Supplementary DATA

Intracellular replication of *Streptococcus pneumoniae* inside splenic macrophages serves as a reservoir for septicaemia

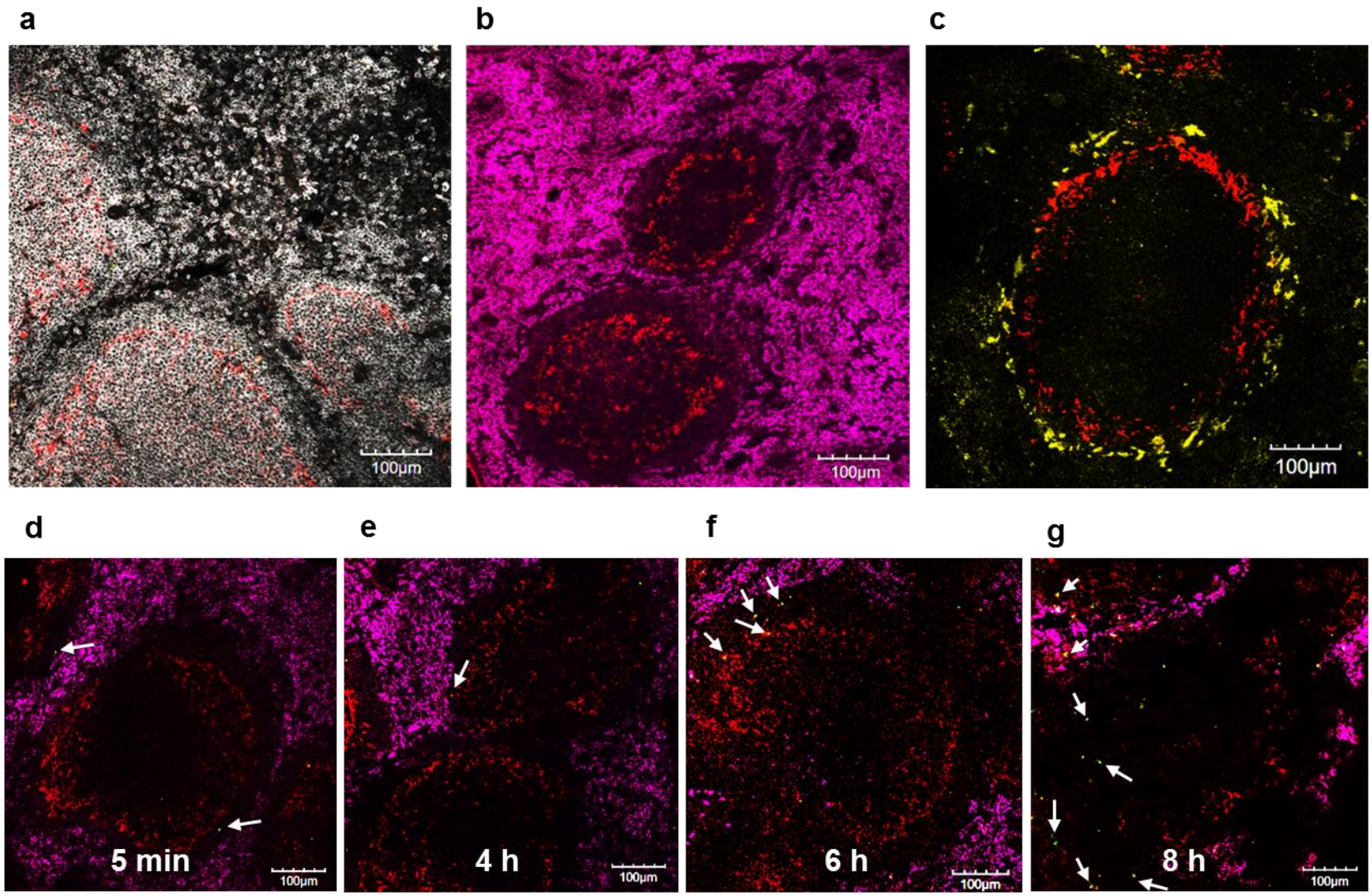
Giuseppe Ercoli[1], Vitor E. Fernandes[2], Wen Y. Chung[3], Joseph J Wanford[1], Sarah Thomson[4], Christopher D. Bayliss[1], Kornelis Straatman[5], Paul R. Crocker[4], Ashley Dennison[3], Luisa Martinez-Pomares[6], Peter W. Andrew[2], E. Richard Moxon[7], Marco R. Oggioni[1,*]

[1] Department of Genetics, University of Leicester, UK
[2] Department of Infection Immunity and Inflammation, University of Leicester, UK
[3] Hepato-Pancreato-Biliary (HPB) Unit, Leicester General Hospital, University of Hospitals of Leicester, NHS Trust, UK
[4] Division of Cell Signalling and Immunology, School of Life Sciences, University of Dundee, UK
[5] Centre for Core Biotechnology Services, University of Leicester, UK
[6] School of Life Sciences, Faculty of Medicine & Health Sciences, University of Nottingham, UK
[7] Department of Pediatrics, University of Oxford, UK

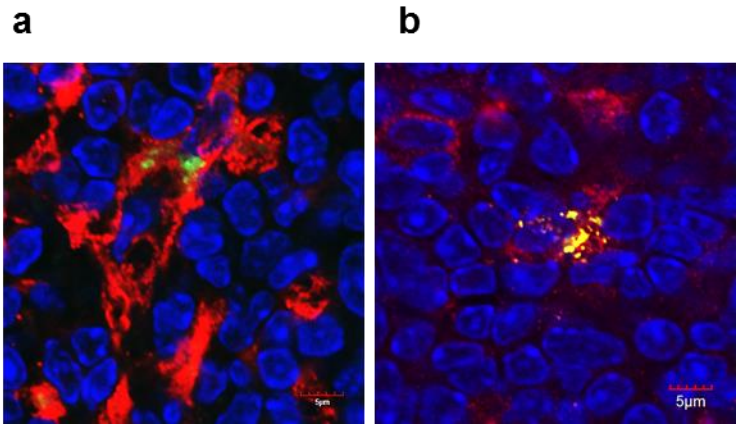
* Address correspondence to Marco R. Oggioni, to Department of Genetics, University of Leicester, LE1 7RH Leicester , UK; email mro5@leicester.ac.uk; phone +44 116 2252261

Index

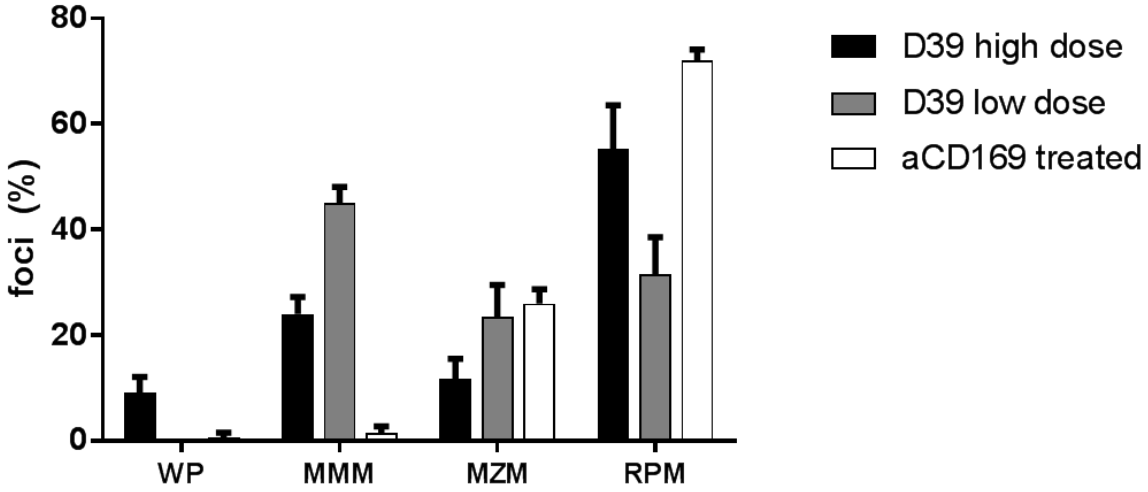
- Supplementary Figure 1: Spleen compartments and infection time-course.
- Supplementary Figure 2: CD169+ splenic macrophages following 8-hour infection with *S. pneumoniae* TIGR4 and a D39 non-encapsulated mutant.
- Supplementary Figure 3: Distribution of foci of infection.
- Supplementary Figure 4: Quantification of mouse spleen compartments.
- Supplementary Figure 5: CD169 blocking and infection of CD169 knockout mouse strain.
- Supplementary Table 1: Antibodies and microscopy reagents .
- Supplementary Table 2: Detection of GFP and RFP labelled bacteria in splenic foci.
- Supplementary Table 3: Primers for construction of strains expressing recombinant *nanA.*


a
b
c
100μm
100μm
100μm
d
e
f
g
5 min
4 h
6 h
8 h
100μm
100μm
100μm
100μm

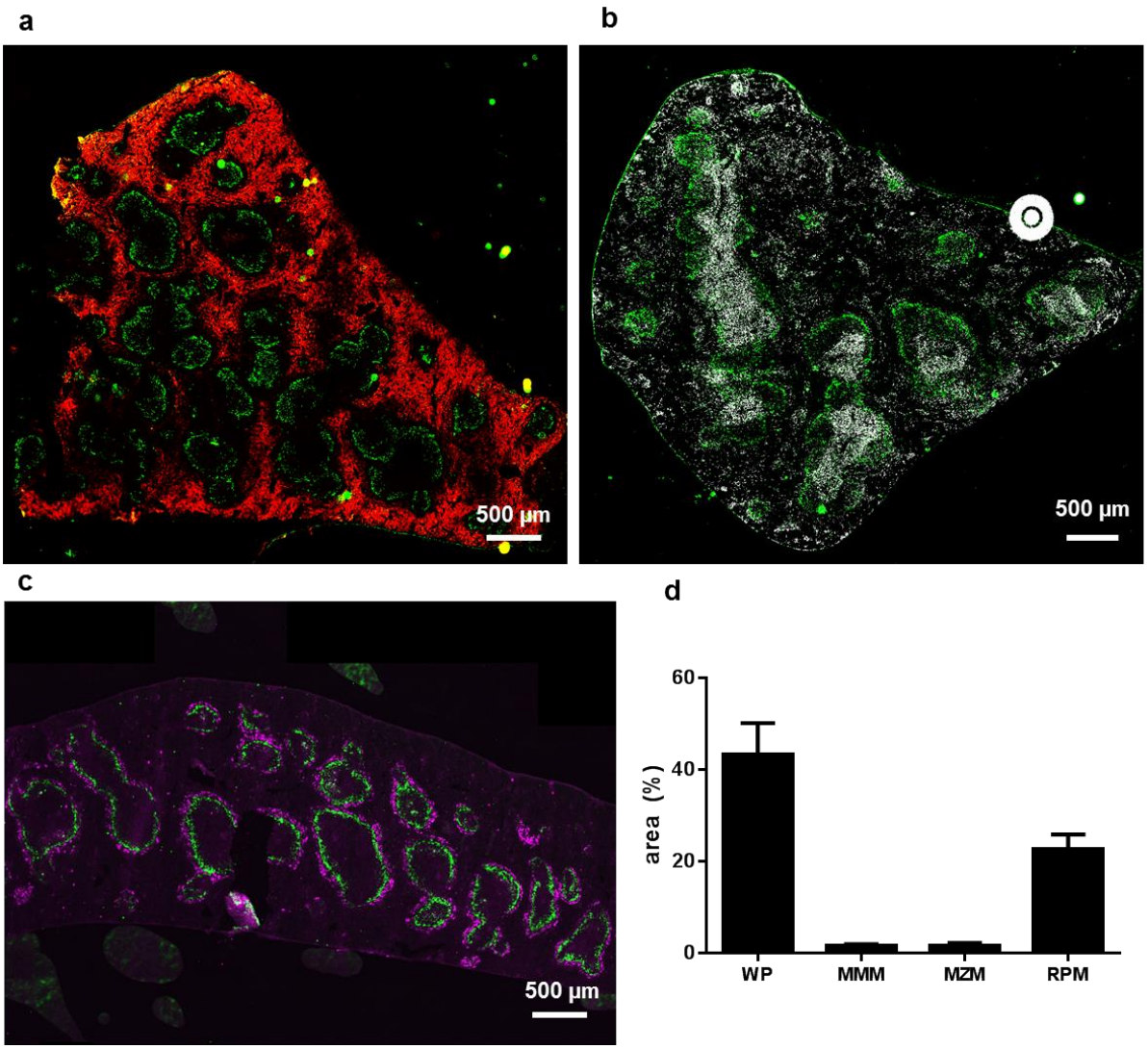
Supplementary Figure 1: Spleen compartments and infection time-course. CD1 mouse spleen sections have been stained with three different antibody combinations in order to show the spleen compartments. In panel a metallophilic CD169+ macrophages (red, Cr-Fc, AF568b) and B cells + T cells (white, α-B220+α-CD3, both AF647) are shown, in panel b metallophilic macrophages (red, Cr-Fc, AF568b) and red pulp macrophages (magenta, α-F4/80, AF647), in panel c metallophilic macrophages (red, Cr-Fc, AF568b) and marginal zone macrophages (yellow, aSIGN-R1b, AF488s). Spleen sections of CD1 mice infected by *S. pneumoniae* D39 show an increase of number, and a change in localisation of the foci of infection over time (white arrows indicate bacterial clusters). After five minutes, single bacteria (green) can be observed exclusively in the marginal zone (area not stained between the red pulp in magenta and the metallophilic area in red) (d). At four hours after infection (e), the majority of the bacteria are cleared and there are only a few foci of pneumococci. At six hours post-infection (f) an increase in the number and in the size of the foci is observed in the metallophilic macrophages. At eight hours (g) the number of bacteria in most foci is much higher and clusters of bacteria can be found throughout the spleen. Bacteria were stained in green (α-type2, AF488), red pulp macrophages in purple (α-F4/80, AF647), metallophilic macrophages in red (CR-Fc, AF568) and nuclei in blue (DAPI). All the immunofluorescence images are representative of 5 sections from 3 different samples. Antibody details are in Table S1.



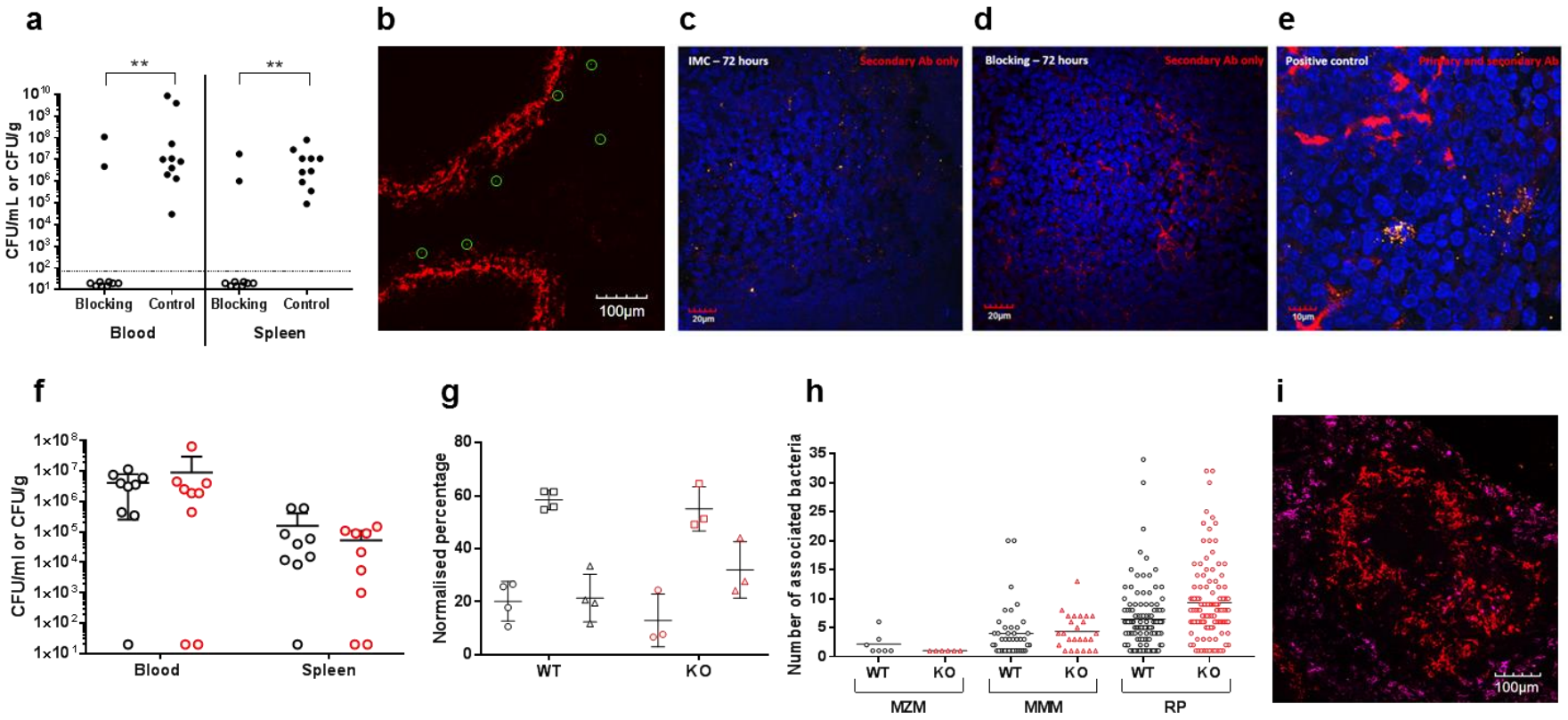
Supplementary Figure 2: CD169+ splenic macrophages following 8-hour infection with *S. pneumoniae* TIGR4 and a D39 non-encapsulated mutant. CD1 mice, infected intravenously with $1x10^6$ CFU of GFP-expressing non-encapsulated pneumococci, develop foci of infection in the metallophilic zone macrophages (a). GFP expressing bacteria are shown in green, CD169+ macrophages in red (CR-Fc, AF568) and nuclei are stained in blue (DAPI). Panel b shows co-localisation of bacteria, and CD169+ macrophages also occurs in TIGR4 infected spleens. Mice were infected with $1x10^6$ CFU of TIGR4 pneumococci (green, α-type4, AF488). Metallophilic macrophages (red) and nuclei (blue) have been stained as above. All the immunofluorescence images are representative of 5 sections from 3 different samples. Antibody details are in Table S1.



Supplementary Figure 3: Distribution of foci of infection. The figure shows the distribution of the foci of pneumococci, at 6 h after infection, in the white pulp (WP), the metallophilic macrophage area (MMM), the marginal zone macrophage area (MZM) and the red pulp area (RP) of the murine spleen. Three groups of infected mice have been considered in this analysis: mice infected with high dose (1 x 10^6 CFU) of wild-type D39 *S. pneumoniae* (black bars), mice infected with low dose (1 x 10^5 CFU) of wild-type D39 *S. pneumoniae* (grey bars) and mice infected with 1x10^6 CFU of the D39 after administration of anti-CD169 mAb (white bars). The data are shown as percentages of the total number of foci counted. The counts were obtained from 30 random microscope fields from three independent infected spleens. Error bars represent Standard deviation.



Supplementary Figure 4: Quantification of mouse spleen compartments. Fluorescent images of infected mouse spleens were acquired using a fully motorised Nikon Eclipse Ti microscope. The representative images were then analysed using ImageJ software to calculate the area of the different spleen compartments. Different staining combinations have been used to calculate the area of the different compartments for the red pulp (red, α-F4/80, AF647), metallophilic area (green, Cr-Fc, AF568b), the marginal zone macrophages (magenta, α-SIGN-R1, AF568) and the white pulp with a combination of B- and T-cell markers (white, α-B220+α-CD3, both AF647). For quantification, at least 5 sections for each staining combination have been analysed. The area of each staining was measured and the proportion with respect to the area of the section was calculated. In panel D the average area percentages (error bars represent standard deviation) of the different spleen compartments are reported. All the immunofluorescence images are representative of 5 sections from 3 different samples. Antibody details are in Table S1.


a
b
c
d
e
**
**
CFU/mL or CFU/g
Blocking
Control
Blocking
Control
Blood
Spleen
100µm
IMC – 72 hours
Secondary Ab only
Blocking – 72 hours
Secondary Ab only
Positive control
Primary and secondary Ab
f
g
h
i
CFU/ml or CFU/g
Blood
Spleen
Normalised percentage
WT
KO
Number of associated bacteria
WT
KO
WT
KO
WT
KO
MZM
MMM
RP
100µm

Supplementary Figure 5: CD169 blocking and infection of CD169 knockout mouse strain. Blocking experiments in CD1 mice are shown in panels a to c while experiments in CD169 KO C57BL/6 mice are shown in panels f to i. The majority of CD1 mice infected i.v. with D39, after blocking the CD169 receptor with a specific antibody (Rat IgG2a,k, Clone: 3D6.112), do not develop systemic infection after 72 hours (a). CFU were counted 72 hours after challenge or earlier if the animal was showing signs of disease (left, Isotype control, n=10, right, CD169 mAb, n=10). ** $P < 0.01$, Fisher's exact test, one tailed. Immunofluorescence on spleen sections 30 minutes after blocking and challenge is shown in panel b. The localisation of the antibody used for the blocking of CD169 was revealed using an Alexafluor 568 secondary antibody (red) showing a specific binding to the metallophilic macrophages ring. In the green circles, pneumococci (stained in green) can be observed to do not co-localise with the metallophilc macrophages. At 72h after antibody treatment the staining of metallophilic macrophages was tested by using only the secondary antibody in samples pre-treated with an isotype matched control antibody (c) and the anti-CD169 blocking antibody (d). A positive control spleen treated with both anti-CD169 antibody, and the secondary antibody are also shown (e). Nuclei are staining with DAPI (blue), Metallophilic macrophages are stained with an alexafluor-567-conjugated secondary antibody (red), and bacteria are staining with an alexafluor-488-conjugated secondary antibody (green). Further antibody details can be found in Table S1. Panels f to i report data on CD169 KO mice. (f) Number of CFU/g of spleen, or CFU/mL of blood from WT (black symbols, n=9) C57BL/6 mice, or sialoadhesin knock-out mice (red symbols, n=9) 6 hour after intravenous infection with *S. pneumoniae* D39. Lines represent the mean while error bars the standard deviation. (g) Shows the relative distribution of infectious foci in WT and KO mice to different splenic compartments, normalised against the total area of the spleen formed from that compartment (5 sections of 3 spleens analysed). Open circles; marginal zone macrophages, open squares; metallophilic macrophages, open triangles; red pulp macrophages. Lines represent the mean while error bars the standard deviation. (h) Shows the number of bacteria in each focus (size of foci) localised to each splenic macrophage compartment in both WT and KO mice (30 microscope fields from 3 different spleens were analysed at 60X magnification). Black symbols; WT mice, red symbols; KO mice. (i) Microscopy on knock-out mice spleen show that despite the lack of CD169, the metallophilic macrophages are still present in a ring-like structure bind to Cr-Fc (red, Cr-Fc, AF568b), in magenta red pulp macrophages are also stained (α-F4/80, AF647). In all the graphs lines represent the mean and error bars the standard deviation. All the immunofluorescence images are representative of 5 sections from 3 different samples. Antibody details are in Table S1.

SupplementaryTables

Supplementary Table 1: Antibodies and microscopy reagents

Antibody	Abbreviation	Specificity	Conjugated	Catalogue	Supplier
Anti-mouse/human CD45R/B220	α-B220	B cells	no	103211	Biolegend
Anti-mouse CD3	α-CD3	T cells	no	ab33429	Abcam
Anti-mouse CD169 (Siglec-1)	α-CD169	Metallophilic MΦ[a]	no	142401	Biolegend
Rat IgG2a control	IMC	N/A	no	400501	Biolegend
CR-Fc	CR-Fc	Metallophilic MΦ	no		[66]
Anti-mouse F4/80	α-F4/80	Red pulp MΦ	no	14-4801-81	eBioscience
Anti-mouse SIGN-R1 [ER-TR9]	α-SIGN-R1	Marginal zone MΦ	no	ab37220	abcam
Anti-mouse [ER-TR9] to SIGN Related 1 (Biotin)	α-SIGN-R1b	Marginal zone MΦ	biotinylated	ab51819	abcam
Anti-mouse Ly-6G (GR1)	α-GR1	Neutrophils	no	127602	Biolegend
Anti-porcine CD169 3B11/11	α-CD169p	CD169+ MΦ	no	MCA2316GA	Biorad
Anti-porcine CD163 2A10/11	α-CD163	CD163+ MΦ	no	MCA2311GA	Biorad
Anti-human CD3 | CD3-12	α-CD3	T cells	no	MCA1477A488	Biorad
Anti-pneumococcal type 2 capsule	α-type2	Bacteria	no	16745	Statens Serum Institut
Anti-pneumococcal type 4 capsule	α-type4	Bacteria	no	16747	Statens Serum Institut
Chicken anti-Rabbit IgG (H+L)	AF488	Secondary Ab[b]	Alexa Fluor® 488	A-21441	Thermoscientific
Goat anti-Rat IgG (H+L)	AF568	Secondary Ab	Alexa Fluor® 568	A-11077	Thermoscientific
Chicken anti-Rat IgG (H+L)	AF647	Secondary Ab	Alexa Fluor® 647	A-21441	Thermoscientific
Goat anti-Human IgG (H+L)	AF568b	Secondary Ab	Alexa Fluor® 568	A-21090	Life technologies
Goat anti-Mouse IgG (H+L)	AF568c	Secondary Ab	Alexa Fluor® 568	A-11004	Thermoscientific
Streptavidin 488 Conjugate	AF488s	Biotin	Alexa Fluor® 488	S32354	Thermoscientific
Wheat Germ Agglutinin	AF633	Membranes	Alexa Fluor® 633	11550816	Molecular Probes
Phalloidin	pAF647	Actin	Alexa Fluor® 647	A22287	Thermoscientific

[a] MΦ macrophage, [b] Ab antibody

Supplementary Table 2: Detection of GFP and RFP labelled bacteria in splenic foci

sample	GFP bacteria	RFP bacteria	both GFP and RFP
1.1*	1	2	0
1.2	2	1	0
1.3	4	2	0
1.4	2	0	0
1.5	5	6	0
1.6	4	2	0
1.7	0	4	0
2.1	3	2	0
2.2	1	1	0
2.3	3	5	0
2.4	2	3	0
2.5	1	0	0
2.6	1	2	0
TOTAL	29	30	0

* Seven and six samples were analysed respectively from two spleens

Supplementary Table 3: Primers for construction of strains expressing recombinant *nanA*

Name	Sequence (5' – 3')	Target region
Sial_F1	CCAAGAGATTACTATGCACGA	Lectin-like domain
Sial_R1	AGATTATATCACATTATCCATTAAAAATCAAACCGTTTTCTCTGTTAAAGCCGC	
Sial_R1*	CCAATTGAAGGGTTGGAGCCGTTTTCTCTGTTAAAGCCGC	upstream flank
Sial_F2*	GCGGCTTTAACAGAGAAAACGGCTCCAACCCTTCAATTGG	Lectin-like domain
Sial_F2	CAAAAGCATAAGGAAAGGGGCCGCTCCAACCCTTCAATTGG	
Sial_R2	TGTTTCAGGAAGTGCCTGC	downstream flank
Lect_F1	GGATTGAGCAGGAAGTATG	Sialidase domain
Lect_R1	AGATTATATCACATTATCCATTAAAAATCAAACTCGTGCATAGTAATCTCTTGG	
Lect_R1*	CGTTTTCTCTGTTAAAGCCGCTCGTGCATAGTAATCTCTTGG	upstream flank
Lect_F2*	CCAAGAGATTACTATGCACGAGCGGCTTTAACAGAGAAAACG	Sialidase domain
Lect_F2	CAAAAGCATAAGGAAAGGGGCCGCGGCTTTAACAGAGAAAACG	
Lect_R2	GAAGTAGATATTGCCTAGTAATTGG	downstream flank