Staphylococcus aureus drives expansion of low density neutrophils in diabetic mice

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Supplemental Methods:

Neutrophil Gene Expression

HDN and LDNs were purified as described above. Cells purified from 5 mice were pooled and treated as a single sample for analysis. Total RNA was extracted using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol and treated with RNase-free DNase I to remove genomic DNA contamination. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and the quality of RNA was assessed using the Agilent RNA ScreenTape assay in conjunction with a 4200 TapeStation system (Agilent Technologies). Only high-quality RNA samples with an RNA Integrity Number (RIN) greater than 9 were used for microarray hybridization.

RNA samples were analyzed as previously described [48]. Briefly, RNA was amplified and labeled using MessageAmp Premier RNA Amplification Kit (Thermo Fisher Scientific). Total RNA was reverse transcribed to first strand cDNA with T7-oligo(dT) primer using ArrayScript reverse transcriptase, followed by second strand cDNA synthesis to generate double-stranded cDNA (ds-cDNA). Subsequently, the ds-DNA was used as a template for in vitro transcription to synthesize biotin-labeled antisense-RNA (aRNA) molecules. The biotin-labeled aRNA was purified with RNA binding beads and then fragmented at 94°C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.2, 100 mM Potassium Acetate and 30 mM Magnesium Acetate). Fragmented aRNA (10 µg) was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 Array (Thermo Fisher Scientific) at 45 °C for 18 hrs. Affymetrix GeneChip Fluidics Station 450 was used for washing and staining of the arrays, and hybridized arrays were scanned using a GeneChip Scanner 300 7G (Thermo Fisher Scientific) according to the manufacturer's user guide. Array files were analyzed using packages from R Bioconductor: - normalized using the RMA method implemented in affy package; - probe IDs were converted into Entrez gene IDs and filtered to IRQ=0.33 using method nsFilter in genefilter package; - analysis for differential gene expression was performed

with limma package; - ranking of genes from limma analysis was used as input to gene set enrichment analysis method gsePathway implemented in ReactomePA; - per-sample pathway activities for the heatmap visualization were estimated with GSVA package; heatmaps were shown with ComplexHeatmap package; - MGSAT R software was used implement the entire analysis pipeline. These data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE122195.

Membrane protein preparation

Low density (LDN) and high density (HDN) neutrophils from 10 mice were pooled. Three pooled LDN and HDN samples were lysed in a 500µl buffer comprised of 50mM HEPES, 0.24M sucrose, 25mM NaCl, 1mM EDTA, pH 7.0, cocktail protease inhibitor (Sigma-Aldrich). Lysates were further homogenized using Dounce homogenizer by 30 manual piston strokes. Samples were then sonicated briefly 3 times for 5 sec at 20% amplitude using a Branson sonicator and centrifuged at 1500 x g for 10min at 4°C. Membrane fraction was pelleted from the supernatant using a Sorvall™ MTX 150 Microultracentrifuge at 100,000 x g for 96 min at 4°C. The pellet was resuspended in 0.1M Na_2CO_3 at 4°C for 15 min and repeated the ultracentrifugation step. The resulting pellets containing membrane proteins were used for protein digestion.

Protein digestion and tandem mass tag (TMT) labeling

The protein pellets were dissolved in 50% trifluorethanol containing 10mM tris(2-carboxyethyl)phosphine in 200 mM triethylammonium bicarbonate (TEABC) and heated at 55°C for 15 min. Samples were cooled to room temperature and cysteine residues were modified using 40 mM iodoacetamide for 15min at dark. Samples were then digested using trypsin/Lys-C mix, Mass Spec Grade from Promega Corporation in 40mM ammonium bicarbonate for 15h at 37°C.

Resulting peptides were acidified with trifluoroacetic acid and desalted using an Oasis HLB 96-well plate (Waters #WAT058951). The dried samples were dissolved in 100 µL of TEABC and labeled with 41 µL of TMT reagents according to manufacturer's protocol (Life-Technologies). Six samples comprised of three high density neutrophils and three low density neutrophils were randomized and labeled with six different TMT reagents. TMT labeled samples were then combined and fractionated on an Oasis plate under basic conditions. Initially 20 different elutions were collected by using step gradient of acetonitrile containing 10mM TEABC. Two distant fractions were then pooled to generate 10 final samples for mass spectrometry analysis.

Nanoflow LC-MS/MS analysis

LC-MS/MS analysis of TMT labeled peptides was carried out on an Orbitrap Fusion Tribrid™ (Thermo Fisher Scientific) mass spectrometer interfaced with Dionex 3000 RSLCnano system. Peptides were captured on a 2 cm x 75 µm C18 trap column (ReproSil-Pur 120 C18-AQ 7µm) and separated on a 30cm x 75µm C18 (ReproSil-Pur 120 C18-AQ 2.4µm particle) analytical column. Reversed-phase solvent gradient consisted of 0.1% formic acid with increasing levels of organic solvent B (80% acetonitrile in 0.1% formic acid) over a period of 120 minutes. Peptide separation was carried out using a linear gradient of solvent B from 5-24% for 82min, 24-32% for 30min, 32-45% for 28min, 45-90% for 20min and 95% for 7min. Each of 3 seconds data dependent acquisition (DDA) cycle contained a MS survey scan between 400-1600m/z at 120,000 resolutions at 200m/z followed by higher energy collisional dissociation (HCD, 36% collision energy) MS2 scans on most intense precursors. Peptides were sprayed at 2600 volts, precursor AGC target was set at 3.0e5 and isolation width of 1.2Th with 0.345Th offset. Already fragmented precursors were excluded for subsequent MS/MS analysis of a period of 30sec. MS2 scan

resolution was set to 30,000 at 200m/z; maximum injection time, 250ms and MS2 AGC target of 5.0e4. Easy-IC ETD ion was used for constant internal calibration of precursors.

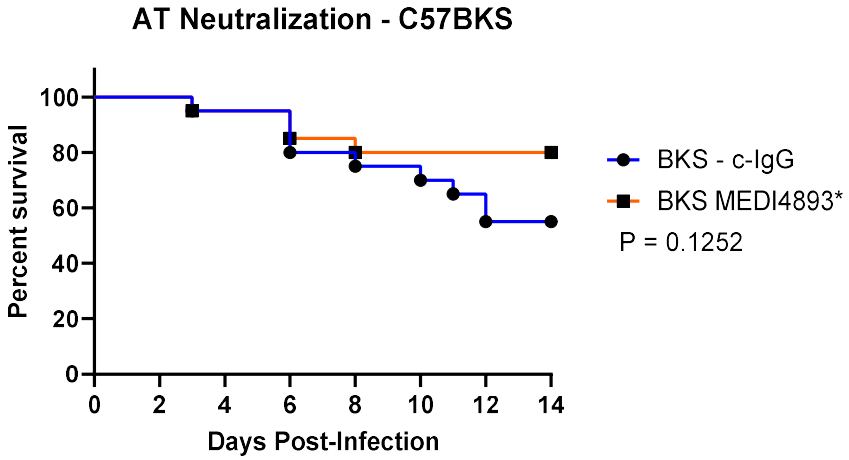
Each fraction was also analyzed in multinotch MS/MS/MS (MS3) mode to overcome the interference by co-isolated TMT labeled precursors. Parameters used in this method are same as above except a collision induced fragmentation (CID) MS2 scan was performed with ion trap detection followed by multisynchronous isolation of 10 fragment ions for HCD fragmentation at 15,000 resolution.

Data analysis

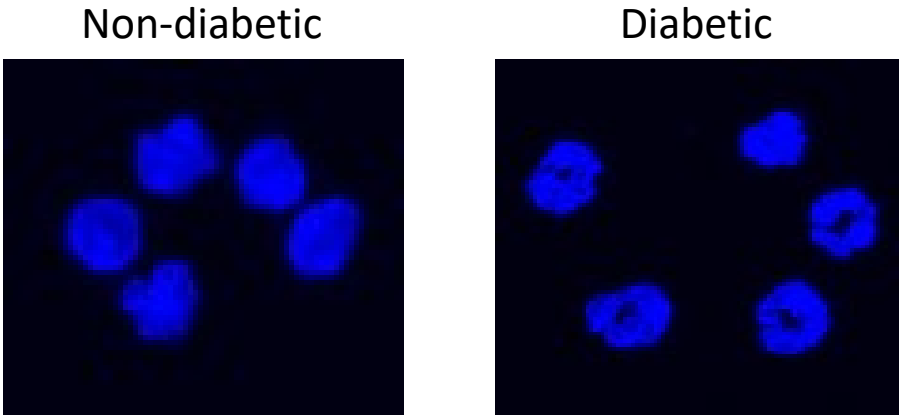
Mass spectrometry data was analyzed using Proteome Discoverer 2.2 (Thermo Fisher Scientific) software with search engines Mascot (version 2.6.0). Data was searched using latest Uniprot Mus musculus 2017 protein database with common laboratory contaminants included. Unfragmented precursor and TMT reporter ions were removed using non-fragment filter in the PD 2.2 workflow. Search parameters included 3 missed cleavages for trypsin, oxidation (M) and deamidation (N, Q) as variable modifications. Tandem label (229.163Da) at N-terminus and lysine residue and carbamidomethylation on cysteine residue were set as fixed modifications. The mass tolerances on precursor and fragment masses were set at 20 ppm and 0.05 Da, respectively for MS2 analysis. For MS3 analysis, precursor and fragment masses were set at 20 ppm and 0.5 Da, respectively. Percolator node in PD2.2 calculates posterior error probabilities and q values for identified peptides. False discovery rate (FDR) cutoff value was set at 0.01 and PSMs with delta Cn value better than 0.05 were automatically selected. Consensus step in PD2.2 included several nodes for spectrum, peptide and protein grouping and FDR calculation. Peptide spectrum matches (PSMs) with more than 30% co-isolation interferences were excluded from quantitation. Reporter ions for TMT labeled peptides were quantified using the PD quantitation node and peak integration tolerance was set at 20 ppm by considering most confident centroid peaks. Signal to

noise values were calculated in addition to measurement of intensities of the TMT reporter ion for peptide and protein quantitation.

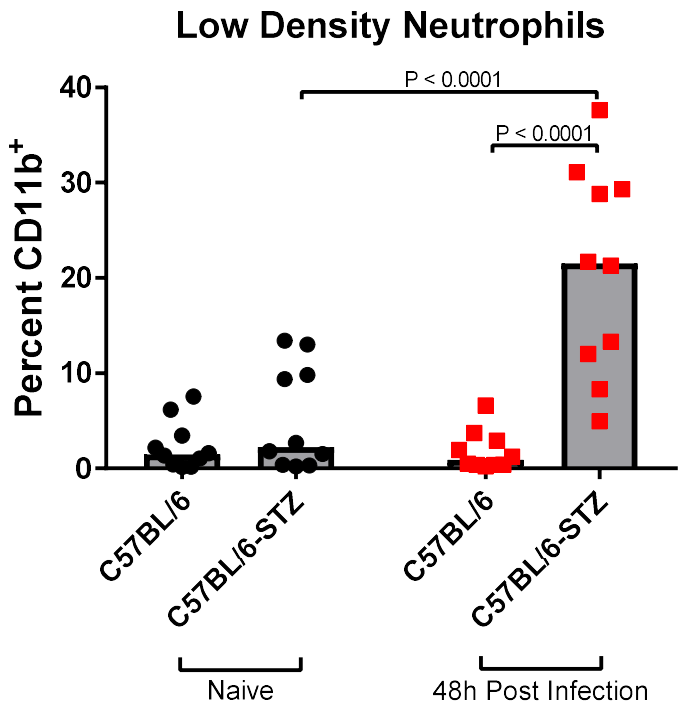
Supplemental Figures



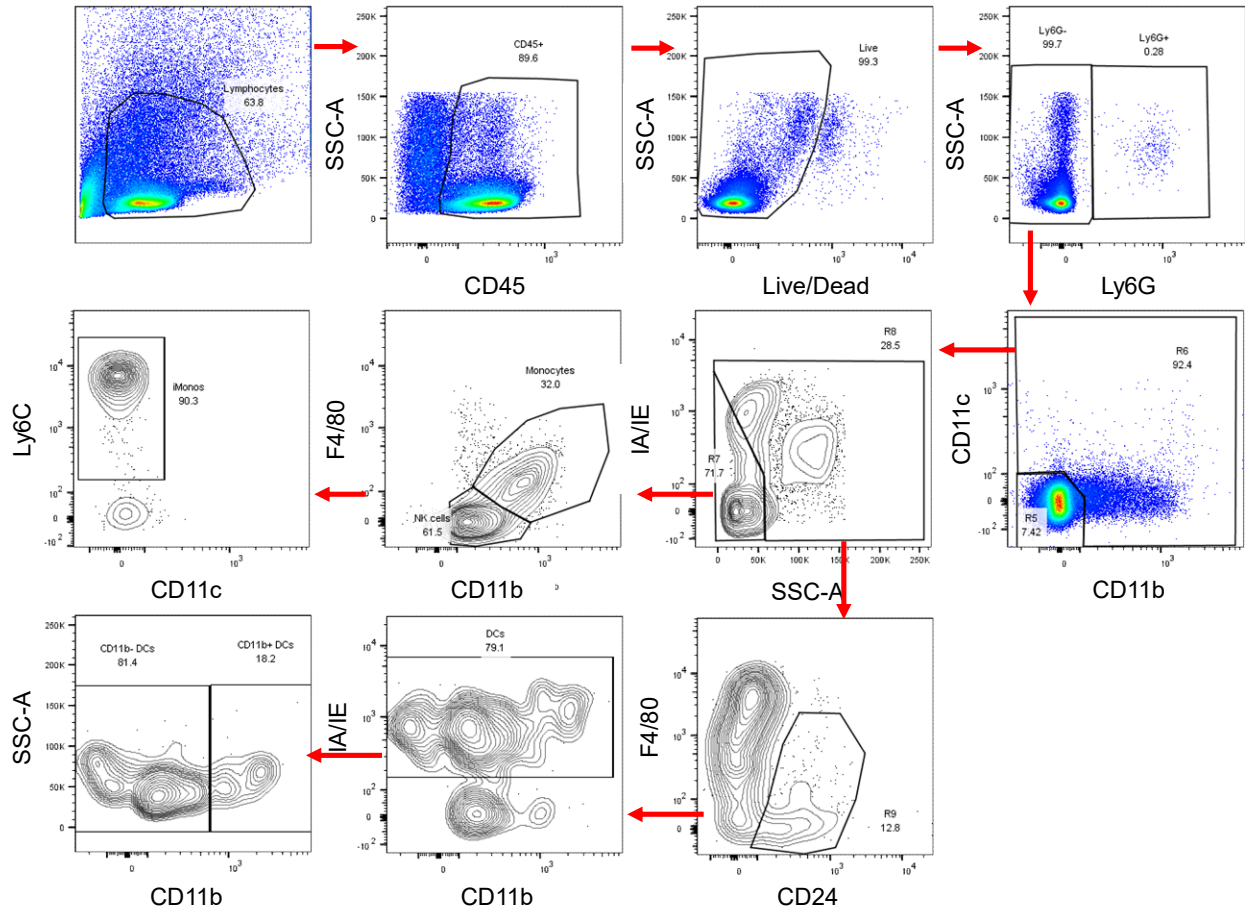
Supplemental Figure 1: Survival of C57BKS mice prophylactically treated with MEDI4893* or c-IgG (15mg/kg, 24h prior to infection) and infected with 5e7 CFU *S. aureus*. N = 20 per group.



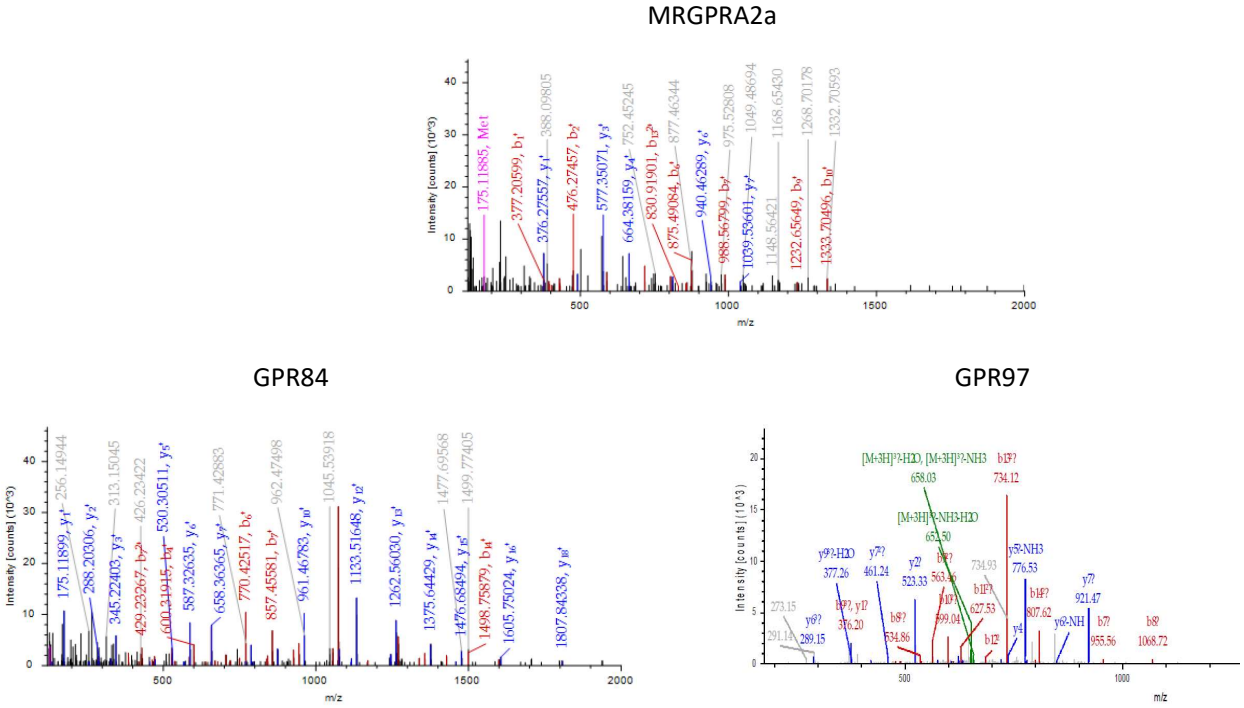
Supplemental Figure 2: Nuclear structure differs in neutrophils isolated from non-diabetic and diabetic mice. DAPI staining of purified neutrophils, representative of 3 independent experiments. Magnification 63x.



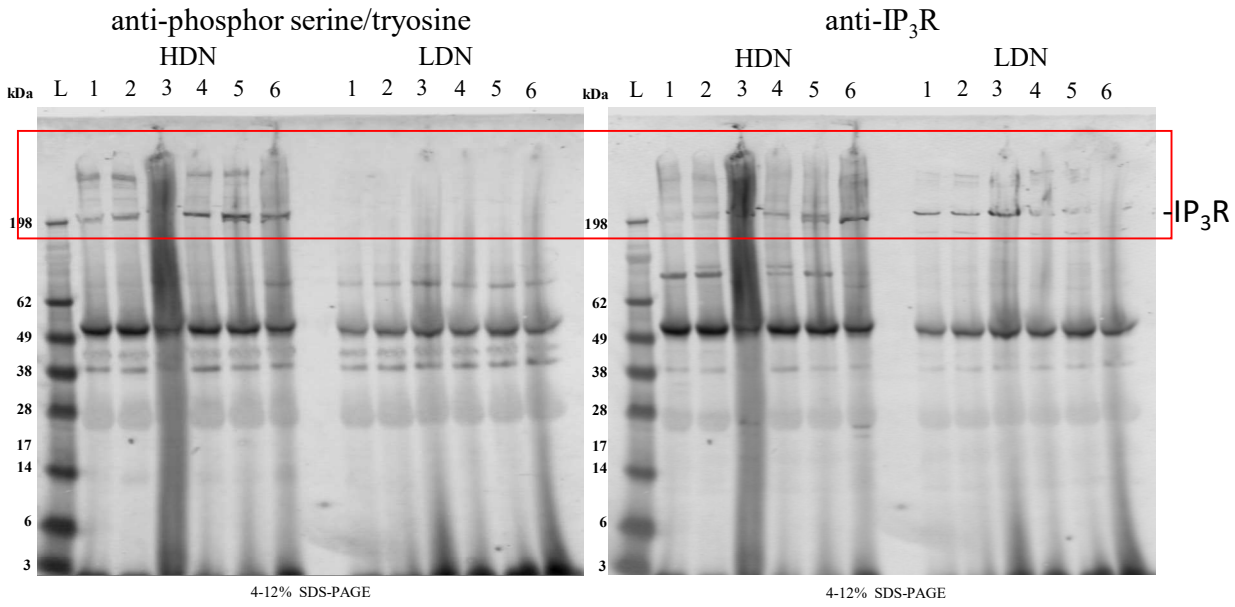
Supplemental Figure 3: FACs analysis of LDNs in the blood of C57BL/6 and C57BL/6-STZ mice. Representative of 3 independent experiments, data were analyzed by Kruskal-Wallis followed by Dunn's test.



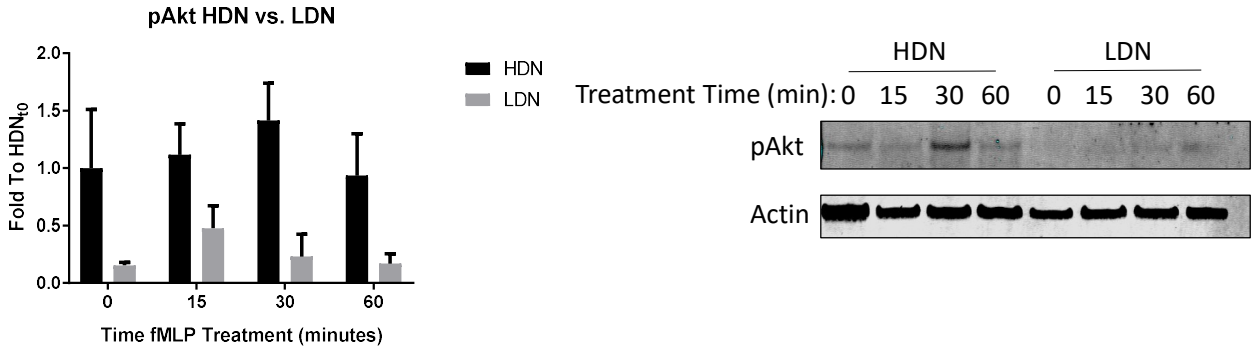
Supplemental Figure 4: FACs gating of immune cells in the liver.



Supplemental Figure 5: MS/MS spectra of GPCRs. Representative of three independent experiments.



Supplemental Figure 6: Phosphorylation of IP_3R. IP_3R was immunoprecipitated from HDN and LDN lysates obtained from the blood of mice 48h following infection with *S. aureus*. Membranes were probed with antibodies against phosphor-Ser/Thr, stripped and re-probed with anti-IP_3R. Representative of 3 independent experiments.



Supplemental Figure 7: Akt activation in LDN and HDNs. Western blot analysis of HDN and LDN restimulation with fMLP. Cells were purified from the blood of infected (48h) *db/db* mice and restimulated ex vivo with 100 nM fMLP. Representative of three independent experiments.

	logFC	AveExpr	P.Value
Mrgpra2a	3.3708	10.4832	0.0049
Gpr84	2.1716	7.4400	0.0004
Gpr97	2.9777	7.5257	0.0001

Supplemental Table 1: RNA expression of GPCRs showing the fold change (logFC) between LDN and HDNs.