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ORIGINAL ARTICLE

Fibrocyte-like cells recruited to the spleen support innate and adaptive immune responses to acute injury or infection

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Abstract Bone marrow (BM)-derived fibrocytes are a population of $CD45^+$ and collagen Type I-expressing cells that migrate to the spleen and to target injured organs, such as skin, lungs, kidneys, and liver. While $CD45^+Col^+$ fibrocytes contribute to collagen deposition at the site of injury, the role of $CD45^+Col^+$ cells in spleen has not been elucidated. Here, we demonstrate that hepatotoxic injury (CCl_4), TGF-β1, lipopolysaccharide, or infection with *Listeria monocytogenes* induce rapid recruitment of $CD45^+Col^+$ fibrocyte-like cells to the spleen. These cells have a gene expression pattern that includes antimicrobial factors (myleoperoxidase, cathelicidin, and defensins) and MHC II at higher levels than found on quiescent or activated macrophages. The immune functions of these splenic $CD45^+Col^+$ fibrocyte-like cells include entrapment of bacteria into extracellular DNA-based structures containing cathelicidin and presentation of antigens to naïve $CD8^+$ T cells to induce their proliferation. Stimulation of these splenic fibrocyte-like cells with granulocyte macrophage-colony stimulating factor or macrophage-colony stimulating factor induces downregulation of collagen expression and terminal differentiation into the dendritic cells or macrophage. Thus, splenic $CD45^+Col^+$ cells are a population of rapidly mobilized BM-derived fibrocyte-like cells that respond to inflammation or infection to participate in innate and adaptive immune responses.

Keywords Liver fibrosis · Antimicrobial response · Antigen presentation

Abbreviations

Sp. F	$CD45^+Col^+$ fibrocyte-like cells in spleen
MΦ	Macrophages
SC	Hepatic stellate cells

Introduction

Fibrocytes are a population of bone marrow (BM)-derived cells expressing CD45 and collagen type I ($CD45^+Col^+$) that are implicated in the pathogenesis of fibrotic diseases in diverse organs including liver, lungs, skin, and kidneys

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[1–5]. $CD45^+Col^+$ fibrocytes possess dual characteristics of hematopoietic cells and fibroblasts, and are capable of differentiating into myofibroblasts [1, 6]. Fibrocytes have been reported to contribute between 5% and 25% of the myofibroblasts in different types of experimental and clinical organ fibrosis [5].

In addition to the target organ, recruitment of $CD45^+Col^+$ fibrocytes to spleen has been documented during liver [7] and kidney fibrotic injury [8], but the biological significance of this splenic population of cells is not understood. $CD45^+Col^+$ cells are capable of differentiating according to their microenvironment, giving rise to different subtypes of fibrocyte-like cells, which have distinct roles during tissue repair and fibrosis [9]. For example, $CD45^+Col^+$ fibrocytes have been reported to function in antigen presentation and priming of T cell responses [10, 11]. Fibrocytes are also linked to inflammatory responses in colitis and bronchial asthma [12, 13], where they acquire an organ-specific phenotype (e.g., round shape). Moreover, a population of $CD45^+Col^+$ fibrocyte-like cells has been recently identified within the BM. When freshly isolated, these BM $CD45^+Col^+$ cells exhibit a round shape and phenotypically resemble $CD11b^+CCR2^+LyC6^+CD115^+$ monocytes, but rapidly obtain a spindle-like shape in culture, and in response to TGF-β1, differentiate into (myo)fibroblasts (submitted). Thus, a growing body of evidence suggests that fibrocyte function is not limited to inducing fibrosis in injured organs.

While the contribution of fibrocytes to wound healing and fibrosis has been extensively studied, our present study examines the functional properties of $CD45^+Col^+$ cells in the spleen. We demonstrate that a splenic population of $CD45^+Col^+$ cells possesses fibrocyte-like features and is recruited from the BM to the spleen in response to lipopolysaccharide (LPS), bacterial infection, or the release of the fibrogenic cytokine TGF-β1. In the spleen, these cells adopt a myeloid cell phenotype and gene expression pattern consistent with a role in the immune response. Further analysis reveals that splenic fibrocyte-like cells possess additional functions, causing direct antimicrobial activity through release of DNA-based extracellular traps and serving as antigen-presenting cells (APCs) to stimulate adaptive immune responses. Finally, under further stimulation from growth factors, the $CD45^+Col^+$ cells downregulate collagen expression and acquire cell markers and phenotypes consistent with terminally differentiated macrophages or dendritic cells.

Materials and methods

Mice C57BL/6, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ ($CD45.1^+$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I), C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II), C57BL/6-Tg (ACTB-OVA)916Jen/J, and B6.C-*H2*^{bm1}/ByJ (bm1) were kindly provided by Dr. Schoenberger (La Jolla, CA, USA). Collagen α1(I)-GFP mice were generated [14]. BMT chimeric Col-into-wt mice were generated by transplantation of collagen-α1(I)-GFP BM into lethally irradiated (1,200 Rad) C57BL/6 wild-type mice [7]. Mice were maintained and studied at the University of California San Diego and the La Jolla Institute of Allergy and Immunology under the guidelines and approved protocols of the respective Institutional Animal Use and Care Committees.

Liver injury Liver injury was induced in mice by i.p. injections with carbon tetrachloride CCl_4 (1:4 dilution in corn oil, 60 μl×14 injections [15]. Splenectomy was performed as described [16].

Flow cytometry Cell sorting of $CD45^+GFP^+$ cells was performed on a MoFlo (Beckman Colter, Fullerton, CA, USA). Phenotyping of BM and spleen was performed on Canto (BD Bioscience Flow Cytometry Systems, BD, San Jose, CA, USA). Antibodies used in this study are listed in the Supplementary Methods (eBioscience, San Diego, CA, USA).

Fluorescent and electron microscopy Fluorescent microscopy was performed on cryosection from tissues fixed in 4% PFA and 30% sucrose. Electron microscopy was performed as described ([17] see Supplementary Methods) and viewed using a JEOL 1200EX II (JEOL, Peabody, MA, USA) transmission electron microscope and a Gatan digital camera (Gatan, Pleasanton, CA, USA).

Whole mouse genome gene expression microarray Splenic $CD45^+Col^+$ cells from LPS (6 μg/g)-treated mice, quiescent B-1 cells from peritoneal lavage, and quiescent and activated macrophages from thioglycollate-stimulated mice (Supplementary Methods) were compared using whole mouse genome microarray (Agilent, Santa Clara, CA, USA). Total RNA was isolated using RNeasy columns (Qiagen, Valencia, CA, USA), labeled, and hybridized to a Microarray 4x44K 60 mer slide. Slides were scanned using the Agilent GZ505B Scanner and analyzed using the Gene Spring Software (Agilent).

In vivo infection with Listeria monocytogenes Col-into-wt mice were intravenously infected with *L. monocytogenes* (strain 10403s, 4×10^6 cfu). Twenty-four hours later, liver, spleen, and kidney were removed and evaluated by fluorescent microscopy or homogenized and plated to enumerate bacterial colony-forming units. The results are shown as surviving colony-forming units per well±SEM.

In vitro bacterial killing assay Splenic $CD45^+Col^+$ cells from LPS-treated mice (1×10^6) were co-cultured in DMEM

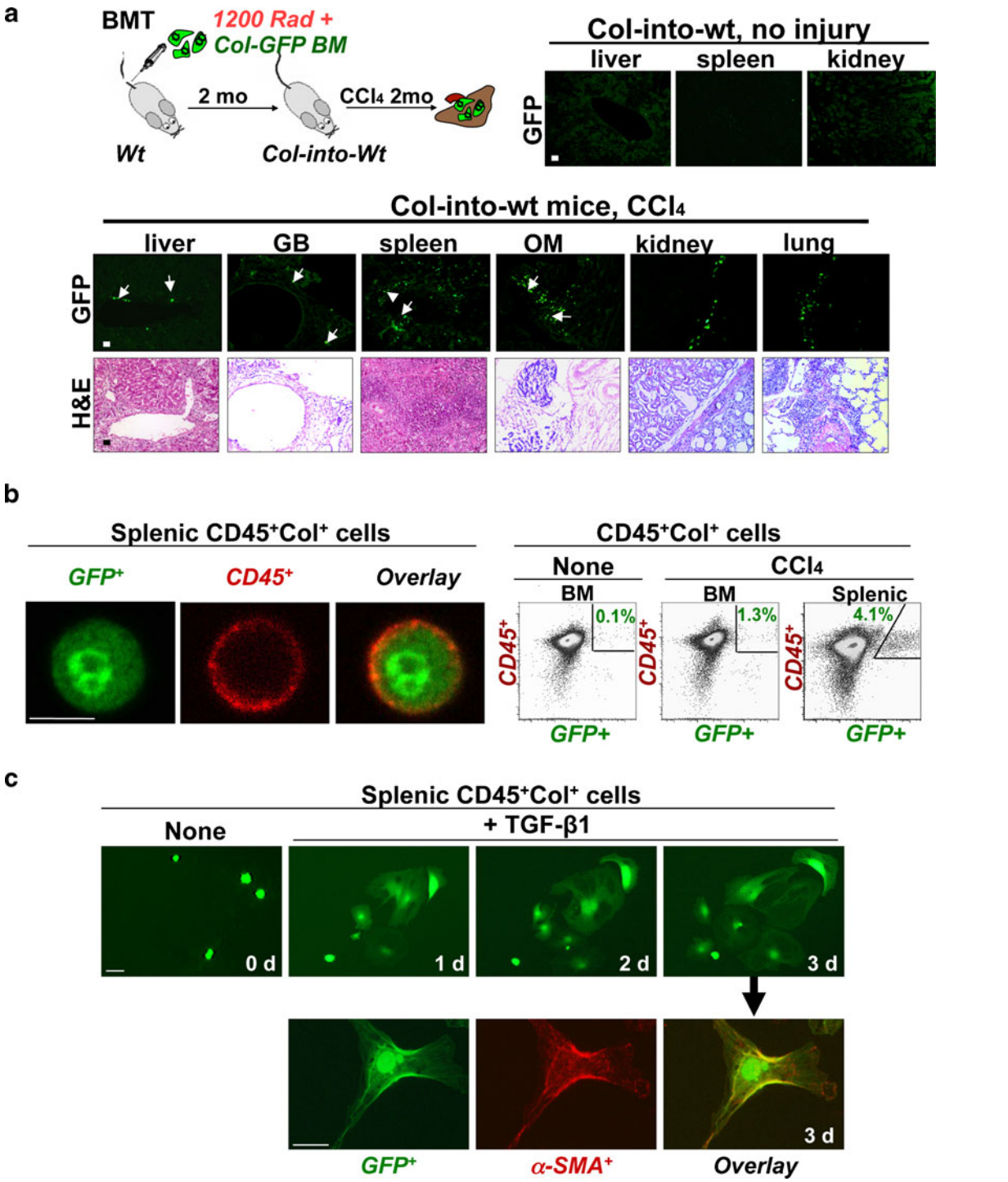
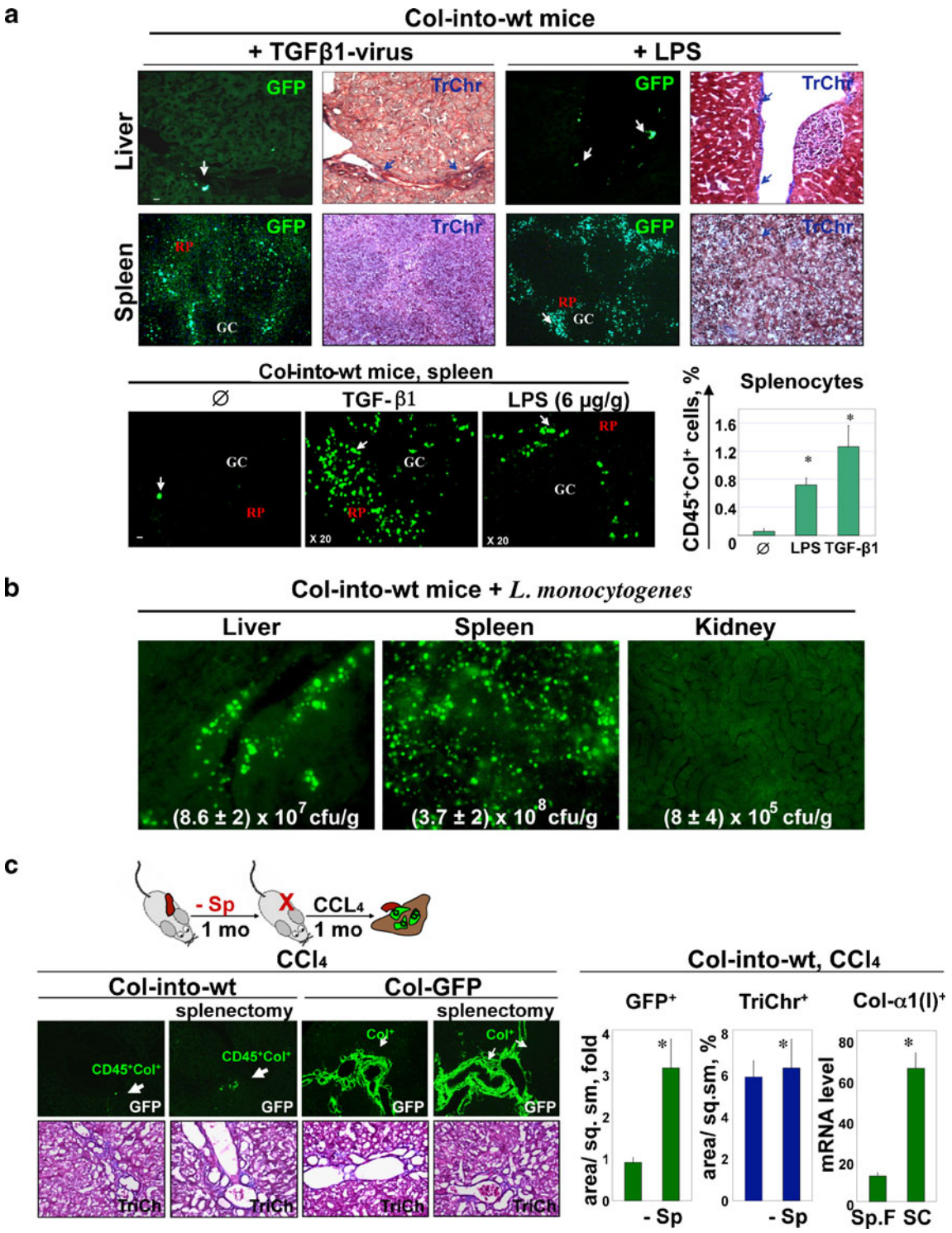


Fig. 1 $CD45^+Col^+$ cells migrate to spleen in response to CCl_4. **a** Outline of the experiment: lethally irradiated wild-type mice are transplanted with Col-GFP^+ BM, and 2 months later subjected to liver injury by CCl_4. $CD45^+Col^+$ were not detected in the liver or spleen of Col-into-wt mice without injury or stress (n=15), *upper panel*. The organs populated by $CD45^+Col^+$ cells in Col-into-wt mice in response to CCl_4-induced liver injury are liver, gall bladder (GB), spleen, mesentery lymph nodes (LN), and omentum majus (OM), as analyzed by fluorescent microscopy and H&E staining, *lower panel* (n=25). *Scale bars* represent 50 μm. **b** Col^+ cells co-express CD45, as detected by immunofluorescence or flow cytometry analysis. In response to CCl_4, the number of $CD45^+Col^+$ cells reached 1.3% in the BM and 4.1% in spleen. Without injury, $CD45^+Col^+$ cells constituted 0.1% in the BM and were undetectable (0.0%, not shown) in spleen. *Dot plot* is representative of ten independent experiments. **c** Splenic $CD45^+Col^+$ cells, cultured on plastic in RPMI+10% FCS+TGF-β1 (5 ng/ml), differentiate into α-SMA^+ myofibroblasts cells. Representative images of three independent experiments are shown



supplemented with 10% FCS with *L. monocytogenes*, strain 10403s, multiplicity of infection (MOI) of 0.01 bacteria: cell, for 30 min at 37°C+5% CO_2. At indicated time points (30, 60, 90, and 120 min), a small aliquot was removed, and serial dilutions were plated to enumerate surviving colony-forming units. The results are represented as surviving colony-forming units per time point±SEM. Alternatively, splenic $CD45^+Col^+$ cells (2×10^5 cells) were co-cultured with bacteria, MOI 2:1 bacteria:cell, for 30 min, and live/dead bacteria were visualized by Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) according to the manufacturer (see Supplementary Methods).

◀**Fig. 2** TGF-β1, LPS, and *Listeria monocytogenes* induce migration of $CD45^+Col^+$ cells to spleen and liver in vivo. **a** Col-into-wt mice are i.v. infected with TGF-β1-expressing adenovirus (1×10^8 pfu) or control adenovirus, or injected with LPS (6 μg/g). After 72 h, GFP^+ cells (*white arrows*) are detected by immunofluorescence (GC-germinal center, RP-red pulp) or flow cytometry (mean fluorescent intensity±SEM (n=5 mice/group, *p<0.001). Collagen deposition was estimated by TriChrome staining (*blue arrows*), which shows lack of collagen deposition in spleen. **b** Mobilization of $CD45^+Col^+$ cells in response to *L. monocytogenes* infection. Col-into-wt mice are infected with *L. monocytogenes* (4×10^6 cfu) for 24 h. GFP^+ cells are detected in livers and spleens but not kidneys, as visualized by fluorescent microscopy using a ×10 objective, *left panel*. The bacterial load of organ homogenates is calculated for liver ($8.6\pm2\times10^7$ cfu/g), spleen ($3.7\pm2\times10^8$ cfu/g), and kidney ($8\pm4\times10^5$ cfu/g). The data represent the average of three mice. **c** The effect of splenic $CD45^+Col^+$ cells on liver fibrosis is studied in splenectomized mice. Outline of the experiment: spleen is surgically removed (−Sp) in experimental group of mice. *Left panel*, fluorescent micrographs of liver obtained from splenectomized or sham-operated CCl_4-treated Col-into-wt mice and Col-GFP mice. Phase contrast micrographs are TriChrome staining of liver tissues, obtained for each group of mice. *Right panel*, liver tissues from splenectomized or sham-operated Col-into-wt mice are analyzed for GFP^+ or TriChrome-positive areas. mRNA levels of collagen α1(I) expression in splenic $CD45^+Col^+$ cells was compared with hepatic stellate cells (SC), as detected by RT-PCR. The data are presented as mean±SEM, *p<0.001

Visualization of the extracellular DNA traps Splenic $CD45^+Col^+$ cells (2×10^5 cells) were co-cultured with *L. monocytogenes* (MOI 1:0.1) on poly-L-lysine (Sigma)-coated glass cover slides. Fixed cells were stained with rabbit anti-murine CRAMP (gift of Dr. R.L. Gallo), anti-following by secondary Alexa fluor 568 (Invitrogen), and embedding in ProlongGold antifade+Dapi (Molecular Probes). Mounted samples were analyzed by confocal laser-scanning 2-photon microscope Olympus Fluoview FV1000 using a 60×/1.42 PlanApo oil objective and Fluoview™ Spectral Scanning technology (Olympus; see Supplementary Methods for details).

T cell proliferation assays $CD8^+$ and $CD4^+$ T cells and DCs were purified using αCD8α, αCD4, or αCD11a MACS microbeads (Miltenyi Biotec, Auburn, CA, USA), respectively, and labeled with 2 μM CFSE (Invitrogen, Carlsbad, CA, USA). For proliferation studies in vitro, 1.5×10^5 T cells were co-cultured with 5×10^4 DCs or splenic $CD45^+Col^+$ cells loaded with 1 μM $OVA^{257–264}$ (SIINFEKL) or 10 μM $OVA^{323–339}$ (ISQAVHAAHAEINEAGR) peptides (Abgent, San Diego, CA, USA) for 1 h, 37°C. For proliferation studies in vivo, 1×10^6 OT-I/bm1 $CD8^+$ T cells were injected i.v. together with 1.5×10^5 DCs or splenic $CD45^+Col^+$ cells into Act-mOVA/bm1 mice. Four days later, proliferation of $CD8^+$ T and $CD4^+$ T was analyzed by flow cytometry.

Differentiation of splenic fibrocyte-like cells into macrophages and DCs In vitro, total BM cells or splenic $CD45^+Col^+$ cells were cultured for 6 days in RPMI 1640 medium containing 10% FCS, 1 mM sodium pyruvate, HEPES, penicillin, streptomycin, and β-mercaptoethanol (RPMI/FCS) supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF; 20 ng/ml; R&D Systems) or macrophage-colony stimulating factor (M-CSF; 30% L-Cell media, gift of Dr. Glass). Harvested cells were analyzed by flow cytometry. In vivo differentiation of splenic fibrocytes was studied in chimeric $CD45.1^+$ mice, generated by adoptive transfer of $GFP^+CD45.2^+$ splenic fibrocytes (1×10^5 cells) into sublethally irradiated $CD45.1^+$ recipient mice.

Phagocytosis assay The vibrant phagocytosis kit (Molecular Probes, Carlsbad, CA, USA) was used to evaluate activity of BM and splenic $CD45^+Col^+$ cell-derived macrophages (1×10^5 cells/ml) or peritoneal macrophages incubated with FITC-labeled *Escherichia coli* (K-12 BioParticles) or flouro-ruby dextran (tetramethylrhodamine 10,000 MW, Invitrogen), at 37°C, followed by a fluorescence quenching of extracellular fluorescence with trypan blue. Phagocytic activity was evaluated by flow cytometry or fluorescent microscopy.

Statistical analyses

Quantitative results are expressed as mean±SEM. The Student's t test was used to determine the significance of differences between means. Statistical significance was estimated at $p<0.05$.

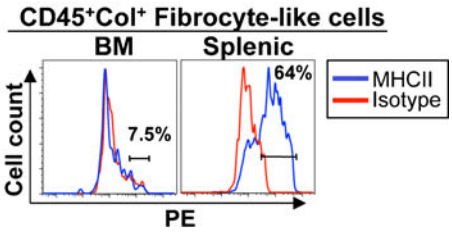
Results

Fibrogenic injury triggers migration of $CD45^+Col^+$ cells to liver and spleen

$CD45^+Col^+$ fibrocytes were studied in chimeric Col-into-wt mice, which were generated by transplantation of collagen α1(I) promoter/enhancer-GFP^+ BM into lethally irradiated wild-type mice [7]. Hepatic fibrosis was induced in these mice by CCl_4 (14 injections/8 weeks; Fig. 1a). Monitored by GFP expression, $CD45^+Col^+$ fibrocytes were detected in fibrotic liver and gallbladder, omentum, lungs, and kidneys of CCl_4-treated mice (Fig. 1a). Remarkably, the greatest numbers of these $CD45^+Col^+$ cells homed to the spleen and lymphoid aggregates in fat. CCl_4 liver injury caused expansion of $CD45^+Col^+$ population in the BM (increasing from 0.1% to 1.3% of total BM cells) and migration to the spleen (increase from 0.0% to 4.1% of total splenocytes; Fig. 1b). Splenic Col^+ cells exhibited a round shape and co-expressed CD45. However, similar to fibrocytes, these cells in culture rapidly obtained a spindle-like shape (Fig. 1c and

a

$CD45^+Col^+$ cells, LPS	Activation	BM, %	Sp, %
	None	0.2 ± 0.1	0.0 ± 0.1
	LPS single	3.2 ± 0.7	0.5 ± 0.2
	LPS multiple	0.3 ± 0.1	4.3 ± 0.9



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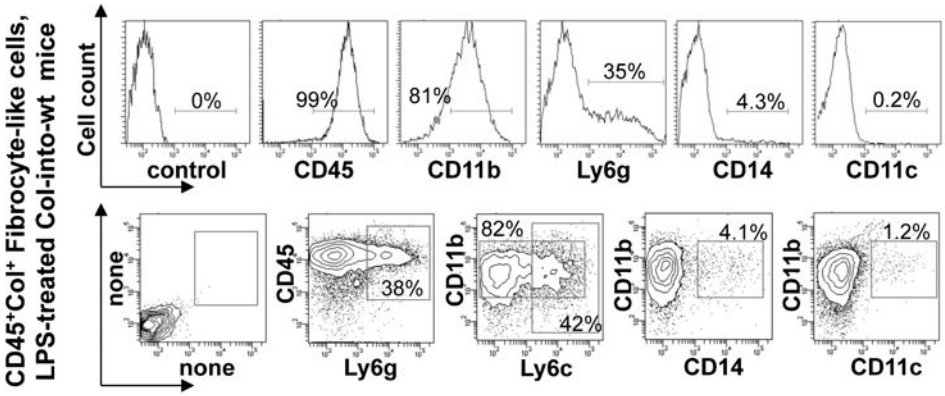

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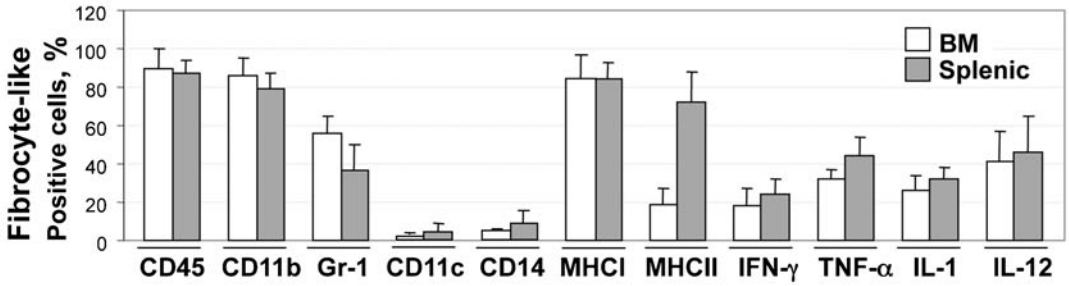

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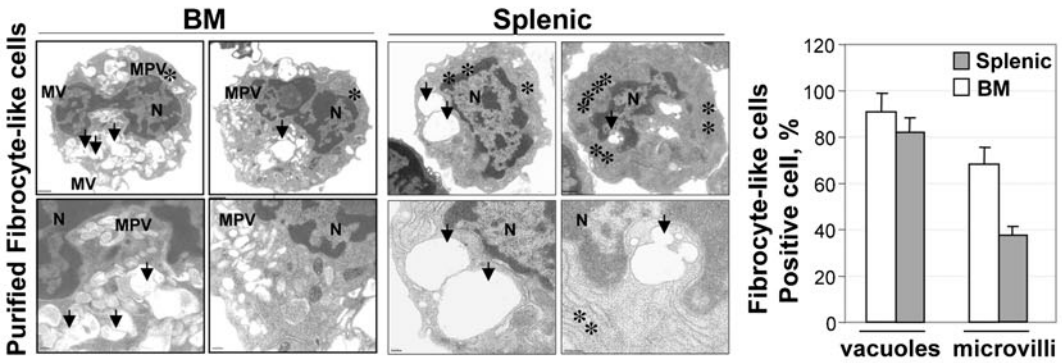

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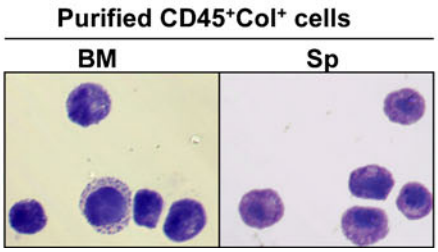


◀**Fig. 3** Phenotyping of splenic $CD45^+Col^+$ cells from LPS-treated mice. **a** Response of splenic $CD45^+Col^+$ cells to LPS. Col-GFP mice are subjected to single or multiple (n=3) LPS injections. The number of $CD45^+Col^+$ cells in the BM and spleen was analyzed 3 or 9 days later, respectively. $CD45^+Col^+$ cells migrated to spleen after single LPS (0.5±0.2%) and multiple LPS injections (4.3±0.9%, of total splenocytes). Mean±SEM are shown (n=13 assays, *left panel*). *Right panel*, histograms show MHC II expression by BM and splenic $CD45^+Col^+$ cells, stained with anti-MHC II antibody (*blue*) or isotype matched control antibody (*red*). Representative *dot plots* are shown. **b** Splenic $CD45^+Col^+$ cells from LPS-treated Col-GFP mice were analyzed by flow cytometry. Representative histograms and *dot plots* are shown. **c** Comparison of surface markers expression by BM and splenic $CD45^+Col^+$ cells. $CD45^+Col^+$ cells from BM and spleen of LPS-treated Col-GFP mice are analyzed by flow cytometry for CD11b, F4/80, Gr-1, CD11c, MHC I, MHC II, surface markers, and intracellular cytokines (IFN-γ, TNF-α, and IL-1). *Bars* represent the mean fluorescent intensity±SEM of three independent experiments. **d** Electron micrographs of the BM and splenic $CD45^+Col^+$ cells. $CD45^+Col^+$ cells are studied by EM at a magnification of ×6,000, *upper panel*, and ×10,000, *lower panel*. Vacuoles are marked with *arrows*, and electron-dense ER is marked with *asterisks*. Other features are listed as N (nucleus), MV (microvili), and MPV (micropinocytotic vesicles). EMs are representative images from ten random fields per sample. **e** BM and splenic $CD45^+Col^+$ exhibited similar morphology, detected by Wright–Giemsa staining, and visualized as round cells, with a basophilic and vacuolated cytoplasm with occasional basophilic granules. Representative images from nine to ten random fields using a ×60 objective

Supplementary Fig. S1) and differentiated into α-smooth actin $(SMA)^+$ myofibroblasts in the presence of TGF-β1 (Fig. 1c), suggesting that splenic $CD45^+Col^+$ cells possess fibrocyte-like features.

TGF-β1, LPS, and bacterial infection induce homing of $CD45^+Col^+$ cells to spleen

Fibrogenic liver injury is associated with the release of fibrogenic cytokines and endogenous bacterial products [15]. Consistent with this, treatment of Col-into-wt mice with LPS (6 μg/g) or with TGF-β1-expressing adenovirus (1×10^8 pfu) induced rapid (within 72 h) migration of $CD45^+Col^+$ cells to spleen (Fig. 2a), where they localized between the red pulp (RP) and germinal center (GC) and contributed 0.7±0.2% and 1.3±0.6% of total splenocytes, respectively (Fig. 2a). Meanwhile, only single $CD45^+Col^+$ cells were detected in the liver (Fig. 2a), the organ primarily targeted by adenoviral infection (e.g., hepatocytes). Thus, our data suggest that $CD45^+Col^+$ cells may traffic through spleen. Splenic homing of BM-derived fibrocyte-like cells was also demonstrated in the context of live bacterial infection (Fig. 2b). Col-into-wt mice were infected with *L. monocytogenes*, a $Gram^+$ bacterial pathogen that infects liver and spleen and causes mobilization of inflammatory monocytes [18]. Recruitment of $CD45^+GFP^+$ fibrocyte-like cells to spleens and livers (but not to kidneys) of Col-into-wt mice was detected 24 h after *Listeria* infection, and directly correlated with the bacterial load, indicating that infection triggered a proportional migration of fibrocyte-like cells to the target organs (Fig. 2b).

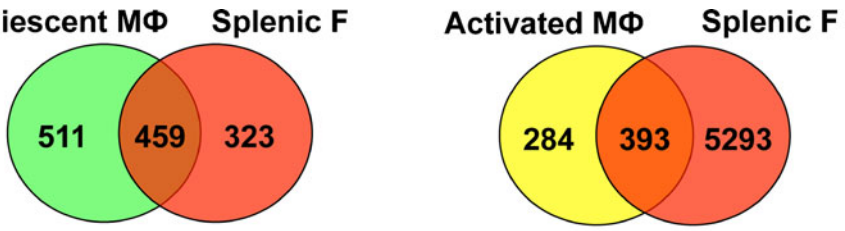
Splenic $CD45^+Col^+$ cells are only minor contributors to hepatic fibrosis

To evaluate the role of splenic $CD45^+Col^+$ cells in liver fibrosis, we eliminated the splenic niche by performing splenectomies in Col-into-wt mice and Col-GFP mice, and 1 month later induced CCl_4 injury in these mice (Fig. 2c). The number of $CD45^+Col^+$ fibrocyte-like cells in the livers of splenectomized animals was increased fourfold in comparison with the sham-operated mice (Fig. 2c).

Splenic $CD45^+Col^+$ cells exhibit myeloid phenotype

The responses of splenic $CD45^+Col^+$ cells to LPS were closely examined and compared to BM $CD45^+Col^+$ cells. A single LPS injection triggered expansion of $CD45^+Col^+$ cells in the BM (3.2±0.7%, Fig. 3a), where they express myeloid markers CD11b (89±7%), Gr-1 (58±8.5%), and F4/80 (47±4%), but lacked maturation markers (CD11c, CD14, and CD68; Fig. 3b, c). On the other hand, multiple LPS injections induced homing of $CD45^+Col^+$ cells to the spleen (4.3±0.9%, Fig. 3a). In the spleen, $CD45^+Col^+$ cells retained expression of myeloid CD11b (82±5%), Ly6c (42±4%), and Ly6g (38±7%, Fig. 3b, c) markers, but strongly upregulated expression of MHC class II (Δ48±8%, Fig. 3a, c). Intracellular cytokine expression (IFN-γ, TNF-α, IL-1, and IL-12) was also increased by splenic $CD45^+Col^+$ cells and was polarized towards Th1 (Fig. 3c and Supplementary Fig. S2). Due to similarity with tissue fibrocytes in surface markers expression ($CD45^+Col^+CD11b^+F4/80^+Gr\text{-}1^+MHC\ II^+$) [1, 19], and the ability to differentiate into myofibroblasts in culture and in response to TGF-β1 (Fig. 1c), we conclude that splenic $CD45^+Col^+$ cells exhibit a fibrocyte-like phenotype. Electron microscopy revealed that both splenic and BM $CD45^+Col^+$ cells possessed similar morphology characterized by numerous intracellular vacuoles and dilation of endoplasmatic reticulum (ER) and the Golgi region [20], indicating extensive protein synthesis (Fig. 3d). Slightly higher number of vacuoles (91±8% vs. 82±5%) and multiple microvilli (64±7% vs. 38±4%) were detected in BM $CD45^+Col^+$ cells, while dilation of ER was more pronounced in splenic $CD45^+Col^+$ cells. Meanwhile, BM and splenic fibrocyte-like cells remained morphologically indistinguishable by Wright–Giemsa staining (Fig. 3e). Our data indicate that in contrast to tissue fibrocytes, splenic $CD45^+Col^+$ cells may acquire distinct features in spleen and

a



b

GO TERM	№ genes	*p* value
Splenic F and quiescent MΦ		
Defense Response	47	$1.2e^{-10}$
Immune Response	42	$1.7e^{-10}$
Inflammatory Resp.	17	$1.2e^{-7}$
Chemotaxis	12	$1.2e^{-5}$
Coagulation	8	$3.9e^{-5}$
Wound Healing	9	$4.1e^{-5}$

GO TERM	№ genes	*p* value
Splenic F and activated MΦ		
Response to biotic stimuli	80	$5.2e^{-37}$
Response to pathogen	30	$3.0e^{-11}$
Response to wounding	21	$7.5e^{-8}$
Cytokine Metabolism	10	$6.5e^{-8}$
Chemotaxis	10	$7.2e^{-5}$
Antigen Presentation	8	$2.5e^{-5}$

c

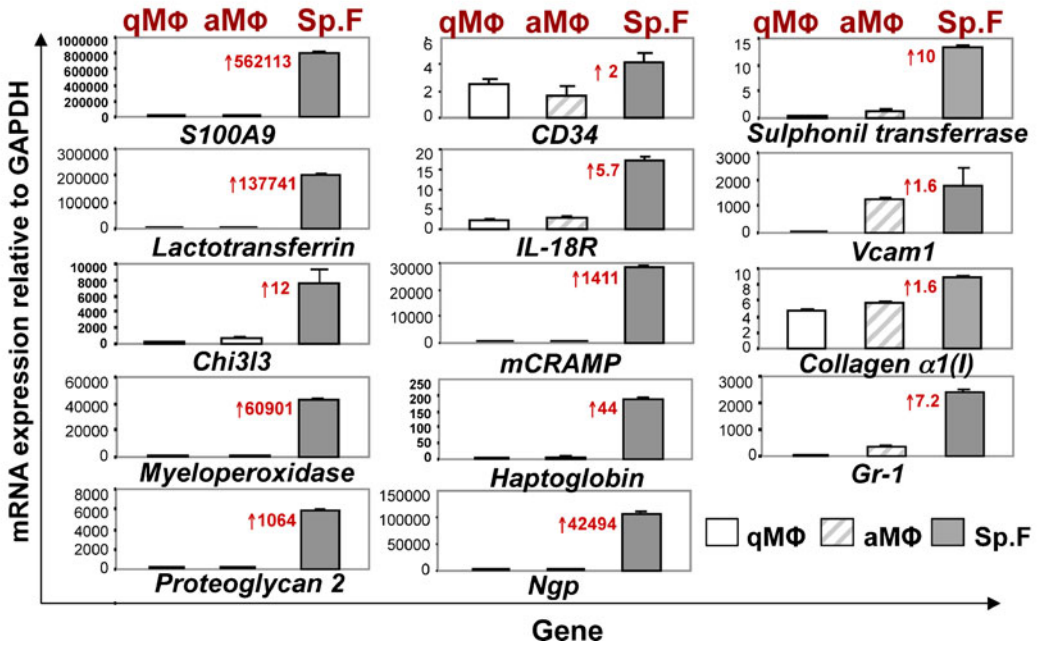


Fig. 4 Comparison of splenic $CD45^+Col^+$ cells and macrophages. **a** Gene expression microarray of splenic $CD45^+Col^+$ cells. Splenic $CD45^+Col^+$ cells from LPS-treated mice and peritoneal macrophages (MΦ) from thioglycollate-stimulated mice, unstimulated (quiescent), or Kdo_2-lipid A-treated (100 ng/ml, 6 h, activated) are compared by gene expression microarray. Venn diagram shows the number of shared and distinct genes. **b** Gene expression overlap identified for each group, presented as GO TERM with the *p* values indicated for each group. **c** Gene expression analysis of fibrocyte precursor-specific genes by RT-PCR. Relative mRNA levels are calculated for qMΦ, aMΦ, and splenic FPs after normalization to GAPDH gene using the ΔΔ CT method. Genes are listed as Chi3l3 (chitinase 3-like 3), Ngp (neutrophilic granular protein), IL-18R (interleukin 18 receptor), mCRAMP (cathelicidin antimicrobial peptide), and Vcam-1 (vascular cell adhesion molecule). Fold induction of gene expression in splenic $CD45^+Col^+$ cells is shown in comparison with the highest value detected in qMΦ or aMΦ, $p>0.0001$

Table 1 Signature genes of splenic $CD45^+Col^+$ cells

Marker	Fold upregulation	Signature genes
Lineage	↑1.6	Col-a1(I)
	–	CD45
	–	CD11b
	↑4	CD34
	↑27	Ly-6c
Surface	↑1,822	S100A9
	↑40	Proteoglycan2 (Prg2)
Antimicrobial	↑318	Myeloperoxidase
	↑186	Lactotransferrin
	↑173	Cathelicidin antimicrobial peptide
	↑90	Neutrophilic granular protein
	↑22	Haptoglobin
	↑11	Chitinase3-like 3/4
	↑10	Spectrin β4
	↑8	Lipocalin 2
	↑5.3	Neutrophil elastase 2
	↑5	Defensin-related cryptidin 3

Genes expressed by splenic $CD45^+Col^+$ cells. Fold upregulation (↑) of $CD45^+Col^+$ cell-specific genes was calculated relative to the highest value of gene expression in quiescent or activated macrophages

therefore may represent a functionally unique myelomonocytic population.

Gene expression profiles link splenic fibrocyte-like cells to innate immunity

Insight into the potential function of splenic $CD45^+Col^+$ fibrocyte-like cells was provided by gene expression microarrays. To estimate the relative levels of gene expression, $CD45^+Col^+$ cells were purified from spleens of LPS-treated Col-GFP mice and compared to quiescent and activated macrophages and B-1 cells (to define a set of "macrophage-specific" genes). Using a threshold defining confident detection of gene expression, splenic fibrocyte-like cells expressed genes that are preferentially found in quiescent and activated macrophages, sharing expression of 459 mRNAs with quiescent macrophages and 393 mRNAs with activated macrophages (Fig. 4a). This set of genes was enriched in Gene Ontology biological process annotations linked to inflammatory responses, chemotaxis, wound healing, and antigen presentation (Fig. 4b and Supplementary Table S1). Similar to fibrocytes, splenic $CD45^+Col^+$ cells retained expression of lineage-specific differentiation markers (CD11b, Gr-1) and progenitor markers (CD34, Thy-1) at a much higher level than macrophages; they lacked markers of maturation (CD68) but expressed fibroblast-like genes (collagen 1α1, collagen 7α1, collagen 11α2, and collagen 14α1 and induced TGF-β1; Supplementary Table S1).

Analysis of the gene expression profile was confirmed for selected genes by reverse transcription-polymerase chain reaction (RT-PCR; Fig. 4c) and implicated splenic $CD45^+Col^+$ fibrocyte-like cells in transmigration, antimicrobial responses, and antigen presentation (Supplementary Table S1 and Reference 1). First, expression of mRNAs encoding S100A9/A8, CD11b, ICAM-1, ICAM-2, VCAM-1, β-integrins, proteoglycan 2, RAGE, CD36, and mannose receptor genes suggest that splenic fibrocyte-like cells are capable of transmigration from the blood stream [21]. Second, splenic $CD45^+Col^+$ fibrocyte-like cells express many genes that encode antimicrobial enzymes and peptides (myeloperoxidase, lactotransferrin, α-defensins, chitinase like 3 and 4, lipocalin, lysozymes, complement 3, and cathelicidin), typically produced by myeloid or other immune cells to restrict bacterial spread and kill microbial pathogens at the site of infection ([22] and Supplementary Table S2 and Reference 2). Third, consistent with earlier findings [10, 11], expression of H2-K1, H2-Q8, H-13, CD83, CD86, and CD11b genes, associated with antigen presentation, links splenic $CD45^+Col^+$ fibrocyte-like cells to adaptive immunity (Supplementary Table S2 and Reference 3) [23]. This comparative analysis identifies highly upregulated "signature genes" that link splenic $CD45^+Col^+$ fibrocyte-like cells to antimicrobial immunity (Table 1).

Splenic fibrocyte-like cells are non-phagocytic but elaborate antimicrobial extracellular traps

The behavior of splenic fibrocyte-like cells in response to live bacteria was further studied in vitro. Splenic $CD45^+Col^+$ cells were isolated from LPS-treated mice and co-cultured with *L. monocytogenes* for 30 min. Although splenic fibrocyte-like cells lacked phagocytic activity (Fig. 5a),

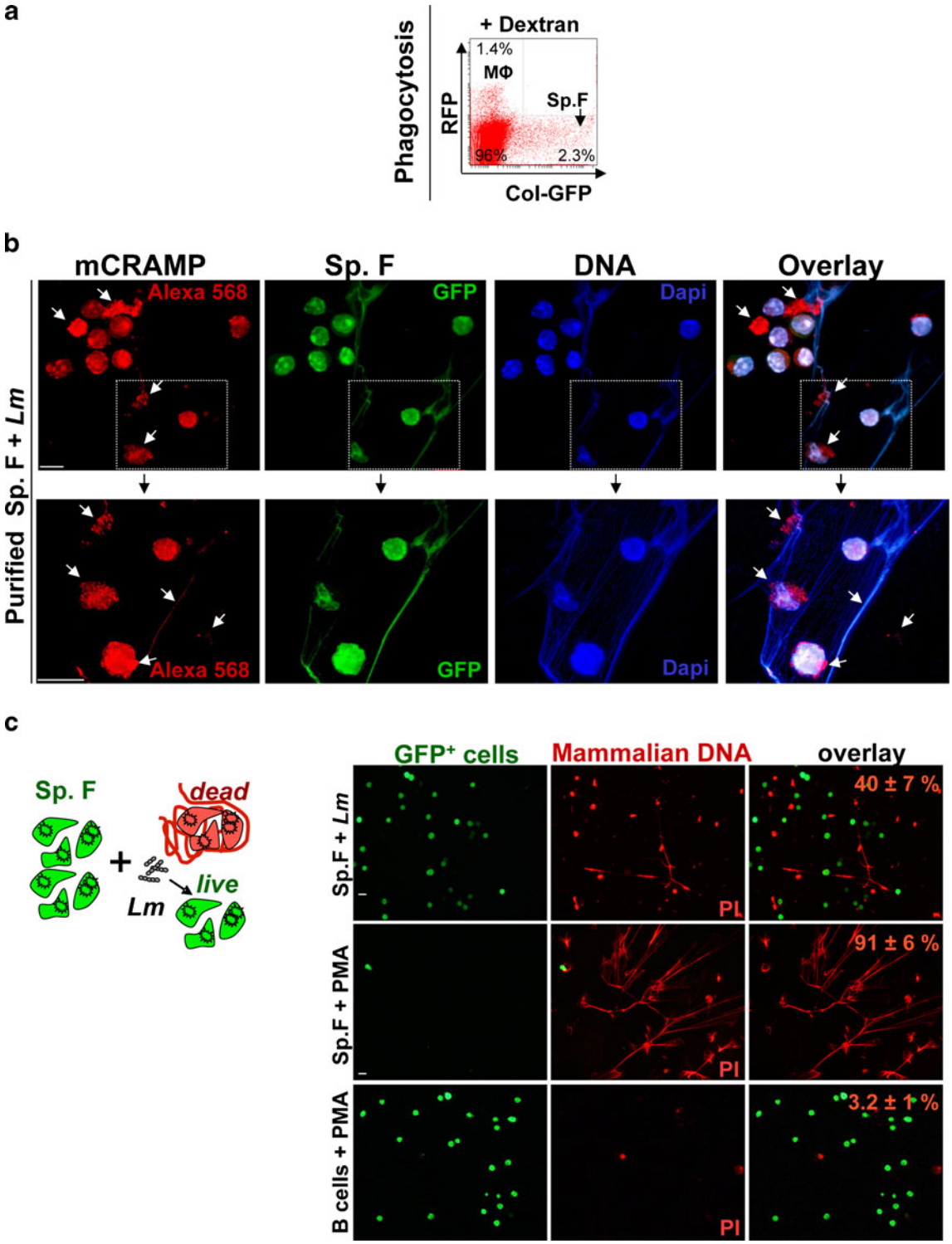
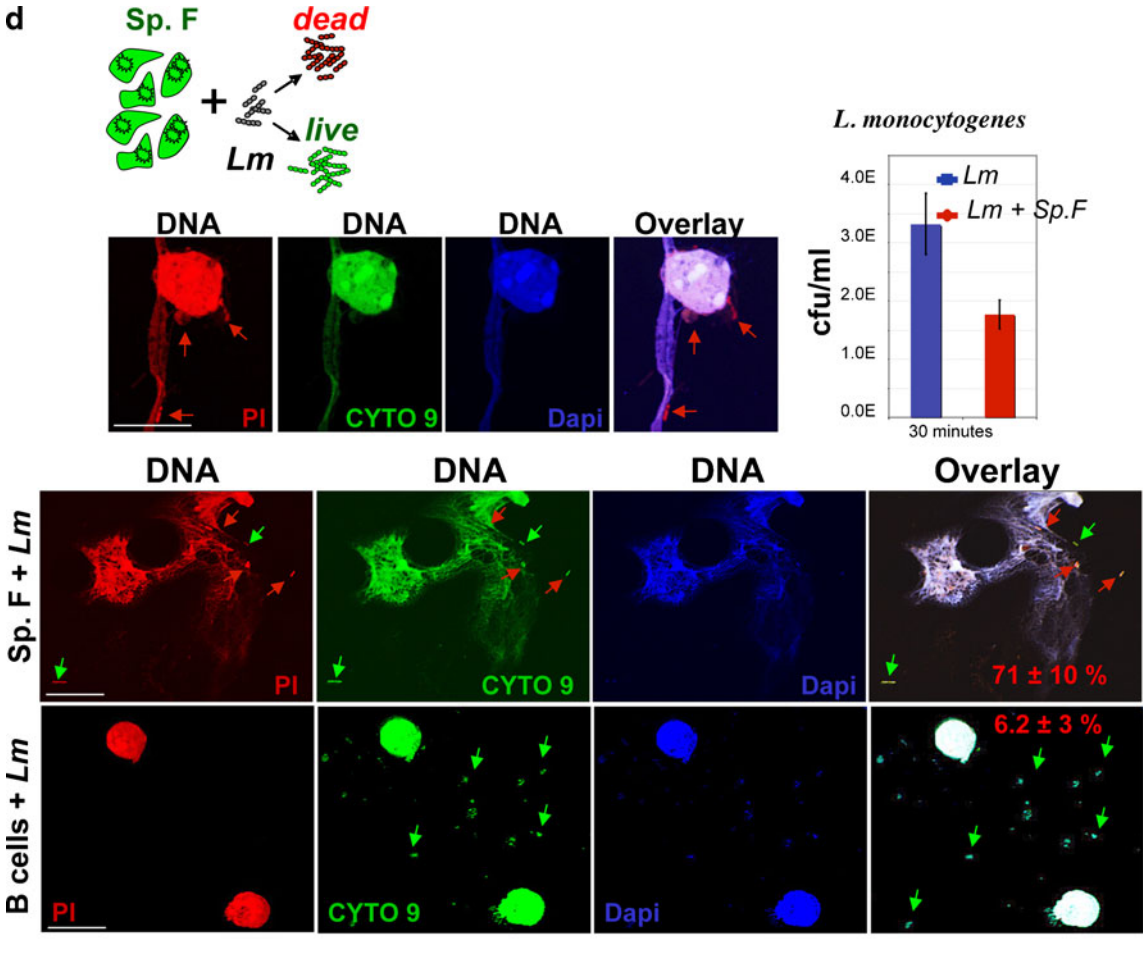


Fig. 5 Splenic CD45$^+$Col$^+$ cells possess anti-microbial properties. **a** Splenic CD45$^+$Col$^+$ cells lack phagocytic activity. Splenic MΦ but not splenic CD45$^+$Col$^+$ fibrocyte-like cells (Sp. F) phagocytosed flouro-ruby dextran (10,000 MW), as detected by flow cytometry after 15 min of co-culturing. *Dot plot* is representative of three experiments. **b** Splenic CD45$^+$Col$^+$ cells release antimicrobial extracellular DNA-traps to entrap and kill *L. monocytogenes* (*Lm*). CD45$^+$Col$^+$ cells co-incubated with *L. monocytogenes* are stained with anti-mCRAMP Ab, visualized in *red* (Alexa fluor 568). DNA traps are visualized in *blue* (Dapi); fibrocytes-like cells are visualized in *green* (GFP). *Bar* represents 20 μm. **c** Release of extracellular traps is associated with death of splenic CD45$^+$Col$^+$ cells. Splenic CD45$^+$Col$^+$ cells are co-incubated with *L. monocytogenes* or PMA for 30 min and analyzed for live/dead cells using Mammalian Cells Viability Kit, *right panel*. Splenic CD45$^+$Col$^+$ cells are compared to PMA-treated GFP$^+$ B cells (from β-actin-GFP mice). Dead cells and DNA traps are visualized in *red* (PI). *Bar* represents 20 μm



e

Percent per total cell number, %	Splenic F			B cells
	None	*LM*	*PMA*	*LM*
DNA traps per total cell number	14 ± 2	31 ± 10	65 ± 17	1 ± 0.3
Intracellular mCRAMP+ cells per total cells	93 ± 2.5	87 ± 5	71 ± 6	1.3 ± 1
Dead trap-forming cells per total dead cells	7 ± 4	60 ± 7	91 ± 6	3.0 ± 1
Dead bacteria per total number of bacteria	2.8 ± 1	71 ± 10	ND	6.2 ± 3

Fig. 5 **d** Splenic $CD45^{+}Col^{+}$ cells reduce viability of *L. monocytogenes* in vitro after 30 min of co-incubation, as shown in *upper* and *middle panel*. Dead bacteria are visualized in *red* (PI; *red arrows*) using Live/Dead Bacterial Viability Kit versus live bacteria (*green*). In addition to fluorescence microscopy, surviving bacteria were quantified by plating surviving colony-forming units on agar plates. Data are shown as mean values±SD of surviving colony-forming units per milliliter. *Lower panel*, B cells co-cultured with *L. monocytogenes* serve as control. **e** Characterization of the extracellular DNA structures formed by splenic $CD45^{+}Col^{+}$ cells, untreated or 30 min after incubation with *L. monocytogenes*, PMA. The number of DNA traps, $mCRAMP^{+}$ cells, viability of trap-forming cells, and dead bacteria is evaluated. B cells, isolated from the same mice and cultured with *L. monocytogenes*, served as a negative control. The data are presented as percent of positive cells calculated ±SEM, $p \leq 0.005$

they responded to bacteria by releasing extracellular traps, formed by DNA fibers and containing the antimicrobial peptide cathelicidin (mCRAMP, Fig. 5b). Cathelicidin is a critical effector of mammalian innate immunity against invasive bacterial pathogens including *L. monocytogenes* [18, 22]. Extracellular traps are a mechanism recently demonstrated in neutrophils, mast cell, eosinophils, and macrophages to entrap and kill bacteria [24–27]. Thus, formation of DNA traps was observed in 31±10% of splenic $CD45^{+}Col^{+}$ cells incubated with *L. monocytogenes* (summarized in Fig. 5e). Even higher number of splenic $CD45^{+}Col^{+}$ cells (65±17%) released extracellular DNA in

response to phorbol 12-myristate 13-acetate (PMA), a strong inducer of extracellular traps in neutrophils and macrophages [28]. In addition to secreted and DNA-bound mCRAMP, the cathelicidin peptide was expressed in the cytoplasm of splenic $CD45^+Col^+$ cells (Fig. 5b). Moreover, the presence of histones and, uniquely, collagen Type I was detected in the extracellular traps generated by $CD45^+Col^+$ cells (Supplementary Fig. S3). Similar to neutrophils, formation of these traps was accompanied by death of splenic $CD45^+Col^+$ cells ("ETosis") [29], determined by release of nuclear (vs. mictochondrial) DNA, required for their anti-microbial function (Fig. 5c). Formation of extracellular traps by splenic $CD45^+Col^+$ cells was also associated with reduction of bacterial viability, as demonstrated by staining of live/dead bacteria (71±10%; Fig. 5d) or by enumeration of colony-forming units (50±6%; Fig. 5d). In comparison, B cells purified from the same tissues neither killed bacteria nor formed extracellular traps in the presence of *L. monocytogenes* or PMA (Fig. 5e). Though non-phagocytic in comparison to neutrophils and macrophages [27], our data suggest that splenic fibrocyte-like cells have a capacity to participate in innate antimicrobial responses through this extracellular killing mechanism.

Splenic fibrocyte-like cells present antigens to naïve T cells

We next examined the ability of splenic $CD45^+Col^+$ cells to present antigens to naïve T cells and induce their proliferation. For MHC I ($H2\text{-}K^b$) presentation, splenic $CD45^+Col^+$ cells were pulsed with $OVA^{257–264}$ peptide and co-cultured with CFSE-labeled $CD8^+$ (OT-I) T cells for 4 days. For MHC II (I-Ab) presentation, splenic $CD45^+Col^+$ cells were pulsed with $OVA^{323–339}$ peptide and co-cultured with CFSE-$CD4^+$ (OT-II) T cells. Splenic $CD45^+Col^+$ cells were compared to DCs, professional APCs, cultured under the same conditions. As visualized by dilution of CFSE dye, splenic $CD45^+Col^+$ cells effectively induced proliferation of both $CD8^+$ OT-I and $CD4^+$ OT-II cells in vitro (Fig. 6a) with an efficiency comparable to DCs. The ability of splenic $CD45^+Col^+$ cells to present antigens via MHC I ($H2\text{-}K^b$) was also confirmed in vivo. Splenic $CD45^+Col^+$ cells or DCs were adoptively transferred together with the CSFE-labeled $CD8^+$ (OT-I in bm1 background) cells into Act-mOVA/bm1 mice, which express β-actin-ovalbumin and bear MHC I ($H2\text{-}K^{bm1}$) mutation. Since an $H2\text{-}K^{bm1}$ mutation completely abrogated presentation of the $OVA^{257\text{-}264}$ peptide, adoptively transferred APCs were the only source of $CD8^+$ OT-I^{bm1} cell proliferation (Fig. 6a and Supplementary Fig. S4). As expected, DCs induced proliferation of OT-I^{bm1} cells in spleens, livers, and lymph nodes of Act-mOVA/bm1 mice. Splenic $CD45^+Col^+$ cells induced OT-I^{bm1} cell proliferation in spleen and liver, at ~50% potency of DC, but not in the lymph nodes. Thus, splenic fibrocyte-like cells may contribute to adaptive immunity as non-professional APCs.

Interestingly, strong co-expression of mCRAMP (but not of an isotype-matched antibody control, not shown) was detected in 43±7% MHC II^+ splenic fibrocyte-like cells, suggesting that a single $CD45^+Col^+$ cell may acquire a phenotype for more than one function (Fig. 6b and Supplementary Fig. S5).

Splenic fibrocytes can further differentiate into macrophages or DCs

mCRAMP and other cytotoxic peptides are usually expressed by terminally differentiated macrophages or other effector cells, which mediate their function via phagocytosis or formation of extracellular DNA traps [24, 27, 30]. Since splenic fibrocyte-like cells lack phagocytic activity (Fig. 5a) and lack expression of macrophage or DCs markers (CD14 or CD11c; Fig. 3b, c), they may comprise a population of myeloid progenitors (monocytes) that can further differentiate into fibrocytes and myofibroblasts (Fig. 1c), or mature macrophages and/or mDCs [31]. To test this hypothesis, splenic $CD45^+Col^+$ cells were cultured in the presence of GM-CSF or M-CSF (Fig. 6c). In response to GM-CSF, splenic fibrocyte-like cells downregulated expression of collagen α1(I) and differentiated into $Col^-CD11b^+CD11c^+$ DCs. Similarly, stimulated with M-CSF, splenic fibrocyte-like cells differentiated into $Col^-CD11b^+CD14^+F4/80^+$ macrophages, capable of phagocytosing *E. coli* as efficiently as BM-derived macrophages. Intracellular localization of FITC-labeled *E. coli* within splenic $CD45^+Col^+$ cell-derived macrophages was confirmed by flow cytometry and fluorescent microscopy (Fig. 6c). Furthermore, adoptive transfer of purified splenic $CD45.2^+Col^+$ fibrocyte-like cells into $CD45.1^+$ mice produced $CD45.2^+Col^-$ cells expressing $CD11b^+$ (75%), $CD11b^+CD11c^+$ (2.6%), and $Gr\text{-}1^+$ (9%) cells, indicating that splenic fibrocyte-like cells can further differentiate in vivo (Fig. 6d). These studies indicate that splenic fibrocytes differ from macrophages and DCs, and their differentiation/maturation is associated with decreased collagen expression.

Discussion

Splenic $CD45^+Col^+$ cells exhibit fibrocyte-like phenotype and are capable of differentiating into fibrocytes and myofibroblasts in vitro, but also possess some unique characteristics. Based on gene expression profiles and functional analysis, we demonstrate that splenic $CD45^+Col^+$ fibrocyte-like cells can mediate antimicrobial activity by formation of antimicrobial extracellular traps and contribute to adaptive immunity via antigen presentation to naïve T

cells. Thus, splenic $CD45^+Col^+$ cells represent a rapidly mobilized component of the immune response to injury, inflammation, or bacterial infection.

Differentiation of $CD45^+Col^+$ fibrocyte-like cells in different microenvironments We have observed that fibrogenic liver injury caused migration of $CD45^+Col^+$ fibrocyte-like cells to lymphoid organs (spleen) in addition to the liver. Similar observations have been made in renal fibrosis [8]. These data are corroborated by the fact that fibrocytes can be outgrown from Gr-1^+ splenic monocytes [32]. It has been proposed that fibrocytes differentiate according to their microenvironment, giving rise to different subtypes of fibrocytes, related to each other but playing distinct roles during tissue repair and

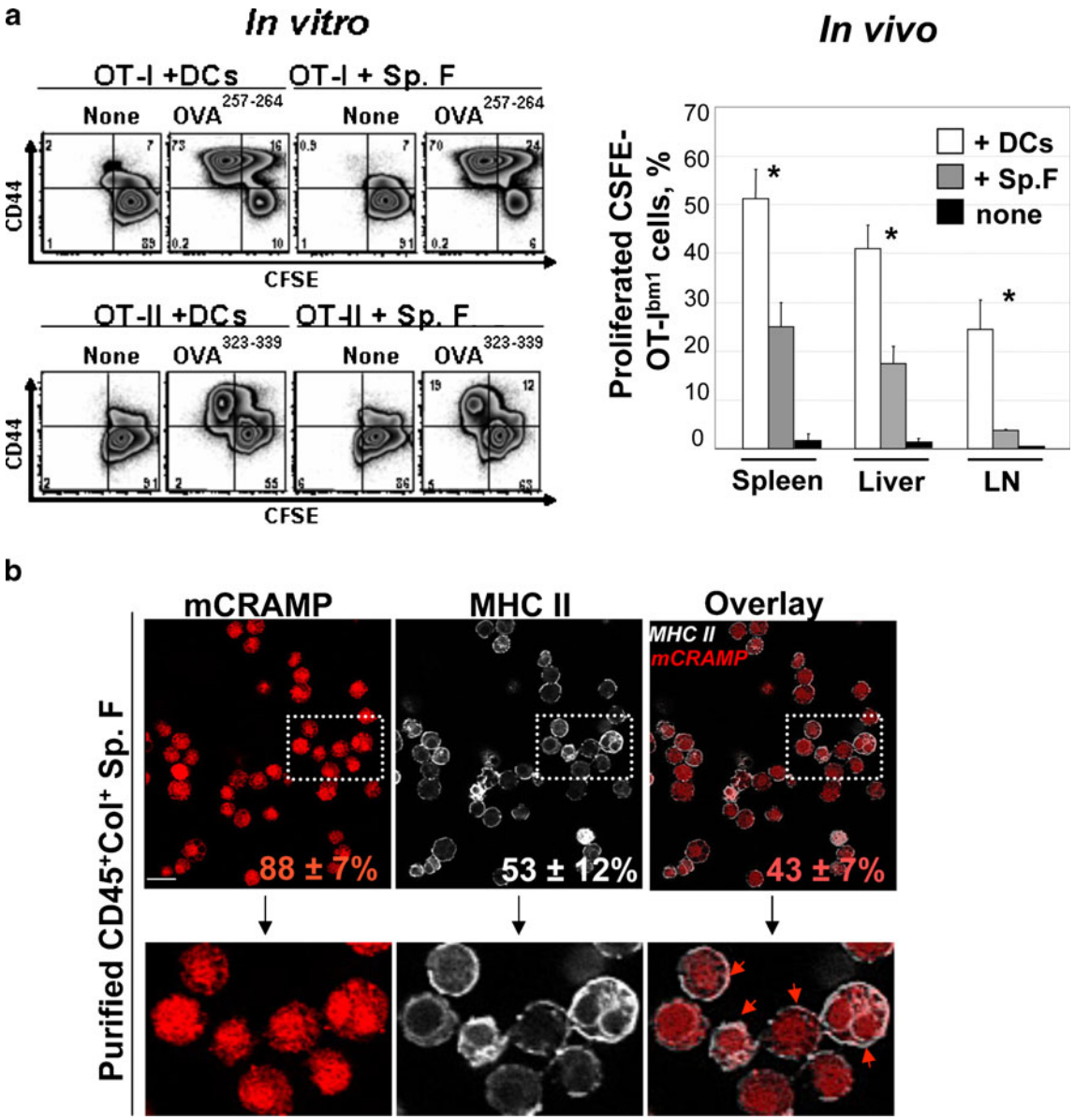


Fig. 6 Alternative functions of splenic $CD45^+Col^+$ fibrocyte-like cells. **a** Splenic $CD45^+Col^+$ fibrocyte-like cells possess properties of antigen presenting (APC) cells. Splenic fibrocyte-like cells present antigens on MHC I (H2-K^b) and MHCII (I-Ab) in vitro, *left panel.* DCs and splenic $CD45^+Col^+$ cells (5×10^4 cells) untreated or loaded with $OVA^{257–264}$ or $OVA^{323–339}$ are co-cultured for 4 days with CFSE-labeled $CD8^+$ OT-I or $CD4^+$ OT-II T cells (1.5×10^5), respectively. Proliferation is measured by CFSE dilution in activated $CD44^+$ T cells. Representative *dot plots* are shown. Splenic $CD45^+Col^+$ cells induce proliferation of adoptively transferred CFSE-OT-I/bm1 $CD8^+$ T cells in Act-mOVA/bm1 mice, *right panel*. Proliferation of CFSE-labeled T cells in the liver, spleen, and peripheral lymph nodes (LN) was analyzed 4 days later by flow cytometry. Data represent mean± SEM. **b** A subset of splenic $CD45^+Col^+$ cells co-express MHC II and mCRAMP. Purified from spleens LPS-treated mice, $CD45^+Col^+$ cells are co-stained with anti-mCRAMP and anti-MHC II antibodies. *Upper panel*, co-localization of MHC II (shown in *white*) and mCRAMP (*red*) is detected in 43±7% of cells. *Bar* represents 30 μm. *Lower panel*, representative images are magnified five times; double positive cells are marked with *arrows*

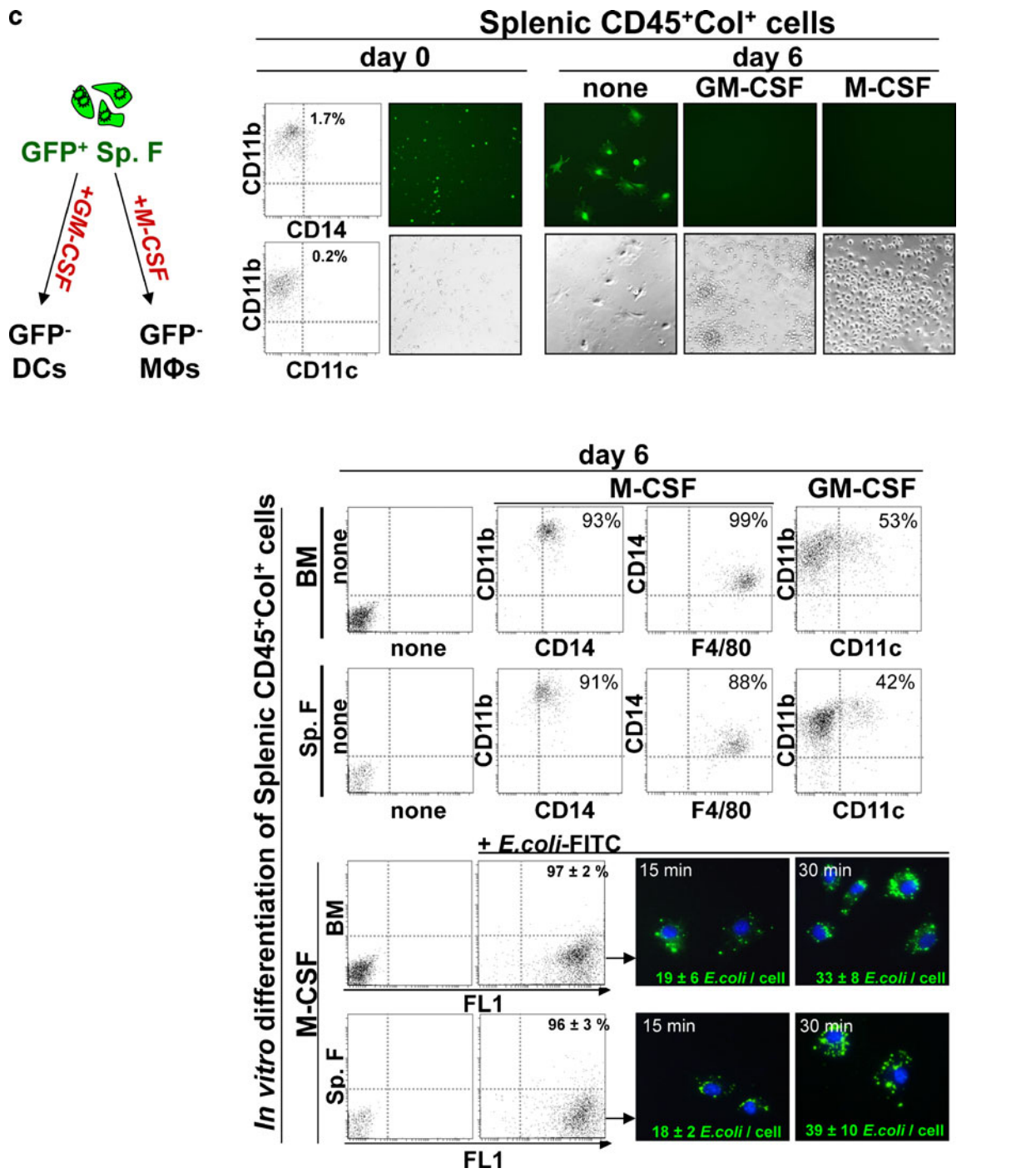


Fig. 6 **c** Splenic $CD45^+Col^+$ cells differentiate into myeloid ($CD11b^+CD14^+F4/80^+$) or dendritic ($CD11b^+CD11c^+$) cells in response to M-CSF (30% L-cell medium) or GM-CSF (20 ng/ml), respectively, detected by flow cytometry, *upper panel*. *Lower panel*, phagocytosis of FITC-labeled *Escherichia coli* by M-CSF-differentiated splenic $CD45^+Col^+$ cells or BM-derived macrophages. The number of phagocytosed *E. coli*-FITC particles increases over time. Representative images of three independent experiments are shown

fibrosis [9]. Thus, fibrocytes cultured in serum-free conditions are implicated in immune response and tissue inflammation, in contrast to serum-cultured fibrocytes [9]. Our findings indicate that recruitment of $CD45^+Col^+$ fibrocyte-like cells is not limited to the damaged (fibrotic) organ and provides a broader linkage of fibrocyte-like cells to immune response. Thus, dependent on the biological niche, $CD45^+Col^+$ fibrocyte-like cells may acquire specific features.

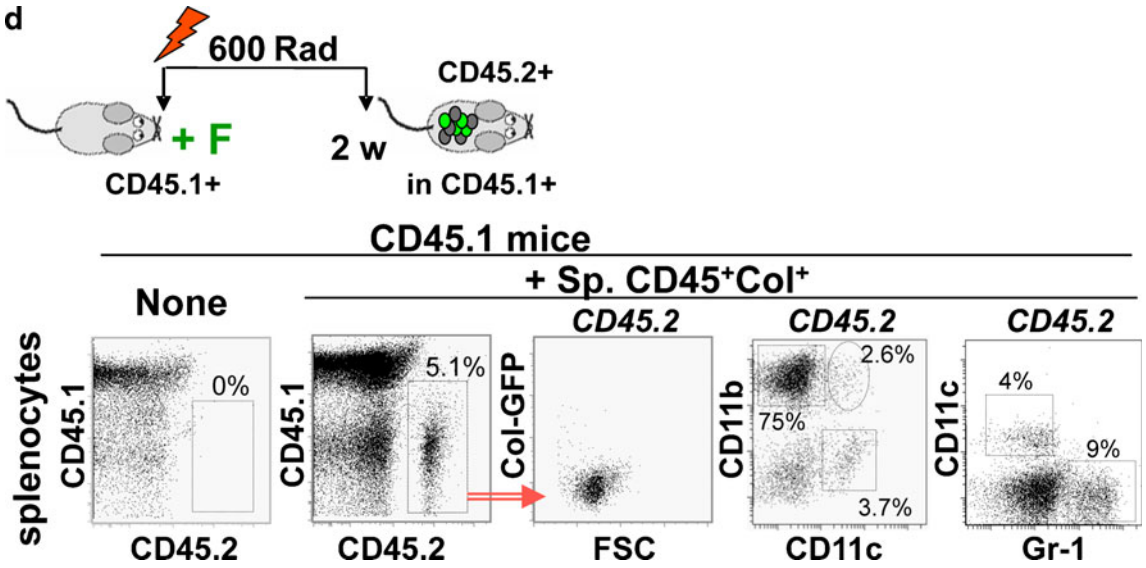


Fig. 6 **d** Splenic fibrocytes give rise to CD11b$^+$ (76%), CD11b$^+$CD11c$^+$ (2.6%), CD11b$^-$CD11c$^+$ (3.7%), and Gr-1$^+$ (9%) cells, when transferred into sublethally irradiated (600 Rad) CD45.1$^+$ mice (n=10). Fibrocyte differentiation is accompanied by the loss of collagen-GFP expression. *Dot blot* analysis: representative images of five experiments are shown

Splenic CD45$^+$Col$^+$ fibrocyte-like cells provide immune defenses Fibrogenic liver injury, LPS, or infection with *L. monocytogenes* triggers migration of CD45$^+$Col$^+$ cells to the spleen and liver. In response to *L. monocytogenes*, splenic CD45$^+$Col$^+$ fibrocyte-like cells are detected only in the infected spleen and liver, indicating a more specific role in innate immunity. Although splenic CD45$^+$Col$^+$ fibrocyte-like cells lack phagocytic activity, they have developed several mechanisms to combat infection. First, splenic fibrocyte-like cells can further differentiate into mature macrophages, which in turn mediate bacterial clearance. Second, splenic fibrocyte-like cells themselves may confine bacterial spread and destroy microbial pathogens locally, at the site of infection, by direct inhibition of bacterial viability. A variety of antimicrobial peptides detected in splenic fibrocyte-like cells is typically produced by myeloid or other immune cells ([22, 27, 33–35] and Supplementary Reference 2). Some of them, such as lysozymes, complement 3, and myeloperoxidase, kill bacteria directly upon contact [36] or make conductive pores in bacterial membranes (such as cathelicidin and α-defensins) [22]. Also, splenic CD45$^+$Col$^+$ fibrocyte-like cells may mediate their immediate bactericidal activity by formation of extracellular traps, a unique mechanism previously identified only in neutrophils, eosinophils, mast cells, and macrophages [24–27]. Here, we show that, like macrophages, neutrophils, and mast cells, splenic CD45$^+$Col$^+$ cells release cathelicidin into the DNA-based framework to aid in bacterial killing. Similarly, formation of extracellular traps is accompanied by death of splenic CD45$^+$Col$^+$ fibrocyte-like cells, suggesting that release of nuclear (versus mitochondrial) DNA mediates this process. Third, fibrocyte-like cells may facilitate adaptive immunity by presenting antigens to naïve T cells [10, 11]. They express MHC I and upregulate MHC II upon migration to the spleen and are capable of antigen presentation via MHC II and I in vivo and in vitro, at levels comparable to DCs.

Splenic CD45$^+$Col$^+$ fibrocyte-like cells differ from macrophages Antimicrobial functions of effector cells are well documented [28, 37]. Despite a similarity in the expression of surface markers (CD11b, Gr-1, and F4/80), the presence of cytotoxic granules, and the ability to form chromatin-rich extracellular structures, splenic fibrocyte-like cells have characteristics that are different from neutrophils and macrophages. Unlike neutrophils, CD45$^+$Col$^+$ cells egress the BM only in response to injury or stress and have a short transit time in the bloodstream. Splenic fibrocyte-like cells lack phagocytic activity and maturation markers (CD68 and CD11c), but under appropriate stimuli can further differentiate into mature macrophages or DCs. Based on these characteristics, splenic CD45$^+$Col$^+$ fibrocyte-like cells may contribute to immune defense by supplying tissues with precursors of macrophages, dendritic cells, and/or effector cells with distinct antimicrobial activities [31]. Supporting this notion is the recent report linking tissue fibrocytes and circulating monocytes [38].

Cytoskeletal proteins and adhesion molecules may facilitate migration of fibrocyte-like cells It is unclear why splenic CD45$^+$Col$^+$ fibrocyte-like cells express collagen Type I and whether this expression is related to their function in acute immune responses. It has been suggested that hematopoietic cells develop specific machinery to migrate through the bloodstream. This includes expression of different types of

intracellular or secreted matrix proteins, which together with adhesion molecules facilitate transmigration of circulating cells [39]. Thus, interaction with the ECM and adhesion is likely mediated in splenic fibrocyte-like cells by S100A9/A8, β-integrins, proteoglycan 2, and mannose receptors [21, 40]. For example, S100A9/A8 proteins (Calprotectins) mediate chemotaxis and transendothelial migration of myeloid cells to the site of inflammation, and restrict bacterial growth through metal chelation [41]. On the other hand, β-integrins and mannose receptors, which represent distinct families of collagen-receptors, contribute to cellular binding and migration through the collagen-rich stroma ([21] and Supplementary Reference 1). We speculate that expression of collagen Type I and other collagens may facilitate migration and cytoprotection of splenic $CD45^{+}Col^{+}$ fibrocyte-like cells. In support of this concept, expression of matrix proteins, including collagens, has been reported in circulating monocytes and macrophages and appear to modulate cell–cell and cell–matrix interactions [42]. In addition, we demonstrated that splenic $CD45^{+}Col^{+}$ cells release collagen Type I into extracellular traps, suggesting that collagen may facilitate trap formation by providing a scaffold-like support.

In conclusion, here, we characterized a rapidly responding population of $CD45^{+}Col^{+}$ fibrocyte-like cells in the spleen that can participate in immediate innate immune defense, stimulate adaptive immunity as APCs, and regulate inflammatory responses in situ by terminal differentiation into macrophages or dendritic cells.

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