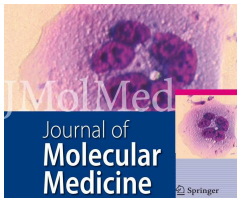

Supplemental Materials for:

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

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Supplemental Methods

Mice:

C57BL/6 (CD45.2 Ly5.2) mice and congenic CD45.1 Ly5.1 (#2014, referred to as "SJL"), β-actin-RFP (#6051) were purchased from The Jackson Laboratory (Bar Harbor, Main). The following mouse strains have been described previously: T cell receptor transgenic animals specific for peptides derived from chicken ovalbumin presented by H2-K^b ($OVA^{257-264}$, Vα2/β5, #3831, referred to as OT-I) (Hogquist et al., 1994) or by I-Ab ($OVA^{323-339}$, Vα2/β5, #4194, referred to as OT-II) [1]; transgenic animals expressing membrane bound chicken ovalbumin under the actin promotor (#5145, Act-mOVA) [2]; animals bearing point mutations in H2-K^b which abrogates the presentation of $OVA^{257-264}$ (H2-K^{bm1}, #1060, referred to as bm1)[3]. OT-I mice were crossed with bm1 and SJL mice (OTI/bm1) and Act-mOVA mice were crossed with the bm1 mouse strain (Act-mOVA/bm1). Mice from both intercrosses were kindly provided by Dr. Schoenberger (La Jolla, CA, USA). Mice were maintained under SPF conditions at the animal facilities of UCSD (San Diego, CA, USA) and LIAI (La Jolla, CA, USA).

Whole mouse genome expression microarray:

The gene expression profile of $CD45^+Col^+$ cells was studied using Whole Mouse Genome Microarray (Agilent). Splenic $CD45^+Col^+$ cells were isolated from spleens of LPS-treated (6 µg/g x 3 injections) Col-GFP mice. B-1 cells ($CD5^+CD19^+$) were sorted from peritoneal lavage of C57BL/6 mice. Peritoneal macrophages were isolated from thioglycollate-stimulated C57BL/6 mice, activated for 6h with Kdo_2-Lipid A (100 ng/ml, activated macrophages, aMΦ), or left intact (quiescent macrophages, qMΦ). Total RNA from each sample was isolated using RNAeasy columns (Qiagen, Valencia, CA), 160 ng of purified RNA per sample was labeled using the LRILAK PLUS, two color low RNA input Linear Amplification kit and hybridized to a Whole Mouse Genome Microarray 4x44K 60 mer slide according to the manufacturer's instructions (Agilent, Santa Clara, CA). Slides were scanned using the Agilent GZ505B Scanner and analyzed using the Gene Spring Software (Agilent).

Quantitative RT-PCR:

Total RNA was isolated from cells using RNeasy columns (Qiagen, Valencia, CA). First strand cDNA was synthesized using SuperScript III and random hexamers (Invitrogen, Carlsbad, CA). Samples were run in 20 ul reactions using an AB1 7300 (Applied Biosystems, Foster City, CA). SYBR Green oligonucleotides were used for detection and quantification of genes. Sequence-specific primers are listed below. Gene expression levels were calculated after normalization to the standard housekeeping gene GAPDH using the ΔΔ CT method as described by the manufacturer (Invitrogen, Carlsbad, CA), and expressed as relative mRNA levels compared with control. The results are represented as average ± SEM, $p<0.0001$

Primers for RT-PCR

Gene/Primers	Forward	Reverse
CD34	AAGGCTGGGTGAAGACCCTTA	AAGGCTGGGTGAAGACCCTTA
mCramp	GCTGTGGCGGTCACTATCAC	TGTCTAGGGACTGCTGGTTGA
Chi3I3	AGAAGGGAGTTTCAAACCTGGT	GTCTTGCTCATGTGTGTAAGTGA
Collagen α1(I)	ACATGTTCAGCTTTGTGGACC	TAGGCCATTGTGTATGCAGC
Carbohydrate sulfotransferase 1	ATGCAATGTTCTTGGAAGGCT	CTCCTCACACAACCGCTCT
Gapdh	AATGTGTCCGTCGT	CATCGAAGGTGGAAGAGTGG

Gr-1	GCAGTGCTACGAGTGCTATGG	ACTGACGGGTCTTTAGTTTCCTT
Haptoglobin	GCTATGTGGAGCACTTGGTTC	CACCCATTGCTTCTCGTCGTT
IL-18r1	ACTTTTGCTGTGGAGACGTTAC	CCGGCTTTTCTCTATCAGTGAAT
Lactotransferrin	TGAGGCCCTTGGACTCTGT	ACCCACTTTTCTCATCTCGTTC
Myeloperoxidase	AGTTGTGCTGAGCTGTATGGA	CGGCTGCTTGAAGTAAAACAGG
Ngp	AGACCTTTGTATTGGTGGTGGC	GGTTGTATGCCTCTATGGCTCTA
Perforin	GCTCCCACTCCAAGGTAGC	TTTGTACCAGGCGAAAACTGT
Proteoglycan 2	TGAAACTTCTGACTCCAAAAGCC	CGGCATTAGCTCTTCCCCT
S100a9	TTACTTCCCACAGCCTTTGC	AGGACCTGGACACAAACCAG
Vcam 1	CCATTGAAGATACCGGGAAAT	TAGCTGTCTGCTCCACAGGAT

Visualization of the extracellular DNA traps. Splenic $CD45^+Col^+$ cells (2 x 10^5 cells) were seeded on Poly-l-lysine (Sigma) coated glass cover slides (8 mm diameter, 1.5 thick, Electron Microscopy Science), infected with *L. monocytogenes* (MOI 1:0.1 cell/bacteria), centrifuged for 5 min at 300g, incubated for additional 40 min at 37°C and 5% CO_2, fixed in 4% PFA, then washed 3 times with PBS, blocked with PBS + 3% BSA and stained with rabbit anti-cathelicidin antimicrobial peptide [4], followed by secondary goat anti rabbit Alexa fluor 568 (Invitrogen). Slides were embedded in ProlongGold antifade + Dapi (Molecular Probes) to visualize the DNA traps. To visualize live/dead bacteria, splenic $CD45^+Col^+$ cells (2 x 10^5 cells) were co-cultured with bacteria (MOI 1:2 cell/bacteria) for 40 min, and analyzed by Live/dead BacLight Bacterial Viability Kit (Molecular Probes) following the recommendations of the manufacturer. After staining, cells were washed 3 times with PBS, fixed with 1% paraformadehyde for 5 min, washed again and mounted onto glass slides using Prolong Gold Dapi. Mounted samples were examined using an inverted confocal laser-scanning 2-photon microscope Olympus Fluoview FV1000 using a 60x/1.42 PlanApo oil objective at calibrated magnifications and Fluoview™ Spectral Scanning technology (Olympus).

Flow cytometry. Antibodies used in this study are as follow: CD3-PE, CD8α-FITC, CD4-FITC, CD11b-PE, CD11c-APC, CD19-APC, Ter119-PE, B220-PE, B220-PE-Cy7.5, IgM-PE, IgD-APC, CD14-FITC, F4/80-APC, c-kit-PE-Cy7, Sca-1-PerCp-Cy5.5, IL-1-PE, IL-4-PE, IL-5-PE, IL-12-APC, TNF-α, IFN-γ (eBioscience, San Diego, CA). Intracellular staining was performed using BD Cytofix/Cytoperm fixation and Permeabilization Solution (BD, San Jose, CA).

SUPPLEMENTAL REFERENCES:

Supplemental References 1 (Transmigration)
[5-10]
Supplemental References 2 (Anti-microbial defense)
[11-22]
Supplemental References 3 (Antigen presentation)
[23-36]

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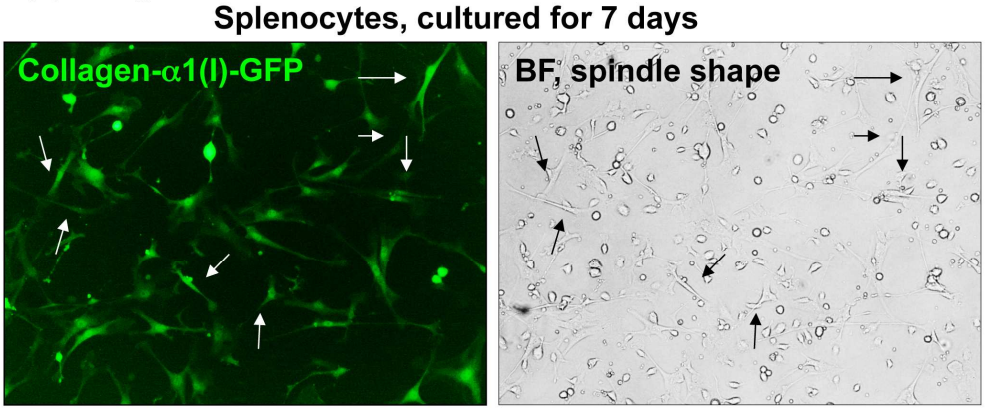
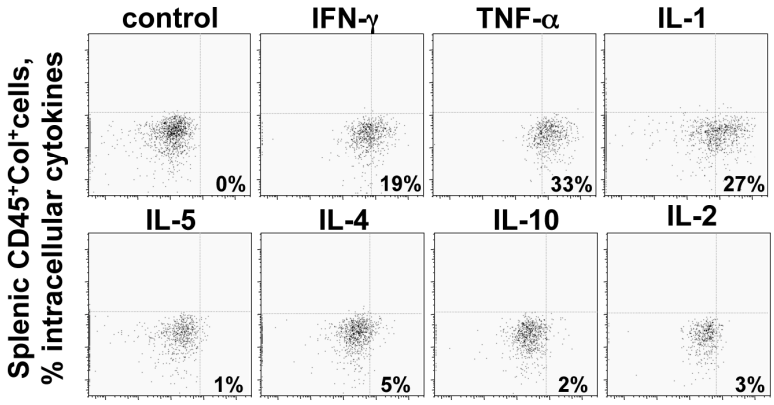
SUPPLEMENTAL FIGURES

Suppl. Figure S1



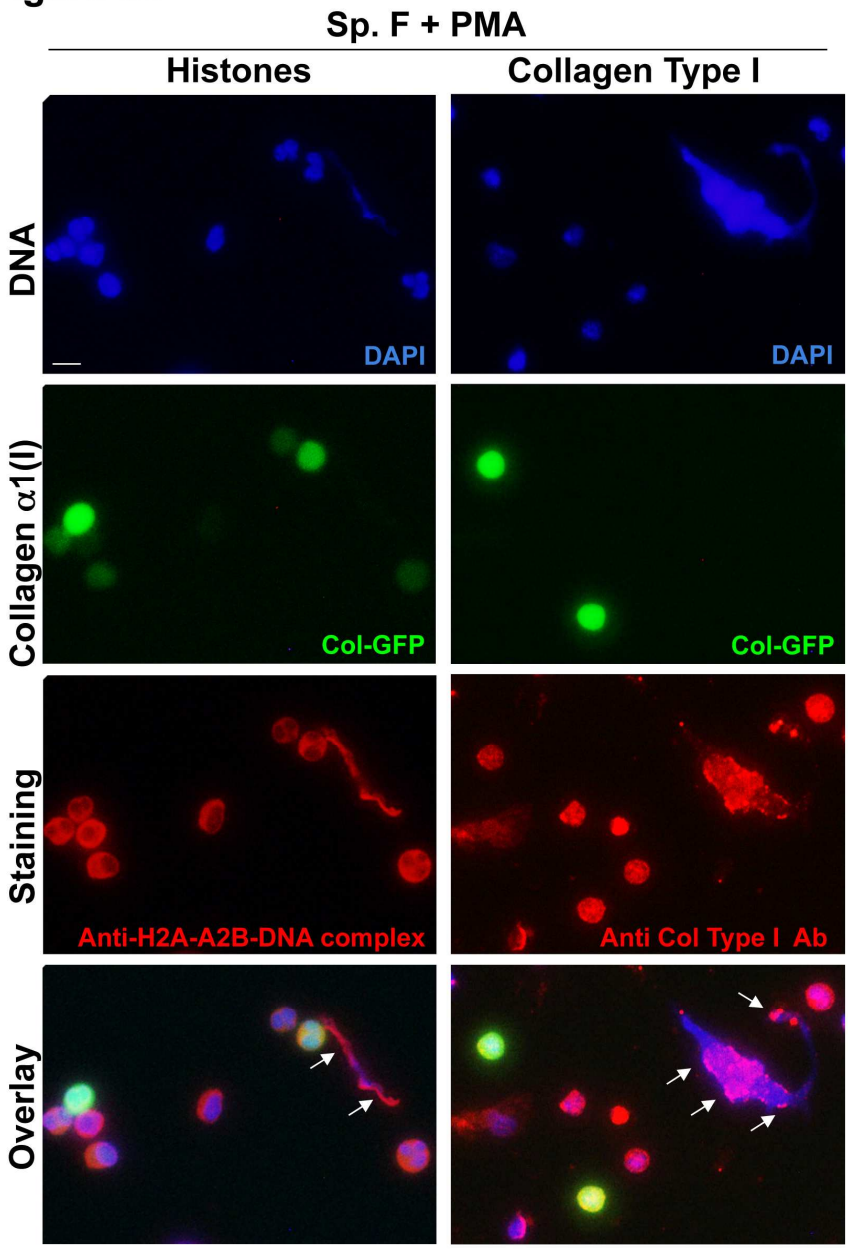
Supplemental Figure S1. Splenic CD45$^+$Col$^+$ cells give rise to fibrocytes *in vitro*.
Splenic CD45$^+$Col$^+$ cells from CCl_4-treated Col-into-wt mice were cultured on plastic in RPMI + 10% FCS for 7 days, and gave rise to spindle-shaped Col$^+$ fibrocytes. Representative images of three independent experiments are shown.

Suppl. Figure S2



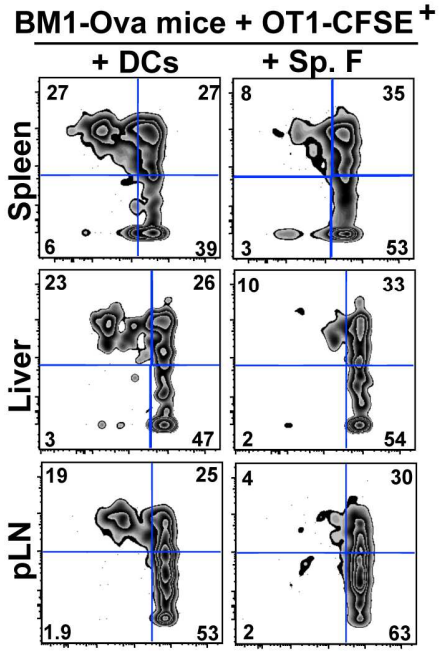
Supplemental Figure S2. Phenotyping of splenic CD45$^+$Col$^+$.
Flow cytometry analysis of splenic CD45$^+$Col$^+$ cells of LPS-treated Col-GFP mice. Dot plot analysis of intracellular cytokines expression revealed that splenic CD45$^+$Col$^+$ upregulated Th1 type of cytokines IFN-γ (19%), TNF-α (33%) and IL-1 (27%). The representative images of three different experiments are shown.

Suppl. Figure S3



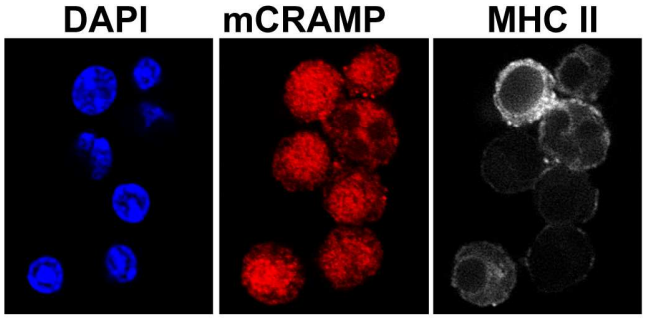
Supplemental Figure S3. Release of antimicrobial extracellular DNA-traps by PMA-treated splenic CD45$^+$Col$^+$ cells. CD45$^+$Col$^+$ cells co-incubated with PMA (25 nM/ml) for 45 min, are stained with H2A-H2B-DNA complex and anti-Collagen Type I antibodies, visualized in red (Alexa fluor 568). DNA traps are visualized in blue (Dapi), fibrocytes-like cells are visualized in green (GFP). Histone-DNA or Collagen Type I-DNA complexes are shown with arrows. Bar represents 20 µm.

Suppl. Figure S4



Supplemental Figure S4. Splenic CD45$^+$Col$^+$ can act as antigen presenting cells. Splenic CD45$^+$Col$^+$ induced proliferation of adoptively transferred CFSE-OT-I/bm1 CD8$^+$ T cells in Act-mOVA/bm1 mice. Proliferation of CFSE-labeled T cells in the liver, spleen and peripheral lymph nodes (LN) was analyzed four days later by flow cytometry. Proliferation of CD8$^+$ OT-I/bm1 cells was evaluated by flow cytometry of CFSE dilution in activated CD44$^+$ T cells. Data is shown as scatter blot analysis.

Suppl. Figure S5



Supplemental Figure S5. 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, and Dapi to visualize nuclei. Co-localization of MHC II (shown in white) and mCRAMP (red) are detected in 43 ± 7% of cells. Bar represents 30 µm.

Supplementary Table 1. Expression of lineage specific markers by splenic fibrocytes.

CHARACTERISTICS OF		Fold	qMΦ	aMΦ	Sp. F
PRECURSOR CELLS					
	CD34	↑ 4	194	279	1111
	CD90 (Thy-1)	↑ 21	384	80	8362
	CD11b		6946	5568	3752
	Gr-1 (Ly6-c)		808	3799	104007
MYELO-GRANULOCYTIC CELLS					
pre-myeloid	GM-CSF2Rα		83405	62347	39336
	GM-CSF2Rβ		31562	24544	4792
	G-CSF3-R		1647	2079	1397
	CD115 (M-CSFR)		ND	5234	3012
	Ly6G		103	195	152
	S100A9	↑1822	294	62	535679
	S100A8	↑ 176	200	495	35240
	CD16 (FcgRIII)		45015	14243	5737
	Myeloperoxidase (Mpo)	↑ 318	116	90	36910
	CD11b	↑ 3.4	501	ND	7753
	Lysozyme (Lyzs)		175542	114205	364293
	Complement 3		1976	32305	30980
	Complement factor properdin (Cfp)		70709	10799	12636
	LPS binding protein (Lbp)		425	232	572
	Treml4	↑ 16	129	146	3497
	S100A1α		4650	289507	589
	S100A4 (FSP-1)		129914	23891	64321
	CD33		734	769	283
	Myd116		795	2376	2983
	Myd88		7291	22298	7011
mature MΦ	CD68		64924	41904	2958
	CD14		11422	32145	7279
	F4/80		41450	61230	8235
	MHC II		500	8756	7752
	CD163		801	58	623
	CD300ε	↑ 3.2	97	444	1430
	Scarb 1		2921	1290	2030
	Msr 1		2781	9813	682
	Msr 2		404	177	220
	Spp1 secreted phosphoprotein		171301	121114	17606
	PI3K 5		25339	14812	11639
	TREM2		107077	69314	7364
	IFN-γ	↑4	77	556	696
	Lactotransferrin	↑195	195	71	36315
	Haptoglobin	↑22	1596	1955	36502
	MΦ expressed gene 1 (Mpeg1)		160405	142407	26035
	Marco		5120	8952	2986
DENDRITIC CELLS					
	CD80		150	598.3	215
	CD83		6793	26213	6894
	CD86		265	1427	410

	CD32 (FcgRII)		15866.9	9882	8616.4
	MHCII Q region (H2-Q8)		500	8756	7752
	Histocompatibility 13 (H13)	↑ 5.5	1541	64	8477
	Histocompatibility (H2-K1)		137851	ND	204021
	CD11c		2147	3319	2410
	CD11b		6946	5568	3752
CYTOTOXIC CELLS					
	CD226		82	60	109
	Granzyme A	↑ 15	81	60	559
	Granzyme B	↑ 4	77	113	459
	Cathepsin W	↑ 14	196	77	2823
	Cathepsin G	↑ 79	158	84	12504
	Perforin	↑ 2	80	61	446
	Proteoglycan 2 (PRG2)	↑ 40	116	80	12168
	MHC II		500	8756	7752
	Killer cell lectin receptor	↑ 23			
	Klra7		223	68	5250
	Klra23	↑ 17	130	61	2176
	Klrd1 (CD94)	↑ 13	261	56	3330
	Leukotreine B4 receptor 1 (Ltb4r1)	↑ 10	471	159	4460
	NK cell group 7 sequence	↑ 16	123	68	1988
	CD244 NK cell receptor 2B4	↑ 2.7	650	290	1757
	CD247	↑ 6	136	83	841
	CD56 (NCAM)	↑ 2.3	77	299	696
	CD69		90	82.7	116
	CD94	↑ 1.2	105	ND	495
	CD16		45015	14243	5737
	CD44		8073	11522	4171
	CD1d		585	1564	5018
	Il-4		100	80	129
	IL-12Rβ1		106	626	492
	IL-18R1	↑ 7.5	138	59	1040
	IL18		678	6738	731
MYOFIBROBLASTS					
	TGFβ induced		8255	13516	17603
	TGF-β1		2711	2718	1020
	TGFβ-RI		3700	2009	1178
	TGF-βR II		11234	2159	5702
	HGF		104	102	73
	PDGFα		12098	2099	1074
	PDGF assoc. protein (Pdap1)		8618	9967	10238
	Smad3		237	156	797
	Smad2		974	1459	572
	Smad4		8955	4190	6030
	Ltbp-2		95	1334	153
	Ltbp-3		5678	3950	1016
	Vimentin		94906	62098	30278
	α-smooth muscle actin		159	2535	206
	Collagen 7α1	↑ 1.5	326	136	995
	Collagen 11α2	↑ 1.4	159	97	482
	Collagen 14α1		84	164	154
	Collagen 4α4		437	852	1264
	Collagen1α1	↑ 1.6	120	58	201
	Cytoplasmic β-actin	↑ 3.7	1616	ND	6952

	Tubulin β3	↑ 2	565	ND	1636
	Tubulin β5		23261	ND	32335
NEUTROPHILS					
	Neutrophil elastase 2 (Ela2)	↑5.3	61	66	403
	Ngp	↑90	94	82	8494
	CD11a		316	8959	10559
	CD16b		45015	14243	5737
	CD24a		4103	1476	16251
	CD32		15866	9882	8616
	CD43		1502	550	17885
	CD66		435	256	617
	CD88		298	292	130
	CD114		1647	2079	1397
	CD116		83405	62347	39336
	CD123		6110	5513	3277
	CD128a		10633	3733	3510
	CD147		1419	1631	896
	CD156		262	128	153
	CD170		169	ND	116
	CD177		102	62	1044

Supplementary Table 1. Expression of lineage specific markers by splenic fibrocytes.

Gene expression microarray of the whole mouse genome was performed to assess function of splenic fibrocytes. Fibrocyte gene expression was compared with expression profiles of quiescent and activated macrophages (qMΦ and aMΦ). The data is presented for five cell types and grouped according to expression of lineage specific genes, characteristic for 1) precursor cells, 2) myelo-monocytic cells, 3) dendritic cells, 4) cytotoxic cells; 5) myofibroblasts, 6) neutrophils. Genes with highest expression in splenic fibrocytes and their fold induction are indicated by pink field.

Supplemental Table 2. Functional properties of splenic fibrocytes.

FUNCTION	GENE	Fold	qMΦ	aMΦ	E cell
TRANSMIGRATION					
S100 proteins	S100A8	↑ 176	200	495	35240
	S100A9	↑1822	294	62	535679
	CCR1		2573.1	ND	3946.6
	CCR2	↑ 2	930	111	1910
	CCR5		3844.5	7369.7	1124.3
	CCR6	↑ 7.3	239	111	1759.8
	CCR7	↑ 7.7	291.7	169	2269
	CXCR3	↑ 22	227.2	92	5135.9
	CXCR4		2224.8	144	3881.5
Adhesion	ICAM-1 (CD54)		1437	19146	7059
	ICAM-2		3398	747	7552
	CD11b		6946	5568	3752
	αL-integrin		316	3199	10559
	β2-integrin like	↑ 15	68	53	1118
	β2-integrin		35242	41041	19215
	β3-integrin		705	1956	1013
	VCAM-1		96	5730	2085
Ca^{2+}channel	Sununit α1 (Cacnα 1s)	↑ 21	144	ND	6913
	Subunit β3 (Cacnβ 3)	↑ 2.6	106	336	892
	Reticulocalbin EF-hand Ca2+ binding protein (Rcn 3)		685	627	1734
Receptors	Proteoglycan 2 (Prg2)	↑ 4.2	58	80	12552
	Mannose receptor, C type 1 (Mrc1)		4329	1846	1267
	RAGE		216	87	157
	CD36		21934	26545	1537
IRON METABOLISM					
	Haptoglobin	↑ 22	1596	1966	36502
	CD163		828	58	641
	Hemoglobin Hbβ-β1	↑ 3	115	4822	87826
	Hbα-α1	↑ 1.7	171	97	29524
	Iron transporter Slc40a1		266	388	2746
	Lactotransferrin	↑ 186	195	71	36315
	Transferrin		13382	5958	2785
	Transferrin R 2	↑ 3	79	63	436
	Ferritin	↑ 2.4	124	120	405
	Mitochondrial Heme binding protein (Hebp1)		5707	2073	3013

	Heme oxygenase (decycling) 1 (Hmox1)		25622	15741	8375
ANTIMICROBIAL RESPONSE					
neutrophils	Lactotransferrin	↑ 186	195	71	36315
	LRP3	↑ 2	67	68	156
	Ngp	↑ 90	94	82	8494
	Neutrophil elastase 2 (Ela2)	↑ 5.3	61	66	403
MΦ	Lysosymes (Lyzm)		175542	114205	364293
	Complement 3		2234	35172	33982
	Myeloperoxidase (Mpo)	↑318	116	90	36910
+ mDCs	Chitinase 3-like 3(Chi3l3)	↑ 11	14780	4925	164447
	Chi3l4	↑ 10	999	427	9558
+ NK	Lectin, mannose binding 1 (Lman1)		6055	6318	14822
	Mannose receptor, C type 1 (Mrc1)		4329	1846	1267
	Lipocalin 2	↑ 8	67	247	2046
	Spectrin β4 (Spn-β4)	↑ 3	666	1385	47392
Peneth cells	Defcr3	↑ 5	3201	8157	114157
	Cathelicidin antimicrobial peptide (Camp)	↑ 175	61	75	13159
CYTOTOXICITY					
Enzymes	Perforin	↑ 2	80	81	446
	Granzyme A	↑ 7	81	56	599
	Granzyme B		139092	85234	39549
	Cathepsin G		158	84	12504
	Serglycin (Srgn)		39766	ND	44186
lysosomal	CD107a (Lamp-1)		121962	52232	10816
proteins	CD107b (Lamp-2)		34989	15883	6406
	CD208 (Lamp-3)		360.1	269	881.1
	CD81		4927	1959	27413
	CD9		42839	37592	13661
	* Secretory leukocyte peptidase inhibitor (Slpi)	↑ 9	49055	17182	453720
	* Serine/cysteine peptidase inhibitor (Serpina 3F)		80	10874	1994

Supplementary Table 2. Functional properties of splenic fibrocytes.

Gene expression microarray of the whole mouse genome was performed to assess function of splenic fibrocytes. Fibrocyte gene expression was compared with expression profiles of quiescent and activated macrophages (qMΦ and aMΦ). The data is presented for three types of cells and grouped according to their functional properties consisting of: 1) transmigration, 2) iron metabolism, 3) antimicrobial response, 4) cytotoxicity. Genes with highest expression in splenic fibrocytes and their fold induction are highlighted in grey field.