Supplemental Materials

Supplemental Methods

Antibodies

Rat-anti-mouse monoclonal antibodies (mAb) against the following antigens were purchased from BD Biosciences (San Diego, CA): CD49d (α4), CD62P (P-selectin), CD29 (β1), CD31 (PECAM), CD54 (ICAM-1) and β7 integrin. Antibodies to CD11a/LFA-1 (Tib 213 antibody), CD62L/L-selectin (MEL-14), PNAd (Meca-79), and MAdCAM-1 (Meca-367) were provided by Dr. Eugene Butcher (Stanford University). Antibodies again CD62E/E-selectin (10E9.6) and CD106/VCAM-1 (MK2.7) were obtained from Dr. B. Wolitzky (Hoffman-La Roche Inc., Nutley, NJ). Dr. Dietmar Vestweber (Max-Planck Institute for Biomolecular Medicine, Münster, Germany) provided P-selectin blocking mAb RB40.34 and anti-PSGL-1 (2PH1). Dr. Jose-Carlos Gutierrez-Ramos (Pfizer Inc., New York, USA) provided the monoclonal antibody to CD34 (clone E8). Supernatant containing the mAb to CD102/ICAM-2 (mIC2/3c4) was a generous gift of Dr. Timothy Springer (CBR, Boston, MA). Monoclonal antibodies obtained from non-commercial sources were purified from hybridoma supernatants and then stored at -70°C in endotoxin-free saline.

Mice

LysEGFP mice were used as breeding pairs and placed together in cages overnight. These mice are also known as neutrophil reporter mice and express enhanced GFP (EGFP) that was knocked into the lysozyme M (lys) locus, leading to the expression of EGFP in myelomonocytic cells [1]. For all matings, the day of plug discovery was considered day 0 of gestation. All mice were housed in a pathogen-free barrier facility, fed a standard diet of mouse chow and sterile water and cared for using guidelines that comply with local regulations for the care and use of laboratory animals. All animal experiments were approved by the local Animal Care and Use Committee, by the Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany, AZ 35-9185.81/G-67/03 and by Regierung of Oberbayern:Az 55.2-1-54-2531-69-07.

Intravital microscopy

A preliminary screening of different injectable fluorophors for use in intravital microscopy experiments revealed that AO rapidly crossed through the placental barrier and permitted fluorescent staining of nucleated cells (leukocytes, endothelium and immature red blood cells) in the fetal circulation while others, including rhodamine 6G, did not sufficiently pass the placenta into the fetal circulation. For wildtype mice, one ~200 µl bolus of acridine orange (AO; 2mg/ml in phosphate-buffered saline) was administered through a carotid artery catheter to the mother and an additional injection (50-100 µl) was given as needed.

Fluorescent fetal cells were visualized in regions of interest in the YS by video-triggered stroboscopic epi-illumination (Rapp Optoelectronics; Hamburg, Germany) through an appropriate filter set. All scenes were recorded on video tape using a CCD camera (model CF8/1, Kappa) for later off-line analysis. Vessel diameter, segment length and blood flow velocity and wall shear rate were measured using a digital image processing system, as previously described [2-4].

Flow cytometry

FACS analysis was performed to assess expression of the following adhesion relevant molecules in a first approach on Gr-1+ fetal blood cells: Mac-1, LFA-1, PSGL-1, and CXCR2. After decapitation of fetuses, fetal blood was collected in Petri-dishes. Red blood cells were lyzed using BD Lysis buffer. Isolated fetal leukocytes were then double-stained with rat anti mouse Ly6G/Ly6C conjugated with PerCP-Cy5.5 (Gr-1, Pharmingen, San Diego, USA) and either rat anti-mouse Mac-1 conjugated with PE, rat anti-mouse LFA-1 conjugated with PE, rat anti-mouse PSGL-1 conjugated with PE, or rat anti-mouse CXCR2 conjugated with PE (all eBioscience, San Diego, USA). Rat IgG2b conjugated with PerCP-Cy5.5 and rat IgG2b conjugated with PE (Pharmingen, San Diego, USA) were used as isotype controls. All antibodies were applied at 5 µg/ml for 45 min on ice and expression (mean fluorescence intensity, MFI) assessed on 10,000 cells/mouse within the Ly6G/Ly6C positive cluster using a four-decade FACScan (Becton Dickinson, San Jose, USA) with FlowJo software package (Version 8.8.6, Tree Star, Ashland, USA). To analyze fMLP-binding capacity, cells were isolated from fetal (<E16 and E16-18) blood and adult bone marrow, incubated for 15 minutes at room temperature in 1.25 µg/ml Mouse BD Fc Block (Pharmingen) and subsequently stained with 2µg/ml PE rat anti-mouse Ly-

6G/Ly-6C (Gr-1, Pharmingen) together with 15nM fMLP-FITC (formyl-Nle-Leu-Phe-Nle-Tyr-Lys, Invitrogen) with or without a 500-fold excess (7.5μM) of unlabeled fMLP for 30 minutes at room temperature. The fMLP-binding capacity of fetal and adult cells was analyzed within the Ly6G/Ly6C high population by comparing the relative mean fluorescent intensities of the fMLP-FITC treated cells alone to fMLP-FITC and excess unlabeled fMLP treated cells (mean fluorescence intensity of the later group was set to 1). We also analyzed selectin binding to fetal and adult neutrophils, as described previously [5],using P- and E-selectin IgG chimeric proteins (3.6 μg/ml each), a secondary goat anti-human Biotin-labeled Ab and a Streptavidin PerCP-Cy5.5 Ab (eBioscience). Cells for selectin chimera binding studies and fMLP binding studies were analyzed using a Gallios Flow Cytometer (Beckman Coulter, Krefeld, Germany) and Kaluza flow cytometry analysis sofware v1.2.

In a second approach, FACS analysis was performed to assess expression of the following adhesion relevant molecules on Ly6G+ fetal and adult blood cells: Mac-1, LFA-1, PSGL-1, and CXCR2. Isolated fetal cells were stained with rat anti-mouse (IgG2a,κ) Ly6G conjugated with Pacific Blue (Biolegend) and either rat anti-mouse (IgG2a) CXCR2 (R&D) and rat anti-mouse (IgG2b) Mac-1 conjugated with PE (eBioscience, San Diego, USA) or rat anti-mouse (IgG2a) LFA-1 (in vivo LGC) and rat anti-mouse (IgG1) PSGL-1 conjugated with PE (Pharmingen). Rat IgG2a,κ conjugated with Pacific Blue (Biolegend) and rat IgG1 (eBioscience) and IgG2b conjugated with PE (Pharmingen) were used as isotype controls. Expression (mean fluorescence intensity, MFI) was assessed on the Ly6G-positive cluster using Gallios FACS (Beckman Coulter) with Kaluza software package (Version 1.2).

Systemic leukocyte count

Systemic blood samples (5 μl) were taken by umbilical cord puncture. Differential counts of Wright-Giemsa stained blood samples (Sigma) were performed using a Zeiss microscope with a ×100/NA1.4 oil immersion objective (Zeiss, Germany). The relative percentages of nucleated RBC and differentiated white blood cells, including lymphoid and myeloid progenitors, neutrophils and lymphocytes were quantitated by visual inspection of blood samples. The phenotypic criteria for cytological differentiation are summarized in Table S1 (supplemental data) and were taken from previous reports [6;7]. The minority of the remaining other nucleated blood cells were counted as others (primordial stem cells, monocytes, basophils, eosinophils).

Quantitative real time RT-PCR

Total mRNA was isolated by NucleoSpin®RNA II Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer´s protocol. Reverse transcription (RT) was carried out with a High Capacity cDNA Reverse Transcription Kit´(Applied Biosystems, Weiterstadt, Germany), according to the protocol in a master-cycler gradient (Eppendorf, Hamburg, Germany). RT conditions were 10min 25°C; 2h 37°C; 5min 85°C and 4°C on hold.

Real-time polymerase chain reactions (RT-PCRs) were performed in quadruplicate in optical 96-well reaction microtiter plates covered with optical films, in a volume of 20µl containing 1µl TaqMan®Gene Expression Assay 20x (Details are presented in Table S2) to detect the mRNA for the following genes: P-selectin glycoprotein ligand (Selpl), integrin alpha L (Itgal), integrin alpha M (Itgam), and interleukin 8 receptor beta (Il8rb). Thermal cycling conditions were set at: 20 sec at 95°C, followed by 40 cycles of amplification with 3 sec at 95°C and 30 sec at 60°C. The ABI PRISM 7500 Fast (Applied Biosystems, Weiterstadt, Germany) was used to perform the PCR assays. Quantification was carried out by the 2-ΔΔCt method using β2-microglobulin (B2m) or glycerolkinase (Gyk) as housekeeping genes (Mm00437762_m1 assay for B2m mRNA detection and Mm00433896_m1 for Gyk mRNA detection, both Applied Biosystems) [8;9].

References

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Supplemental movies:

Supplemental Movie S1: Rolling and adhesion of LysEGFP+ cells in yolk sac vessels of a LysEGFP fetus at E14. Intravital microscopy was used to visualize rolling and adhesion of LysEGFP+ fetal blood cells in exteriorized yolk sac vessels of an E14 LysEGFP fetus. The movie was recorded at a frame rate of 20 frames/s. Note the low number of EGFP+ cells in the fetal circulation and the almost complete absence of endothelium / EGFP+ cell interactions.

Supplemental Movie S2: Rolling and adhesion of LysEGFP+ cells in yolk sac vessels of a LysEGFP fetus at E17. As in movie S1, intravital microscopy was applied to observe rolling and adhesion of LysEGFP+ fetal blood cells in exteriorized yolk sac vessels of an E17 LysEGFP fetus. At E17, many more endothelium / LysEGFP+ cell interactions are observed. In addition, the number of EGFP+ cells is increased in the fetal circulation.

Supplemental Figure 1. FACS histograms on the expression of Mac-1 (A), LFA-1 (B), PSGL-1 (C), and CXCR2 (D) on Gr-1 positive blood cells isolated from <E16 and E16-E18 fetuses and adult mice, including appropriate isotype controls.

Supplemental Figure 2. FACS analysis of adhesion relevant molecules on Ly6G+ blood cells isolated from <E16 and E16-E18 fetuses and adult mice. Expression of the surface molecules CD11b/Mac-1 (A), LFA-1 (B), PSGL-1 (C), and CXCR2 (D) including appropriate isotype controls, are shown as mean fluorescence intensity values (median ± SEM) from at least 3 experiments per group.

Supplemental Tables:

Table S1. Phenotypic markers for progenitor and mature lymphoid, myeloid cells and RBCs in Wright-Giemsa stain

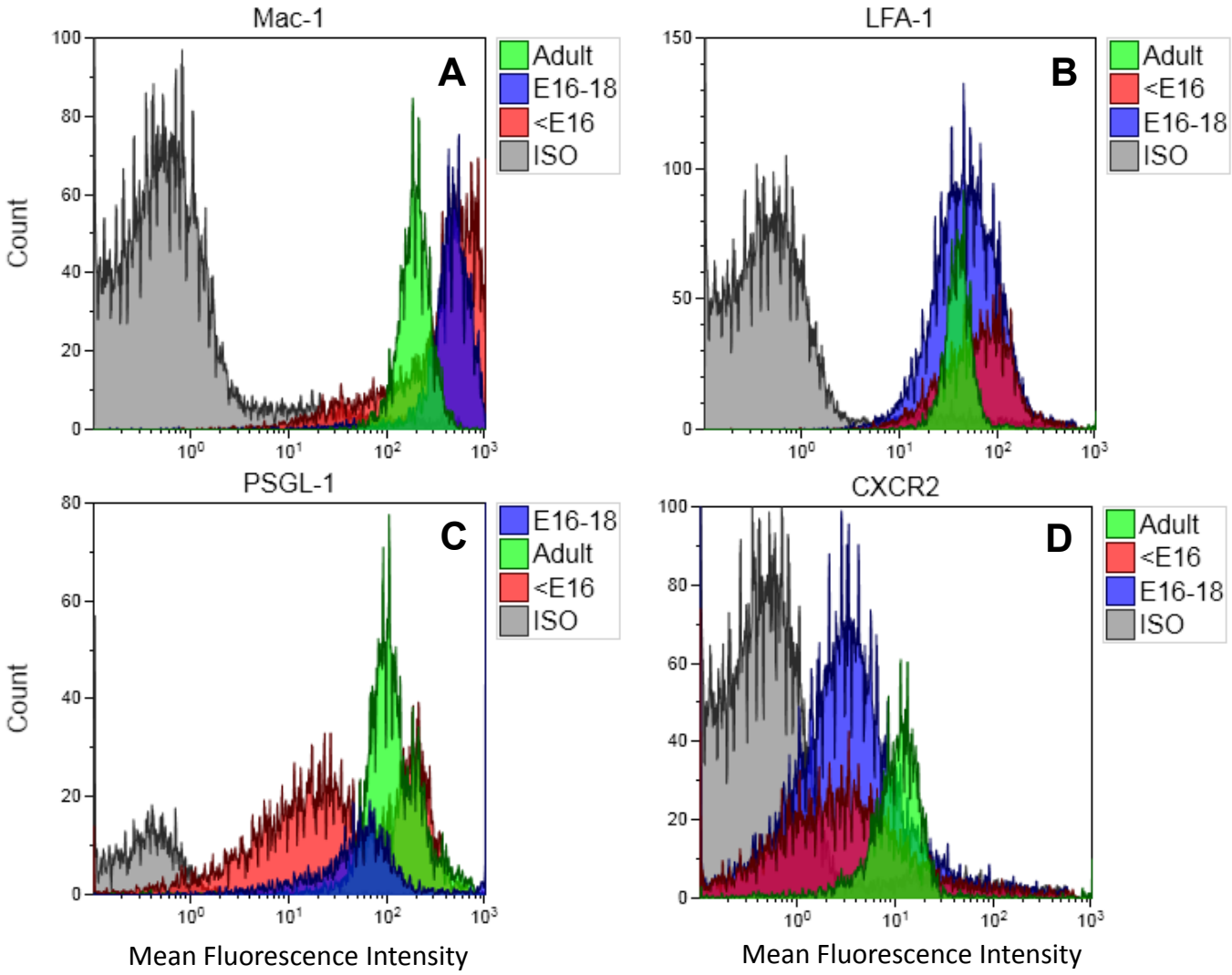
Cell group	**nRBC**	**RBC**	**myeloid progenitors**	**granulocytes**	**lymphoid progenitors**	**Lymphocytes**
Cell types	erythroblasts, normoblasts	reticulocytes, erythrocytes	myeloblasts, promyelocytes, myelocytes, metamyelocytes	neutrophils	lymphoblasts, large lymphocytes	lymphocytes
Diameter [µm]	9-18	7-9	10-30	10-15	10-30	9-14
Cytoplasm	baso- or polychromato philic –orange	eosinophilic	scanty basophilic-azurophilic or primary granules	pink to grey	grey-basophilic	scanty, pale blue
Nucleus	round – pyknotic	No	round-indented-kidney shaped	polymorpho-nuclear, multi-lobed nuclei	round	rounded or oval, densely packed chromatin
Remarks		central pallor	prominent nucleoli in early stages		prominent nucleoli in early stages	

Table S2: Primer and probe details for assay location and exon boundaries.

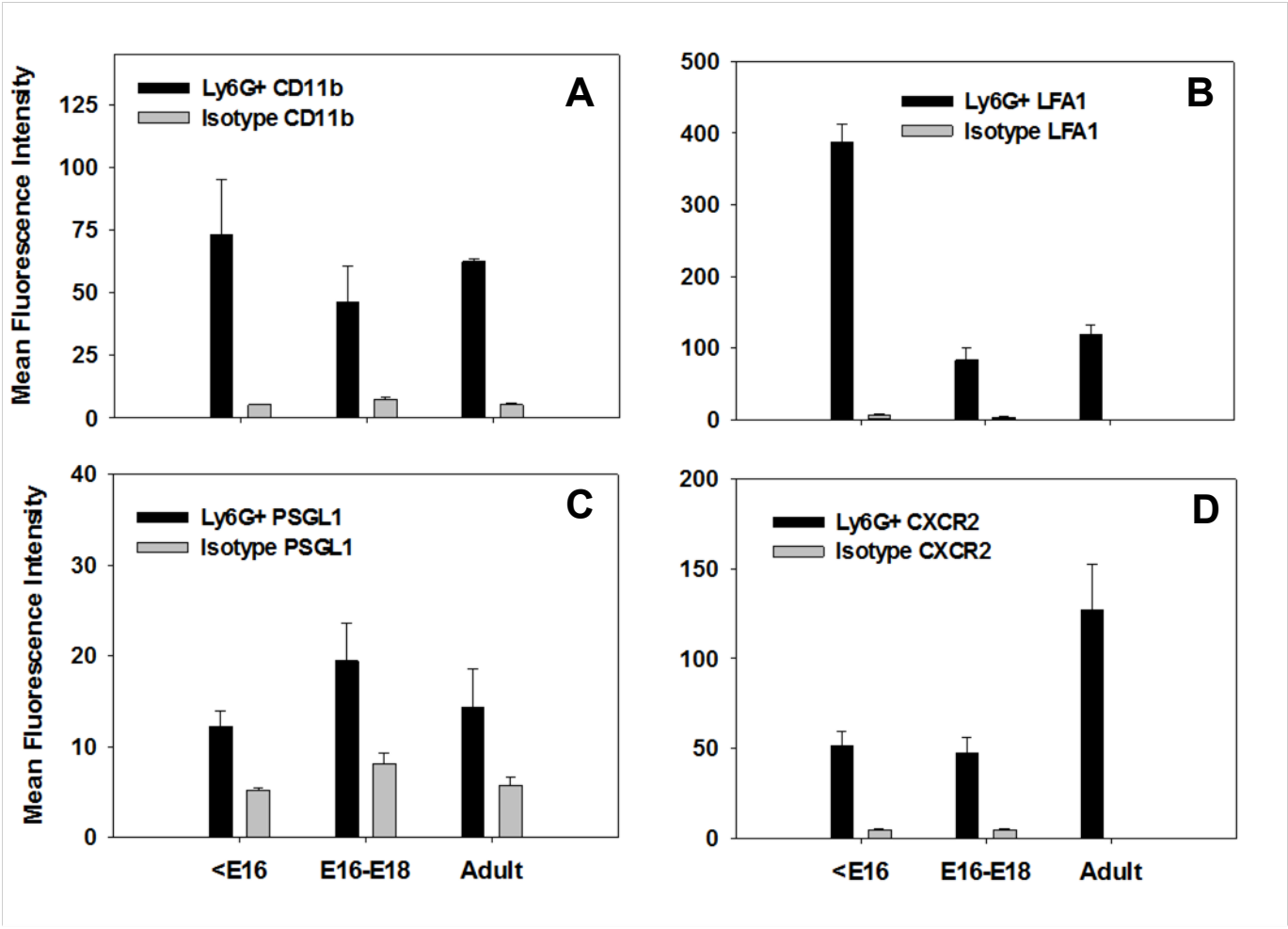
Mouse - Taq Man Primer							
Gene	**Accession-number**	**Abbre-viation**	**Sequence**	**Translated Protein**	**Exon Boundary**	**Assay Location**	**Amplicon Length**
Integrin alpha L	Mm00801807_m1	*Itgal*	RefSeq NM_008400.2	NP_032426.2	6-7	687	119
Integrin alpha M	Mm01271250_m1	*Itgam*	RefSeq NM_001082960.1	NP_001076429.1	14-15	1814	90
Interleukin 8 Receptor beta	Mm00438258_m1	*Il8rb*	RefSeq NM_009909.3	NP_034039.1	1-2	128	67
Selectin, platelet ligand	Mm01204601_m1	*Selplg*	RefSeq NM_009151.3	NP_033177.3	1-2	153	91
Glycosyltransferase	Mm00433896_m1	*GyK*	RefSeq NM_212444.2 NM_008194.3	NP_032220.1 NP_775618.1	7-8 3-3	982 982	110 110
beta2-Microglobulin	Mm00437762_m1	*ß2M*	RefSeq NM_009735.3	NP_033865.2	1-2	115	77

Table S3. Microvascular parameters (vessel diameter, centerline blood flow velocity and wall shear rate) in fMLP-treated yolk sac vessels of LysEGFP fetuses at different days of gestation.

Embryonic Day	15	17	18
Number of fetuses	6	7	8
Number of vessels	8	11	9
Diameter (μm)	56 ± 4	52 ± 3	50 ± 4
V_{mean} (μm/s)	1300 ± 160	1300 ± 140	1300 ± 150
γ (s^{-1})	600 ± 80	650 ± 80	600 ± 80



Supplemental Figure 1



Supplemental Figure 2