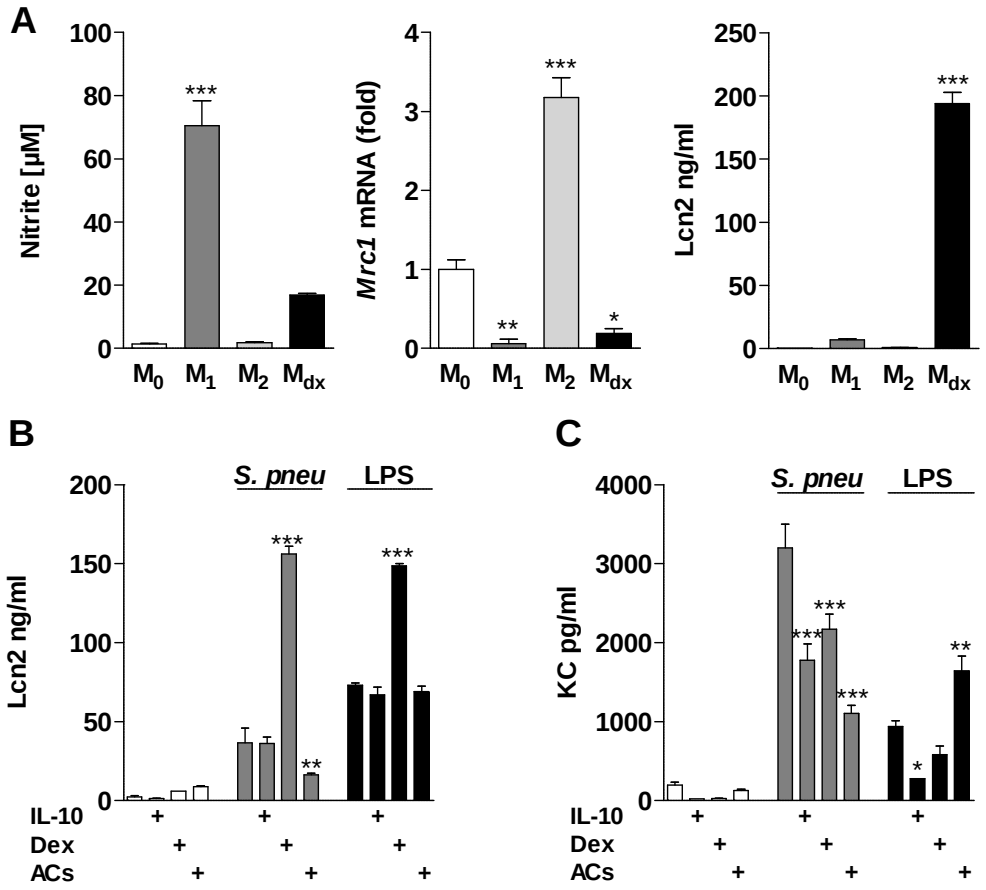
Supplemental Information

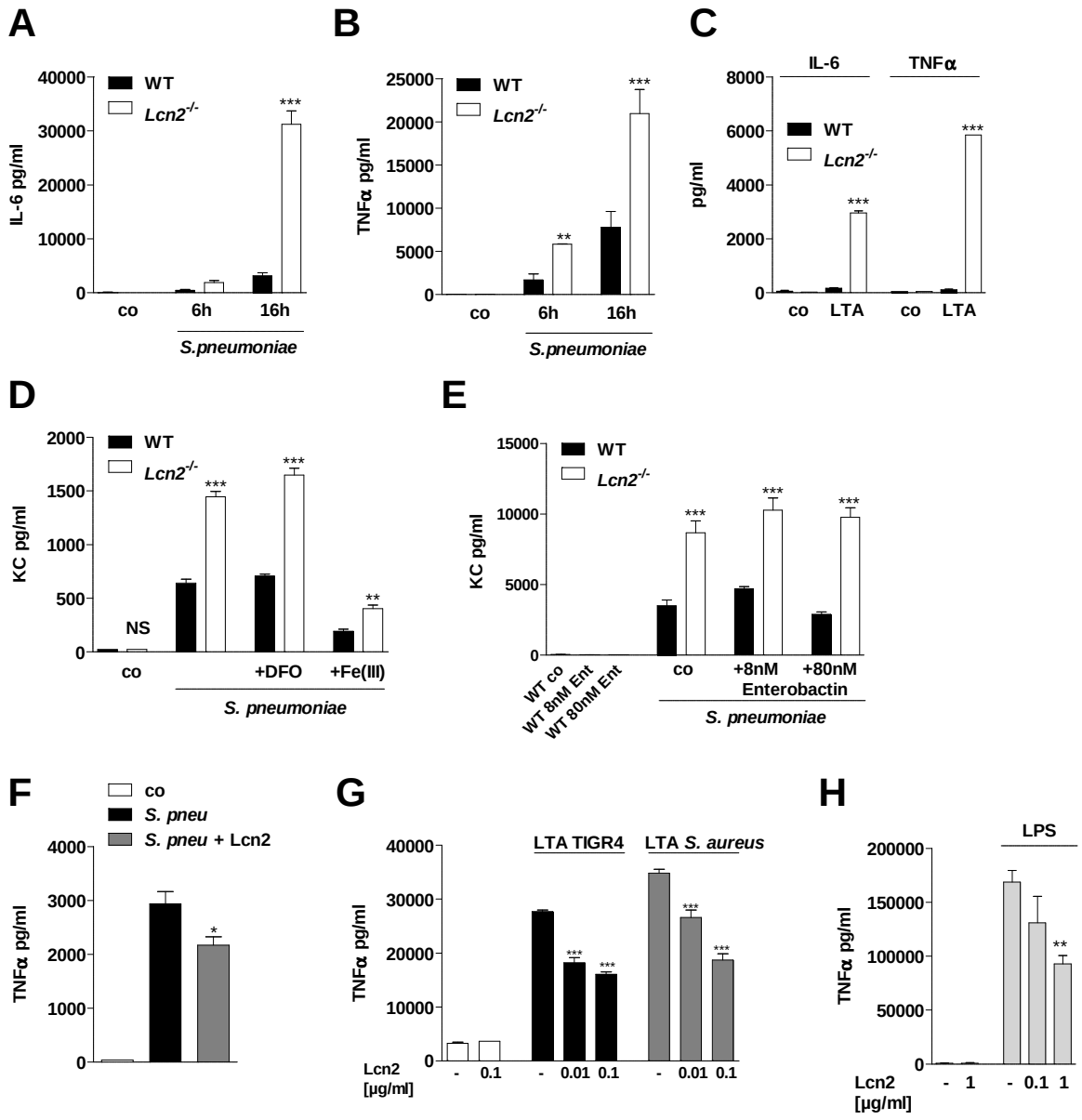
Lipocalin-2 deactivates macrophages and worsens pneumococcal pneumonia outcomes

Joanna M. Warszawska[1,2], Riem Gawish[1,2], Omar Sharif[1,2], Stefanie Sigel[1,2], Bianca Doninger[1,2], Karin Lakovits[2], Ildiko Mesteri[3], Manfred Nairz[4], Louis Boon[5], Alexander Spiel[6], Valentin Fuhrmann[6], Birgit Strobl[7], Mathias Müller[7], Peter Schenk[6], Günter Weiss[4], Sylvia Knapp[1,2]



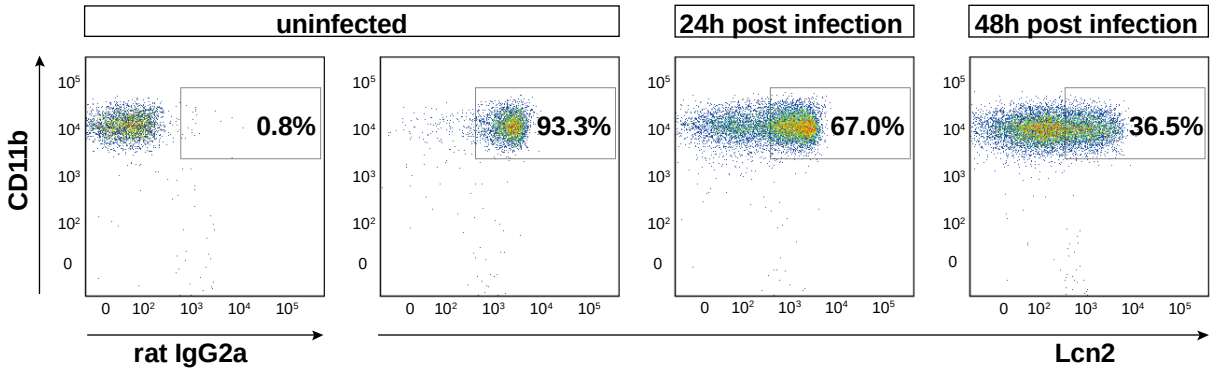
Supplemental Figure 1

Lcn2 is highly expressed in deactivated macrophages. (**A**) MH-S cells were polarized with M_1 (100ng/ml LPS, 200U/ml IFNγ), M_2 (10ng/ml IL-13, 10ng/ml IL-4) or deactivating (M_{dx}; 10ng/ml LPS, 100µM dexamethasone) stimuli for 24h. Nitrite concentration was measured in supernatants and *Mrc1* expression was determined by qRT-PCR. Lcn2 release was assessed in supernatants by ELISA. The mRNA expression data were normalized to HPRT and expressed as a fold change compared to M_0 control cells. (**B, C**) BMDM were treated with $4x10^7$ CFU/ml *S. pneumoniae* (grey bars) or 10ng/ml LPS (black bars) alone or in combination with indicated deactivating stimuli (10ng/ml IL-10, 100µM dexamethasone (Dex), or 10^7/ml apoptotic cells (ACs)) for 16h. Secretions of Lcn2 (**B**) and KC (**C**) were assessed in supernatants by ELISA. Data are presented as mean ± SEM of triplicate (**A**) or quadruplicate (**B, C**) data and representative of at least two independent experiments. */ **/ *** indicate $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ versus M_0 (**A**) or versus respective conditions without deactivating stimuli (**B, C**) (ANOVA).



Supplemental Figure 2

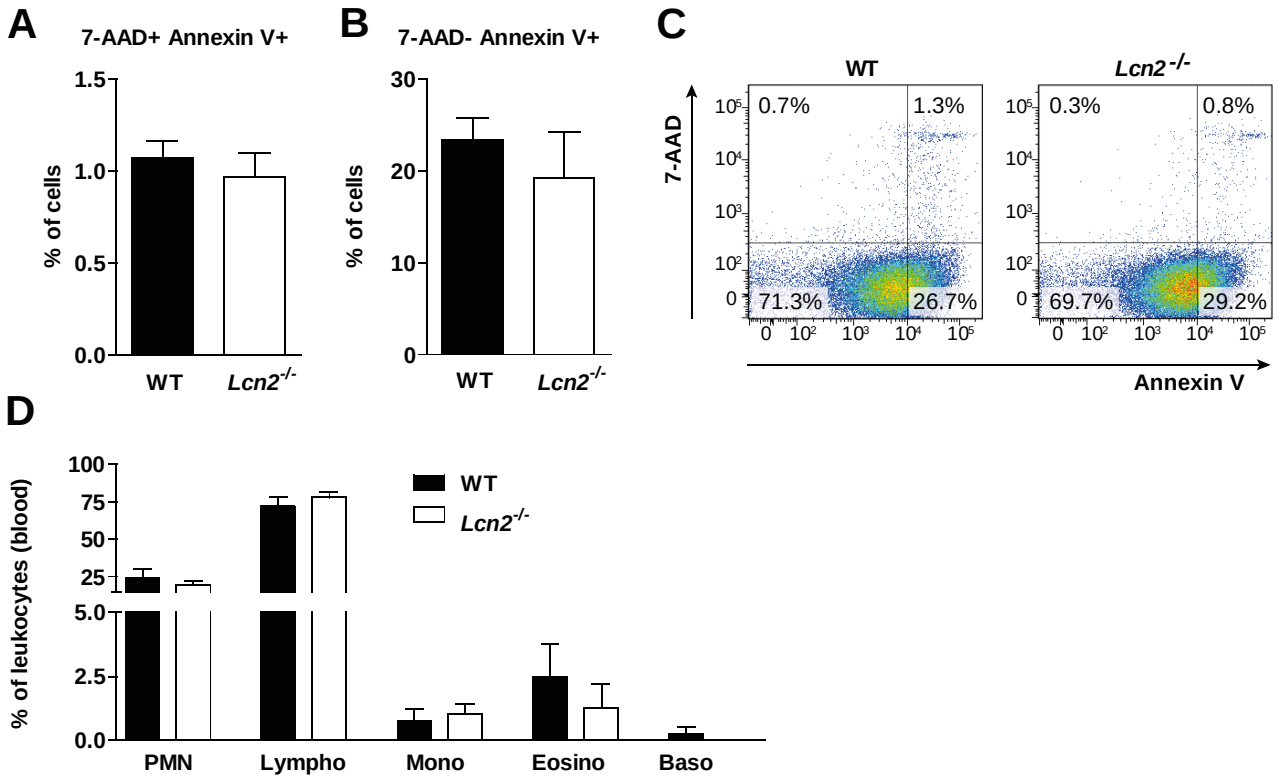
Lcn2 deactivates macrophages. (**A-C**) WT (black bars) and *Lcn2*$^{-/-}$ (white bars) BMDM were stimulated with 4x10^7 CFU/ml *S. pneumoniae* for 6 and 16h (**A,B**) or 10µg/ml LTA from *S. aureus* for 16h (**C**). IL-6 (**A,C**) and TNFα (**B,C**) were measured in supernatants by ELISA. (**D, E**) BMDM from WT (black bars) and *Lcn2*$^{-/-}$ mice (white bars) were exposed to 4x10^7 CFU/ml *S. pneumoniae* in the presence or absence of (**D**) 50µM deferoxamine (DFO) or 50µM Fe (III) ammonium citrate for 6h or (**E**) 8-80nM enterobactin for 16h. KC was assessed in supernatants by ELISA. (**F**) Primary WT AM were treated with 4x10^7 CFU/ml *S.pneumoniae* with or without 100ng/ml Lcn2 for 16h. TNFα levels were quantified in supernatants by ELISA. (**G, H**) MH-S cells were stimulated with 10µg/ml LTA from *S. pneumoniae* (TIGR4), 10µg/ml LTA from *S. aureus* (**G**) or 1µg/ml LPS (**H**) with or without increasing doses of Lcn2 (0.01µg/ml-0.1µg/ml (**G**); 0.1-1µg/ml (**H**)). TNFα was measured after 16h by ELISA. All data are presented as mean ± SEM of quadruplicates and representative of two independent experiments. NS, not significant; */ **/ *** indicate $P < 0.05$/ $P < 0.001$/ $P < 0.0001$ compared to respective WT condition (**A-E**) (ANOVA), *S. pneumoniae* (**F**) (t test), or respective condition without addition of Lcn2 (**G, H**) (ANOVA).



Supplemental Figure 3

Neutrophils release Lcn2 during pneumococcal pneumonia.

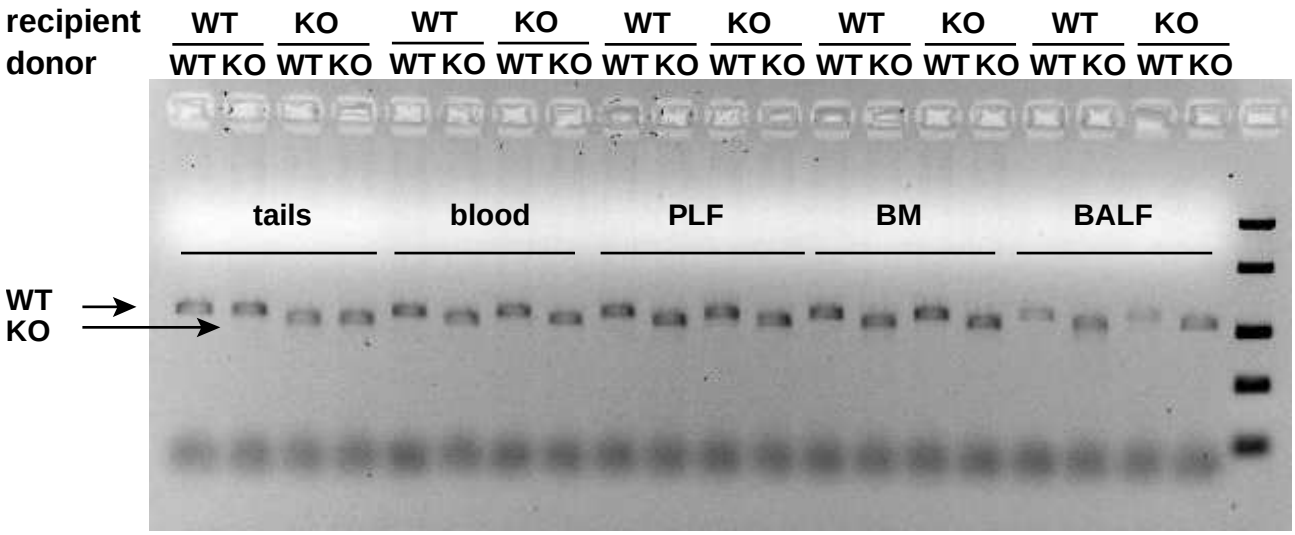
WT mice were infected with 10^5 CFU *S. pneumoniae.* $CD45^+$ $Ly6G^+$ $CD11b^+$ cells were analyzed by flow cytometry for intracellular Lcn2 expression.



Supplemental Figure 4

Apoptosis and a-priori white blood cell counts are similar in WT and *Lcn2*$^{-/-}$ mice.

(**A-C**) WT and *Lcn2*$^{-/-}$ mice were infected with 10^5 CFU *S. pneumoniae* for 12h. Bronchoalveolar lavage was performed and isolated cells were stained with Annexin-V and 7-AAD. The percentage of 7-AAD and Annexin-V double positive or Annexin-V single positive cells is shown in (**A**) or (**B**), respectively. Representative dot plots of WT (left) and *Lcn2*$^{-/-}$ (right) lung cells are shown in (**C**). Data are expressed as mean ± SEM of quadruplicates and representative of two independent experiments. (**D**) Differential cell counts of blood leukocytes from healthy WT and *Lcn2*$^{-/-}$ mice. Data are expressed as mean ± SEM of quadruplicates. PMN – polymorphonuclear cells; Lympho – lymphocytes; Mono – monocytes; Eosino – eosinophils; Baso – basophils.



Supplemental Figure 5

Reconstitution of bone marrow chimeric mice.

WT and KO recipient mice were lethally irradiated and immediately reconstituted with bone marrow from either syngeneic (WT-WT and KO-KO chimeras) or allogeneic donors (WT-KO and KO-WT chimeras). Three months after transplantation, one mouse per group was sacrificed and genomic DNA was isolated from blood cells, peritoneal macrophages (by peritoneal lavage; PLF), bone marrow (BM), alveolar macrophages (by bronchoalveolar lavage (BALF)) and genotyped by PCR. Tail biopsies were tested to ensure proper recipient status. WT-specific PCR products: 471bp, KO specific PCR products: ~450bp.

Supplemental Table 1:

Group	Pathogen	n
Gram-positive; survivors	*Streptococcus pneumoniae*	6
	Staphylococcus aureus	4
	Enterococcus faecium	2
	β-haemolysing *streptoccoci*	2
Gram-positive; non-survivors	*Enterococcus faecalis*	4
	Staphylococcus aureus	2
Gram-negative; survivors	*Escherichia coli*	5
	Klebsiella spp.	5
	Citrobacter freundii	2
	Proteus mirabilis	2
	Haemophilus influenzae	2
Gram-negative; non-survivors	*Acinetobacter baumanni*	2
	Pseudomonas aeruginosa	2
	Legionella pneumophila	2
	Enterobacter cloacae	1

Supplemental Table 2: Primer sequences

Primer name	Full name	Sequence (sense, antisense)
mHPRT	hypoxanthine guanine phosphoribosyl transferase 1	5‘-GTTAAGCAGTACAGCCCCAAAATG-3‘, 5‘-AAATCCAACAAAGTCTGGCCTGTA-3‘
mIL10	interleukin 10	5‘-TGAGGCGCTGTCATCGATTT-3‘, 5‘-CATGGCCTTGTAGACACCTT-3‘
mIL6	interleukin 6	5‘-CCACGGCCTTCCCTACTTCA-3‘, 5‘-TGCAAGTGCATCGTTGTTC-3‘
mKC	chemokine (C-X-C motif) ligand 1	5‘-GACCATGGCTGGGATTCACC-3‘, 5‘-TCAGAAGCCAGCGTTCACCA-3‘
mLcn2	lipocalin 2	5‘-CCTCCATCCTGGTCAGGGAC-3‘, 5‘-TAGTCCGTGGTGGCCACTTG-3‘
mMrc1	mannose receptor, C type 1	5‘-TCTGGGCCATGAGGCTTCTC-3‘, 5‘-CACGCAGCGCTTGTGATCTT-3‘

We generated Lcn2 or GFP expression plasmids by Gateway cloning after adding N- and C-terminal att-sites via PCR. We used pDONRTM201 as a shuttle vector. The destination vector was pCMV StrepIIIHA GW.

Primer name	Sequence (sense, antisense)
attB1 GFP	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGACTGCCATGGTGAGCAAGGGC-3’, 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTTGTACAGCTCGTCCAT-3’
attB1 Lcn2	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGACTGCCATGGCCCTGAGTGTC-3’, 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTGTCAATGCATTGGTC-3’