SAMs, F4/80-staining SAMs were considered to be of BM origin if the nucleus also contained a green fluorescent region due to Y-chromosome paint (Star-FISH; Cambio) used in the supplier’s instruction. A score was assigned blindly by an established scoring system where +4 is established as the maximum score. If the proportion of SAMs of male mice with the same disease was prepared in the same way and scored for the proportion of SAMs with nuclei containing Y-chromosomal DNA. Using this score (62.0%) as a maximum, the proportion of SAMs of male origin in the chimeras was calculated.

**Statistics**

Difference between percentage area measured morphometrically was expressed as mean ± SEM and assessed by ANOVA. Allotted scores were expressed as mean ± 95% confidence intervals and assessed using Wilcoxon ranked sum method.

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