Supplementary Material

"The TLR4-MyD88 signalling axis regulates lung monocyte differentiation pathways in response to *Streptococcus pneumoniae*"

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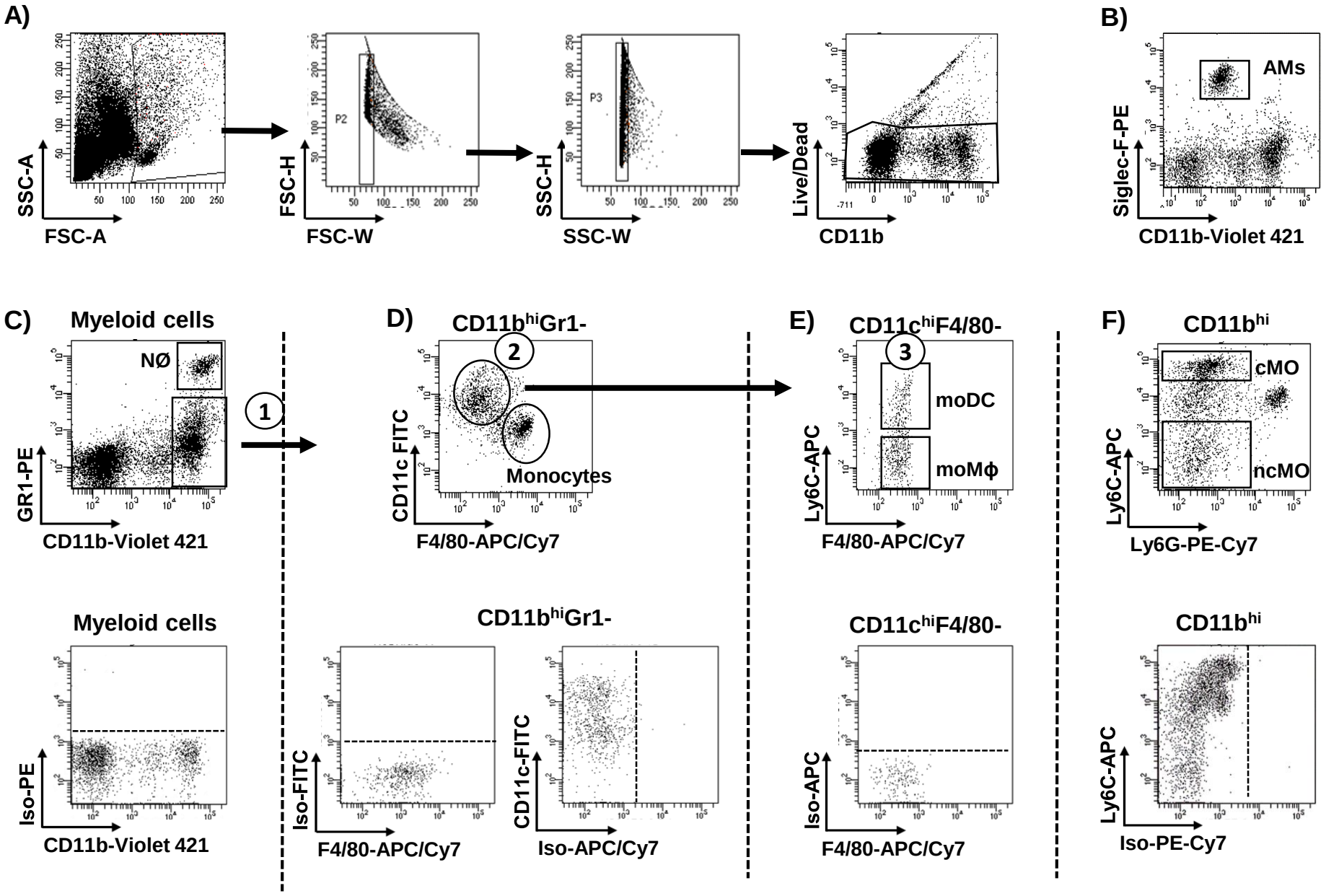
A)
SSC-A
FSC-A
P2
FSC-H
FSC-W
P3
SSC-H
SSC-W
Live/Dead
CD11b
B)
AMs
Siglec-F-PE
CD11b-Violet 421
C)
Myeloid cells
NØ
GR1-PE
CD11b-Violet 421
1
Myeloid cells
Iso-PE
CD11b-Violet 421
D)
CD11b^hiGr1-
2
Monocytes
CD11c FITC
F4/80-APC/Cy7
CD11b^hiGr1-
Iso-FITC
F4/80-APC/Cy7
CD11c-FITC
Iso-APC/Cy7
E)
CD11c^hiF4/80-
3
moDC
moMϕ
Ly6C-APC
F4/80-APC/Cy7
CD11c^hiF4/80-
Iso-APC
F4/80-APC/Cy7
F)
CD11b^hi
cMO
ncMO
Ly6C-APC
Ly6G-PE-Cy7
CD11b^hi
Ly6C-APC
Iso-PE-Cy7

Figure Supplementary 1. Lung suspensions were prepared and stained for flow cytometry analysis. Representative dot plots are shown in the figure, displaying at least 10^5 cells except in the Ly6C/F4/80 dot plot (10^3 cells). **A)** Myeloid-granulocyte populations were electronically gated on the basis of SSC-A and FSC-A . Doublets were discriminated using FSC-Height (H) versus FSC-Width (W) and SSC-H versus SSC-W strategy. Dead cells were discarded by staining with Fixable LIVE/ DEAD violet-510 kit, as a marker of fixed dead cells. **B**) Live cells (10^5-3 X 10^5 cells) were studied using different markers. Alveolar macrophages (AMs) were distinguished as SiglecF+CD11b-. C) A sequential gating strategy was used to identify myeloid CD11b+ populations expressing specific markers (upper dot plots) and gating boundaries of corresponding fluoresecence minus one (FMO) stainings (lower dot plots): 1.- Neutrophils ($CD11b^{hi}$ $GR1^{hi}$) and monocytic cell populations ($CD11b^{hi}$ $GR1^{-}$). D) 2.- Identification of the monocytic cell populations by means of F4/80 and CD11c expression. E) 3.- Monocytes can further differentiate into moMϕ by the differential expression of Ly6C (Gr1-$CD11b^{hi}F4/80^{lo}$CD11c+Ly6C-) and moDC ($CD11b^{hi}F4/80^{lo}$CD11c+Ly6C+). F) CD11b+ gated cells were analyzed for the classical monocytes (cMO) and non-classical monocytes (ncMO) phenotype characterization based on Ly6C and Ly6G expression: cMO ($CD11b^{hi}Ly6C^{hi}$ Ly6G-) and ncMO ($CD11b^{hi}Ly6C^{lo}Ly6G^{-}$). Isotype fluorescence minus one (FMO) controls were displayed in the lower dot plots.

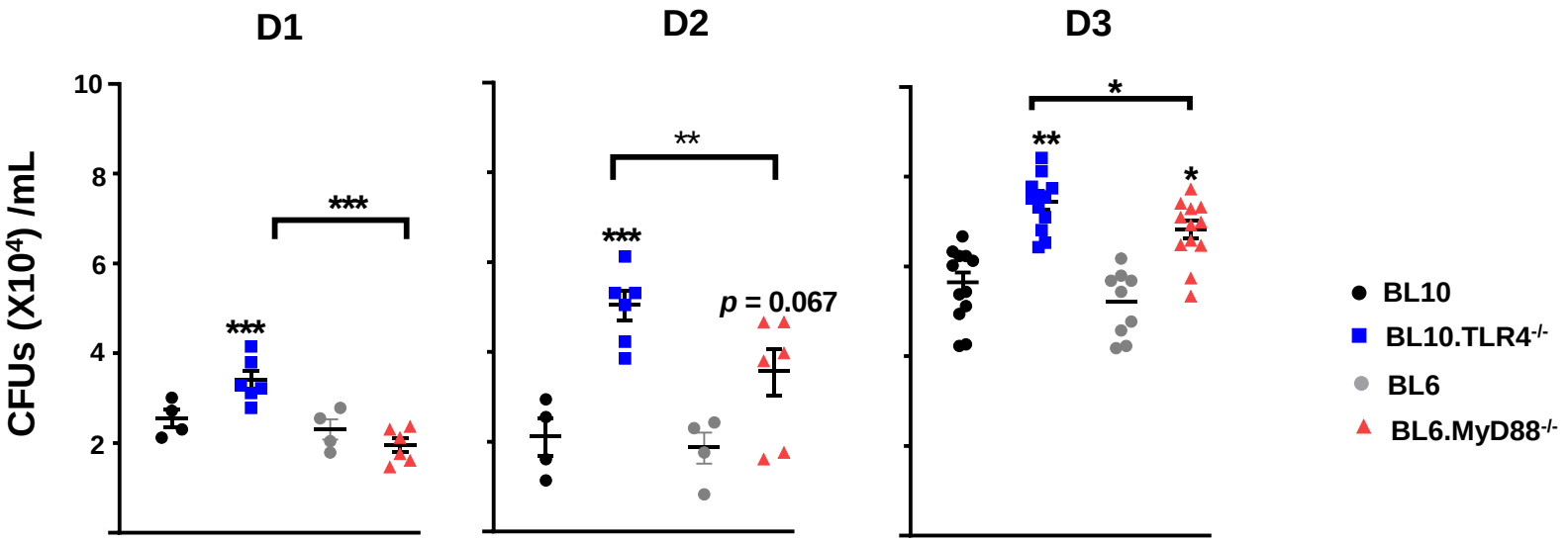

Figure Supplementary 2 CFUs quantitation in lungs infected al 1, 2, and 3 dpi in WT.BL10 and TLR4-/- infected mice and in WT.BL6 and MyD88-/-. The data represent the individual measurements of three independent experiments. Shown inside are means ± SEM: day 1 and 2 dpi (n =6) each mice strain, 3 dpi (n =12) for all mice strains. The comparisons were made using an unpaired two tailed Student`s t-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

SUPPLEMENTARY MATERIALS AND METHODS

Extraction of single cell lung suspensions.

Extraction of adult mouse lungs (C57BL/6) was performed and organs were washed twice in PBS at 4 ºC.

Protocol A: One pulmonary lobe and cell suspensions were prepared by mechanical dissociation, disrupting the tissue and filtering through a 40 µm pore cell strainer (BD Biosciences). The filtrate was then centrifuged for 5 min at 110 g and 4 °C in order to obtain the lung cells.

Protocol B: The other lobe was cut into small pieces that were treated with collagenase D and DNase I in RPMI 1640 for 45 minutes at 37 °C on a shaker as described (*Wijburg et al.; Jungblut et al.*), disaggregating the tissue by pipetting. Suspensions were washed twice before filtering as described in Protocol A.

PROTOCOL	Absolute cell number/lobe (x 10^6)	%Myeloid cells (FSC-A/SSC-A)	%Live cells (IP-)	Abs. Num Myeloid live cells (x 10^3)	CD11b+ Abs. number (x 10^3)
A (n = 4)	81.7 ± 0,2	1.77 ± 0.9	72.2 ± 3	120 ± 15	114 ± 2.5
B (n = 4)	40.3 ± 0.32**	1.2 ± 0.3	68 ± 6	47 ± 25*	43 ± 13**

Comparisons were made using unpaired two-tailed Student`s t-test: *p <0.05, ** p<0.01,

There is an important diminution in the total number of cells and in the frequency of the myeloid cells (gated as described in Supplementary Figure 1) recovered using Protocol B in comparison with Protocol A. Both procedures had similar levels of live cells (gating out propidium iodide positive cells). Absolute numbers of myeloid cells and CD11b+ cells were diminished after Protocol B.

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Supplementary Table 1. Antibodies used on flow cytometry.

ANTIBODY	CLONE	FLUOROCHROME	ORIGIN
CD11b	M1/70	VIOL421	BioLegend
CD11c	N418	FITC, PE/Cy7	eBioscience
CD87 (Ly6C)	HK1.4	APC	BioLegend
Ly6G	1A8	PE/Cy7	BioLegend
CD97 (GR1)	RB6-8C5	PE	BioLegend
F4/80	BM8	APC/Cy7	BioLegend
Siglec-F (CD170)	S17007L	PE	BioLegend
Isotype controls		FITC, PE, APC, PE/Cy7, APC/Cy7, VIOL421	BD
Fixable LIVE/ DEAD kit		VIOL510	ThermoFisher

FITC, fluorescein isotiocyanate; PE, R-phycoerythrin; APC, allophycocyanin; PE/Cy7, R-phycoerythrin conjugated with the tandem Cyanin 7. APC/Cy7, allophycocyanin conjugated with the tandem Cyanin 7.

Supplementay Table 2. List of primers used.

GENE	PRIMER 5´	PRIMER 3´	Temp	Refs
HPRT	GCCTGTATCCAACACTTCGA	TGTCATGAAGGAGATGGGAG	58º	1
TNF-α	GCCCAGACCCTCACACTCAG	AACACCCATTCCCTTCACAG	60º	2
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	60º	3
Nrf2	GATCCGCCAGCTACTCCCAGGTTG	CAGGGCAAGCGACTCATGGTCATC	60º	4
Nox2	CAGGAACCTCACTTTCCATAAGAT	AACGTTGAAGAGATGTGCAATTGT	60º	5

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