Platelet MHC Class I Mediates CD8$^+$ T Cell Suppression During Sepsis

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Supplemental Methods

Human subjects

Patients admitted to the intensive care unit (ICU) with a primary diagnosis of sepsis were prospectively enrolled within 48 (±24) hours of ICU admission. Sepsis was defined using SEPSIS-3 consensus criteria.[1] Patients with systemic infection and life-threatening organ dysfunction were diagnosed with sepsis when total SOFA score was ≥ 2. Septic shock is a subset of sepsis which can be identified as clinical sepsis with persisting hypotension requiring vasopressors to maintain MAP ≥65 mm Hg and having a serum lactate level >2 mmol/L (18 mg/dL). For comparison, healthy donors were also recruited. Healthy donors were defined as medication-free, male and female adults over the age of 21 without any acute or chronic medical conditions, illness, or hospitalization within 30 days prior to enrollment. All healthy donors and patients gave informed consent prior to sample collection (IRB # 51506 and 102638). Supplemental Table 1 shows demographic information for healthy donors and septic patients.

Mice

Wild-type (WT) C57BL/6J male and female mice were obtained from Jackson laboratory. B6.SJL-Ptprc (B6 Cd45.1) mice were obtained either from Jackson laboratory or kindly from the laboratory of Dr. Peter Jensen (Department of Pathology, University of Utah). $B2m^{fl/fl}$-Pf4Cre mice were obtained from Dr. Craig N. Morrell (University of Rochester School of Medicine, NY).[2] OT-I mice were obtained from Jackson laboratory and crossed with $B2m^{fl/fl}$-Pf4Cre and littermate control $B2m^{fl/fl}$ mice to generate the $B2m^{fl/fl}$-Pf4Cre-OT-I (KO-OT-I and WT-OT-I) mice. $Rag2^{-/-}$ OT-I mice were obtained from Taconic Biosciences. All mice were bred and maintained in the University of Utah animal facility under pathogen-free conditions

according to NIH guidelines. All animal experiments were performed according to protocols approved by the institutional Animal Care and Use Committee (Protocol No.18-10012). Male and female mice between 9-16 weeks in age were used in all experiments.

Platelet Isolation

Platelets were isolated as previously described.[3] Briefly, blood was collected into a one-sixth volume of acid/citrate/dextrose (ACD) buffer. Platelet-rich plasma was obtained by centrifugation at 100 × g for 20 minutes or for 10 min using minicentrifuge at room temperature. Platelets were isolated from plasma by centrifugation at 500 × g for 10 minutes and resuspended in Pipes/Saline/Glucose (PSG) buffer containing 20 nmol/L PGE1, $CD45^+$ depleted following Miltenyi Biotec manufacturer's protocol, centrifuged at 500 × g for 10 minutes again in PSG buffer containing 20 nmol/L PGE1, and resuspended in Medium 199.

Megakaryocyte culture and stimulation

$CD34^+$ derived human megakaryocytes were cultured as previously described.[4] $CD34^+$ hematopoietic stem and progenitor cells were isolated from human cord blood and cultured in serum free expansion media supplemented with 25 ng/mL of stem cell factor and 20 ng/mL of thrombopoietin (TPO) for six days. Cells were cultured with 50 ng/mL TPO only from days 6-13. On Day 13, megakaryocytes were harvested, washed, and treated with 50 ng/mL TPO together with either interferon-γ at 250U/mL or lipopolysaccharide (LPS) at 100ng/mL, and lipopolysaccharide binding protein (LBP) and CD14 were added to the cell culture to facilitate LPS binding, both at a final concentration of 100ng/mL. After 20h stimulation, HLA-A, B, C expression on megakaryocytes was measured by flow cytometry.

Cecal ligation and puncture (CLP)

CLP was used to induce polymicrobial sepsis in mice. The mice were first anesthetized with ketamine and xylazine. The lower abdominal area of anesthetized mice was sterilized using iodine swabs. A small incision was made in the lower abdominal area to expose the cecum. The cecum was ligated with a 3.0 silk suture and then carefully punctured a single time with a 21G needle to expose fecal contents into the abdominal cavity. The abdominal cavity was then closed with non-absorbable nylon sutures and the area re-sterilized with iodine. The mice were then given a subcutaneous injection of 600μL saline and returned to clean cages on a heating pad during recovery. Mice received buprenex immediately after the surgery and again a few hours post-surgery subcutaneously. For sham control conditions, all of the above steps were followed except the cecum was not ligated and punctured. Mice were monitored daily for disease severity and euthanized 4 days after CLP unless otherwise stated. Platelets were harvested 4 days after CLP induction.

OVA immunization

To elicit antigen specific $CD8^+$ T cell responses *in vivo*, chicken ovalbumin (OVA) at 2mg/mL was mixed with equal volume of either complete Freund adjuvant (CFA) or incomplete Freund adjuvant (IFA) and injected subcutaneously at 50uL per site at the left and right hock (100uL/mouse). For *in vivo* OVA treatment (pulse) experiments, mice were given OVA without adjuvants intraperitoneally at 3mg/mouse/day for 3 consecutive days.

DQ-OVA and OVA pulse assay

Platelets (2×10^7/mL) in Medium 199 were treated (pulsed) with DQ-OVA at 100ug/mL at 37°C for 1 hour, followed by CD41 surface staining at room temperature in the dark for 15-30 min and acquired on flow cytometry immediately. For negative controls, platelets were stained for CD41, fixed with equal volume of 4% PFA, and then pulsed with DQ-OVA for 1h. Proteasome inhibitors MG-132, Bortezomib, lactacystin and corresponding vehicles were added to platelet suspension at a final concentration of 40uM for 30 min, followed by DQ-OVA pulse for 1h. CD41 staining was added during the last 30 min of DQ-OVA pulse. Samples were acquired on flow cytometer immediately. To confirm the successful inhibition of proteasome activity in platelets, the 20S Proteasome Activity Assay kit was used according to the manufacturer's recommendations (Millipore Sigma). Briefly, platelets isolated from control mice were used for the indicated treatment and prepared in the cell lysis buffer following the manufacture's manual and measured for their 20S proteasome activity. For OVA pulse, platelets (2×10^7/mL) in Medium 199 were pulsed with OVA at 1mg/mL at 37°C with gentle rotation for 6 hours, washed and then stained for CD41, H-$2K^b$, and SIINFEKL:$H2K^b$ surface molecules. For SIINFEKL peptide pulse, platelets were pulsed with SIINFEKL peptide (Ovalbumin257-264 (chicken)) at 25μM for 2h and washed two times.

Aspartate Aminotransferase (AST) Activity Assay

To biochemically evaluate the disease severity in our CLP sepsis model, AST blood levels were measured using the commercial AST Activity Assay Kit from Millipore Sigma following manufacture's protocol. Briefly, mouse blood was collected by cardiac puncture without anticoagulants. After clotting for 30 min undisturbed at room temperature, blood

samples were centrifuged at 2000×g 10min at 4°C. Serum was collected and centrifuged at 12000×g 2 min to further remove debris and saved for the AST activity assay.

Murine CD8$^+$ T cell isolation and *in vitro* labelling

Single cell suspensions of splenocytes from Rag2$^{-/-}$OT-I mice were lysed with ACK red blood cell lysis buffer and then washed with PBS. CD8$^+$ T cells were isolated by negative selection using a CD8$^+$ T cell isolation kit (mouse) from Miltenyi Biotec following the manufacturer's instructions. The purity and viability of isolated CD8$^+$ T cells were measured by flow cytometry (all isolations were >95% purity and >95% viability). For proliferation assays, CD8$^+$ T cells were washed with PBS twice and then stained with Violet Proliferation Dye 450 (V450) following the manufacturer's instructions.

CD8$^+$ T cell proliferation assay

The isolated, V450 stained, CD8$^+$ T cells from Rag2$^{-/-}$OT-I mice mentioned above were used for the proliferation assay. To induce CD8$^+$ T cell activation and proliferation in the absence of stimulation or an exogenous cytokine like IL-2, we added fixed APCs which stimulates CD8$^+$ T cell proliferation weakly.[5] As to the preparation of fixed APCs, we pretreated CD45.1 splenocytes with SIINFEKL at 25uM in the presence of LPS, LBP and CD14 all at 100ng/mL for 6h. After stimulation, the splenocytes were fixed with 0.8% PFA for 5 min, washed and added to cell culture to provide a consistent stimulatory signal for CD8$^+$ activation.

Platelets from indicated conditions were used at a ratio of platelet:CD8=50:1 (comparable to physiological ratios). Fixed CD45.1 were used at equal numbers to CD8$^+$ T cells. For CD3/CD28 antibody-stimulated conditions, we coated the cell culture plate with a CD3 antibody at 1μg/mL

overnight at 4°C, washed with PBS prior to the experiment, and then added a CD28 antibody at 2μg/mL at the time of the experiment.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from platelets lysed in TRIZOL reagent. Quantitative real-time PCR analysis was then performed with Quanta Perfecta SYBR Supermix kit. HLA-A mRNA expression was normalized to human TUBB1. Thermal cycling conditions were as follows: 2 min at 95°C, 40 cycles of 30 sec at 94°C, and 30 sec at 54°C, 45 sec at 72°C, followed by 10 min at 72°C. Primers used for HLA-A and TUBB1 are: HLA-A Forward 5'-GATTACATCGCCTTGAACGAGG-3', HLA-A reverse 5'- GCAGGGTAGA AGCTCAGGG-3'; TUBB1 forward 5'- GCGTGTACTACAACGAAGCCTAC-3', TUBB reverse 5'-AAAACTGTCGGGTTGAAAGAGAG-3'.

Flow cytometry

For the MHC-I expression assay, platelets were incubated with anti-human HLA-A, B, C, anti-mouse H-2D, H-2K, or anti-mouse SIIN:H-2K^{b} antibodies (depending on the experiment) at room temperature in the dark (20-30 min). Platelets were then fixed with an equal volume of 4% paraformaldehyde for 15 min. Samples were acquired on a flow cytometer immediately or, in some experiments, washed with platelet binding buffer after fixation and acquired on a flow cytometer within 2 hours. For CD8^{+} T cells, dendritic cells, and monocytes, single cell suspensions of PBMCs, lymph nodes, and splenocytes, cells were Fc blocked using rat anti-mouse CD16/32 (clone2.4G2, BD 553141). Cells were then incubated with the appropriate antibody (depending on the experiment) or the viability dye at room temperature for 30 min, and

then fixed and washed. For the tetramer staining, cells were incubated with either isotype tetramer control or a specific tetramer at 4°C for 30 min after Fc blocking. Cells were then washed, and stained for CD45, TCRβ, CD8, followed by fixation. Please see Supplemental Table 2 for antibody information. The intracellular interferon-γ (IFN- γ) staining was carried out following BD Fixation/Permeabilization Solution Kit protocol. Briefly, cells were stimulated with eBioscience Cell Stimulation Cocktail for 4-6h *in vitro*, followed by surface staining including TCRβ, CD8, CD44 and CD62L, fixation/permeabilization and intracellular staining for IFN-γ.

Western blotting

Washed platelets were saved in lysis buffer, separated by gel electrophoresis, electrotransferred onto PVDF membranes, and then blocked in TBS, 0.1% Tween 20 and 5% dry milk. Western blots were probed with antibodies against B2M (Abcam), human MHC-I (Santa Cruz) or mouse MHC-I (Bio-Rad). Beta-actin (Santa Cruz) was used as a control for protein loading and densitometric quantification.

Immunofluorescence microscopy

For platelet MHC-I staining, platelets ($0.5\text{-}1\times10^8$/mL) suspended in Medium 199 were fixed with an equal volume of 4% PFA and cytospun onto a cover glass. Platelets were then washed, permeabilized with 0.1% Triton-X 100 in HBSS, blocked with 10% goat serum, and incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 2 hours. For platelet and $CD8^+$ T cell co-culture and staining, chamber wells were pre-coated with either fibrinogen or poly-L-lysine. For fibrinogen coating,

chamber wells were coated with fibrinogen at 100μg/mL at 37°C for 30 min or overnight at 4°C and blocked with 2% serum albumin. For poly-L-lysine coating, chamber wells were coated with poly-L-lysine at 100μg/mL overnight at 4°C. OVA or SIINFEKL pulsed platelets were then washed and seeded onto the fibrinogen coated chamber wells at a density of 4×10^7 platelets/mL for 30-40 min. The wells were then carefully washed to remove nonadherent platelets. $CD8^+$ T cells isolated from *Rag2*$^{-/-}$OT-I mice were next added to the chamber wells at a density of 4×10^6/mL $CD8^+$ T cells/well (platelet: $CD8^+$ T cell ratio=10:1). After 30 minutes of co-incubation, unbound cells were washed off. The remaining cells were fixed with 2% PFA, followed by permeabilization with 0.1% Triton, blocking with 2% serum albumin, and finally stained with primary and secondary antibodies. Alternatively, SIINFEKL pulsed platelets were seeded onto poly-L-lysine coated chamber wells. Since platelets remain quiescent on poly-L-lysin, platelets were seeded at a higher concentration of 6.6×10^7 platelets/mL for 1 hour. The nonadherent platelets were carefully removed. $CD8^+$ T cells from *Rag2*$^{-/-}$OT-I mice were added at a platelet: $CD8^+$ T cell ratio of 10:1 for 1 hour. Samples were fixed with 2% PFA and then washed. Cells were allowed to settled overnight at 4°C, washed again the following day, treated with ammonium chloride 50mM for antigen retrieval (15 minutes), and continued for further permeabilization with 0.1% Triton-X 100 in HBSS, blocking with 10% goat serum, incubation with primary antibodies at 4°C overnight, and immunofluorescent secondary antibodies at room temperature for 2 hours. Antibodies used include anti-beta2 microglobulin antibody, anti-CD8a antibody, anti-integrin αIIb antibody, anti-GPIbβ derivative DyLight649, DAPI and secondary antibodies (please see Supplemental Table 2). 2D and Z-stack images were acquired using an Olympus IX81, FV300 (Olympus, Melville, NY) confocal-scanning microscope equipped with 60×/1.42 N.A. and 40×/1.35 N.A. oil objectives. Four laser excitation wavelengths were used

(405, 488, 568 and 640nm). Z-stack images were used for the reconstructed 3D structure, for 3D videos, and max projection images.

Statistical Analyses

Statistical analyses were performed using Prism 8 software (GraphPad Software Inc.). Two-tailed Student t test or ANOVA with post hoc tests were used to compare continuous variables when data were normally distributed. The Mann-Whitney test was used to compare non-normally distributed variables. Log rank (e.g., Mantel-Cox) survival test was used for survival. Two-tailed P values of <0.05 were considered statistically significant.

Supplemental Tables

Supplemental Table 1. Demographic data from septic patients and healthy donors. SOFA score refers to the Sequential Organ Failure Assessment score, and is an index of illness severity in septic patients.

	Septic Patients	Healthy Donors	
Total	n=46	n=20	
Age, years (mean±SD)	57.6 (±14.3)	38.7 (±12.7)	P<0.0001
Male Sex, n (%)	18 (39.1%)	8 (40%)	p=0.23
SOFA Score	5.9 (±3.3)	--	--

Supplemental Table 2. List of antibodies and reagents.

Antibodies and reagents	Provider	Catalog No.
Antibodies		
Anti-CD41 antibody	Abcam	ab11024
Anti-MHC class I antibody [ER-HR 52]	Abcam	ab15681
Mouse IgG2a, kappa monoclonal	Abcam	ab18415
Rat IgG2a, kappa monoclonal [RTK2758]	Abcam	ab18450
Anti-HLA Class I antibody [W6/32]	Abcam	ab23755
Mouse IgG1 monoclonal	Abcam	ab81032
Anti-beta 2 microglobulin antibody	Abcam	Ab75853
PE-Cy7 Mouse IgG2a, κ Isotype Control	BD Biosciences	552868
Purified Rat Anti-Mouse CD16/CD32	BD Biosciences	553142
FITC Mouse Anti-Human CD41a	BD Biosciences	555466
PE Mouse Anti-Human CD41a	BD Biosciences	555467
PE-Cy™7 Mouse Anti-Mouse CD45.2	BD Biosciences	560696
Human BD Fc Block	BD Biosciences	564220
Pacific Blue™ anti-mouse H-2Kb antibody	Biolegend	116514
PerCP/Cyanine5.5 anti-mouse H-2Kb antibody	Biolegend	116516
Alexa Fluor 488 anti-mouse CD11c Antibody	Biolegend	117311
APC anti-mouse H-2Kb bound to SIINFEKL antibody	Biolegend	141606
Alexa Fluor® 488 anti-human CD41 Antibody	Biolegend	303724
APC anti-human HLA-A,B,C Antibody	Biolegend	31110
APC Mouse IgG1, κ Isotype Ctrl Antibody	Biolegend	400119
Alexa Fluor® 488 Mouse IgG1, κ Isotype Ctrl Antibody	Biolegend	400132
Pacific Blue™ Mouse IgG2a, κ Isotype Ctrl Antibody	Biolegend	400235
PerCP/Cyanine5.5 Mouse IgG2a, κ Isotype Ctrl Antibody	Biolegend	400257
Alexa Fluor® 488 Armenian Hamster IgG Isotype Ctrl Antibody	Biolegend	400923
MHC Class I antibody 2G5	Bio-Rad	MCA2189
H-2D(b) LCMV GP var C41M 33-41 KAVYNFATM Brilliant Violet 421-Labeled Tetramer	NIH Tetramer Core Facility	NA

H-2K(b) chicken ova 257-264 SIINFEKL Brilliant Violet 421-Labeled Tetramer	NIH Tetramer Core Facility	NA
Integrin beta 3/CD61 Antibody (BV4)	NOVUS	NB600-1342
Integrin αIIb Antibody (H-160)	Santa Cruz	sc-15328
MHC class I Antibody (W6/32)	Santa Cruz	sc-32235
β-Actin Antibody (C4)	Santa Cruz	sc-47778
CD41a Monoclonal Antibody, PE-Cyanine7	ThermoFisher	25-0411-82
CD41a Monoclonal Antibody, FITC	ThermoFisher	11-0411-82
CD44 Monoclonal Antibody, FITC	ThermoFisher	11-0441-85
CD45.2 Monoclonal Antibody, FITC	ThermoFisher	11-0454-85
Rat IgG1 kappa Isotype Control, FITC	ThermoFisher	11-4301-81
Mouse IgG2a kappa Isotype Control, FITC	ThermoFisher	11-4724-81
CD8a Monoclonal Antibody, PE	ThermoFisher	12-0081-83
Rat IgG2a kappa Isotype Control, PE	ThermoFisher	12-4321-80
CD8a Monoclonal Antibody	ThermoFisher	14-0081-82
CD3e Monoclonal Antibody (145-2C11), APC	ThermoFisher	17-0031-83
CD19 Monoclonal Antibody, APC	ThermoFisher	17-0193-82
Rat IgG2a kappa Isotype Control, APC	ThermoFisher	17-4321-81
Armenian Hamster IgG Isotype Control	ThermoFisher	17-4888-81
MHC Class I (H-2Db) Monoclonal Antibody (28-14-8), APC,	ThermoFisher	17-5999-82
CD4 Monoclonal Antibody, PE-Cyanine7,	ThermoFisher	25-0041-82
CD11c Monoclonal Antibody, PE-Cyanine7	ThermoFisher	25-0114-82
Armenian Hamster IgG Isotype Control, PE-Cyanine7	ThermoFisher	25-4888-81
Armenian Hamster IgG Isotype Control, PerCP-Cyanine5.5	ThermoFisher	45-4888-80
TCR beta Monoclonal Antibody, PerCP-Cyanine5.5	ThermoFisher	45-5961-82
CD62L (L-Selectin) Monoclonal Antibody, APC-eFluor 780	ThermoFisher	47-0621-82
Rat IgG2a kappa Isotype Control, APC-eFluor 780	ThermoFisher	47-4321-80
CD4 Monoclonal Antibody (GK1.5), eFluor 450	ThermoFisher	48-0041-82
CD41a Monoclonal Antibody, eFluor 450	ThermoFisher	48-0411-82
IFN gamma Monoclonal Antibody	ThermoFisher	50-7311-82
Rat IgG2b kappa Isotype Control, eFluor 450	ThermoFisher	48-4031-80

Rat IgG1 kappa Isotype Control, eFluor 660	ThermoFisher	50-4301-80
Rat IgG1 kappa Isotype Control, eFluor 450,	ThermoFisher	48-4301-80
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A-11029
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	ThermoFisher	A-11030
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	ThermoFisher	A-11081
Donkey anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A-21208
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A-21245
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A-21247
DAPI	ThermoFisher	D1306
Wheat Germ Agglutinin, Alexa Fluor™ 555 Conjugate	ThermoFisher	w32464
CD3 Monoclonal Antibody (17A2), Functional Grade	ThermoFisher	16-0032-85
CD28 Monoclonal Antibody (CD28.2), Functional Grade	ThermoFisher	16-0289-85
Anti-platelet antibody	Emfret	R300
Negative control for #R300	Emfret	C301
Anti-GPIbbeta derivative	Emfret	X649
Chemicals, Peptides, Proteins and Glycolipids		
albumin human solution	Baxalta US Inc	NDC code. 0944-0492-02
Adjuvant, Complete *(Freund)*	BD	263810
Adjuvant, Incomplete *(Freund)*	BD	263910
Recombinant Human TPO	PeproTech	300-18
Fibrinogen, Human Plasma	Millipore Sigma	341576
Albumin from chicken egg white	Millipore Sigma	A5503
Lipopolysaccharides from *Escherichia coli* O55:B5	Millipore Sigma	L6529-1MG
Bortezomib	Millipore Sigma	5.04314
MG-132	Millipore Sigma	M7449-200UL
Lactacystin	Millipore Sigma	L6785-.2MG

Human CD14 Recombinant Protein	FisherScientific	383CD050
Human LBP Recombinant Protein	FisherScientific	870LP025
Human IFN gamma Recombinant Protein	ThermoFisher	RP-8607
SCF (C-Kit Ligand) Recombinant Human Protein	ThermoFisher	PHC2116
DQ Ovalbumin (DQ-OVA)	ThermoFisher	D12053
eBioscience Cell Stimulation Cocktail	ThermoFisher	00-4975-93
Commercial Assays		
CD8a+ T Cell Isolation Kit, mouse	Miltenyi Biotec	130-104-075
Fixation/Permeabilization Solution Kit	BD	554714
AST Activity Assay Kit	Millipore Sigma	MAK055
20S Proteasome Activity Assay	Millipore Sigma	APT280

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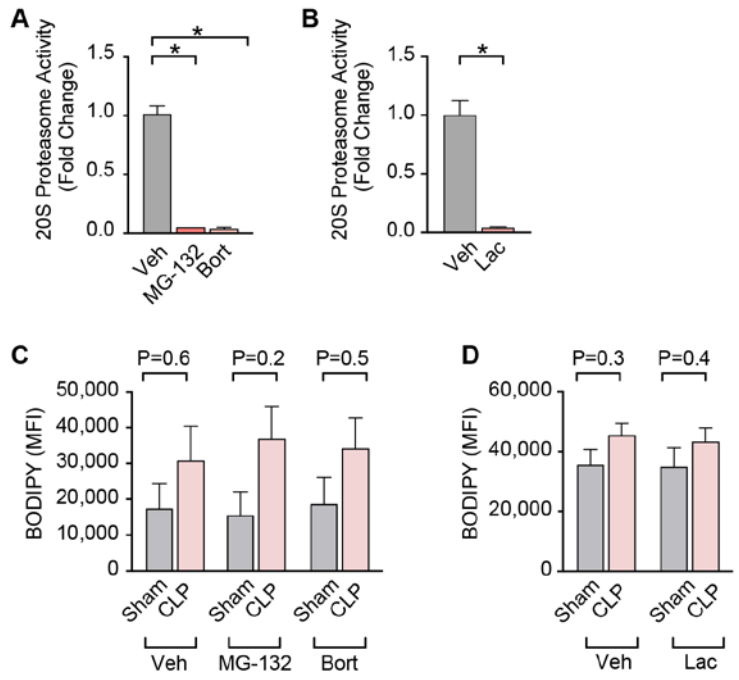
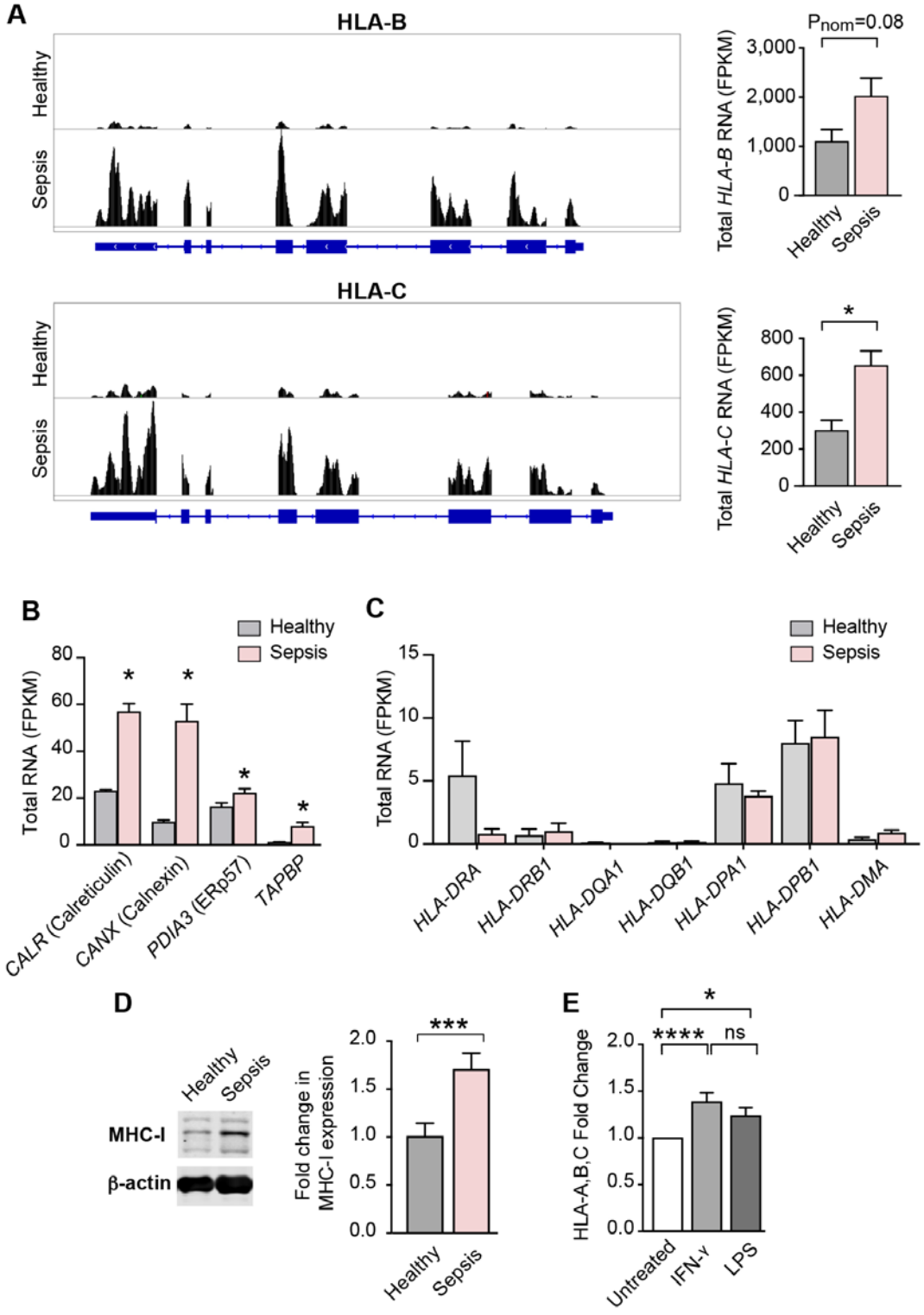
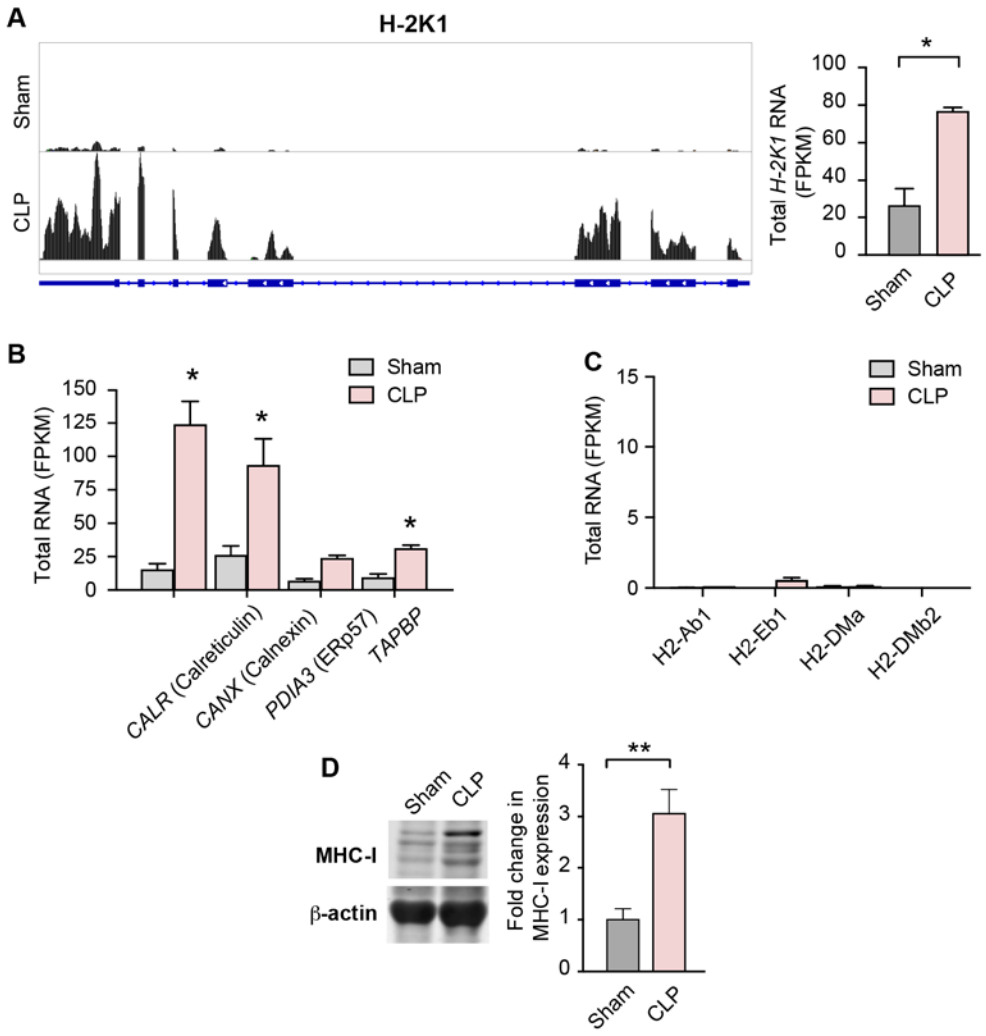
Supplemental Figures



Supplemental Figure 1. Proteasome independent processing of DQ-OVA by platelets. Unstimulated, washed platelets isolated from wild-type mice were treated with three different proteasome inhibitors: MG-132, bortezomib (Bort), or lactacystin (Lac) at 40μM final, or with corresponding vehicle control (Veh) for 30 min at 37°C. **(A-B)** All three inhibitors blocked platelet 20S proteasome activity under basal conditions (i.e., non-septic conditions). **(C-D)** Wild-type mice were subject to either CLP sepsis or sham control, as described in the methods. Washed platelets isolated from either sham or CLP mice on day 4 were pre-treated with one of the three proteasome inhibitors (MG-132, Bort, or Lac) for 30 min at 37°C, then pulsed with DQ-OVA at 100μg/mL for 60 min and then stained for CD41. BODIPY signal was measured in platelets immediately (MFI: mean fluorescence intensity). One-way ANOVA with Sidak's post hoc test and paired t-test were used. (n=3-13, *P<0.05).

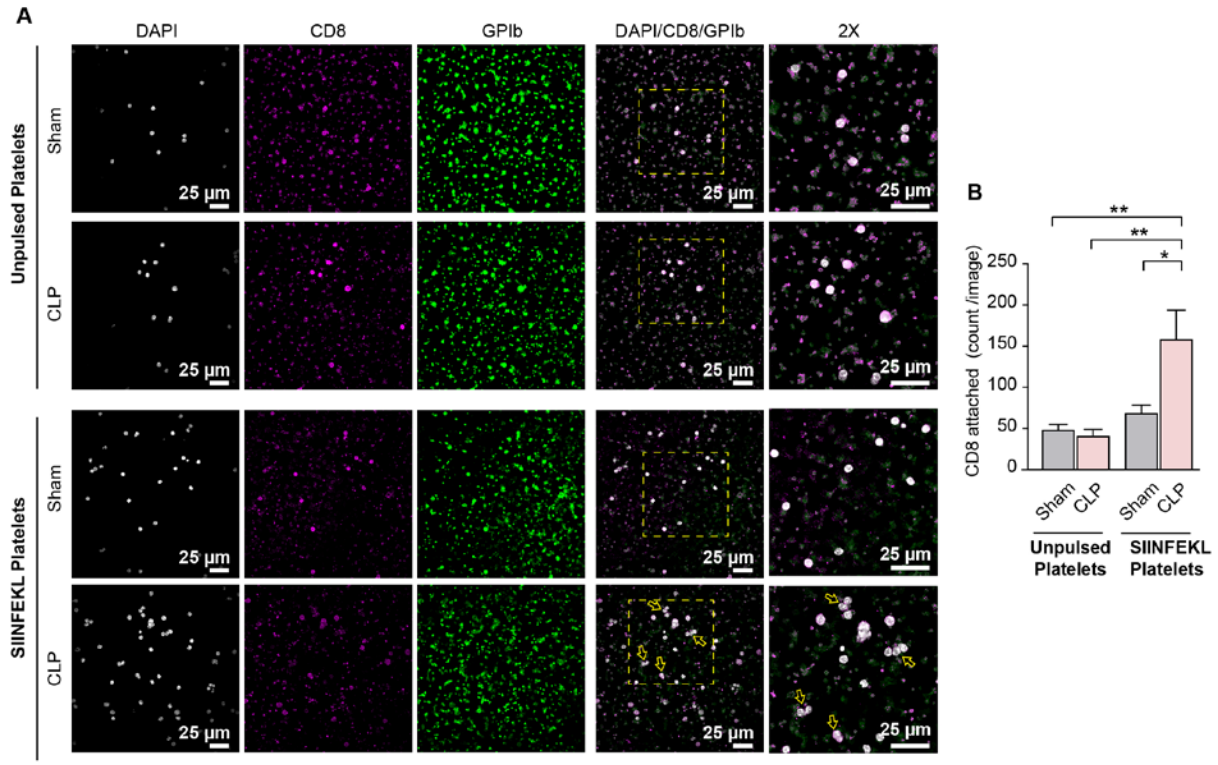

A
HLA-B
Healthy
Sepsis
Pnom=0.08
Total HLA-B RNA (FPKM)
3,000
2,000
1,000
0
Healthy
Sepsis
HLA-C
Healthy
Sepsis
*
Total HLA-C RNA (FPKM)
800
600
400
200
0
Healthy
Sepsis
B
Healthy
Sepsis
Total RNA (FPKM)
80
60
40
20
0
*
*
*
*
CALR (Calreticulin)
CANX (Calnexin)
PDIA3 (ERp57)
TAPBP
C
Healthy
Sepsis
Total RNA (FPKM)
15
10
5
0
HLA-DRA
HLA-DRB1
HLA-DQA1
HLA-DQB1
HLA-DPA1
HLA-DPB1
HLA-DMA
D
Healthy
Sepsis
MHC-I
β-actin
***
Fold change in MHC-I expression
2.0
1.5
1.0
0.5
0.0
Healthy
Sepsis
E
*
****
ns
HLA-A,B,C Fold Change
2.0
1.5
1.0
0.5
0.0
Untreated
IFN-γ
LPS

Supplemental Figure 2. MHC-I pathway genes are selectively upregulated in human platelets during sepsis. (**A, left**) Representative IGV browser images illustrating the RNA expression of MHC-I alleles HLA-B and HLA-C in platelets from a healthy donor and a septic patient. (**A, right**) Bar graphs with average RNA expression of MHC-I alleles HLA-B and -C in platelets from healthy donors and septic patients. (**B**) Genes involved in the MHC-I pathway in platelets from healthy donors and septic patients. (**C**) The RNA expression of genes involved in the MHC-II pathway in platelets from healthy donors and septic patients. (**D**) MHC-I protein in human platelets from healthy controls or septic patients was quantified by western blotting, with representative gels on the left. (**E**) Day 13 $CD34^+$ derived megakaryocytes were either untreated, or treated with IFN-γ 250U/mL or LPS 100ng/mL for 20h. The cell surface expression of MHC-I (HLA-A,B,C) on megakaryocytes was measured by flow cytometry. The fold increase of MHC-I MFI was calculated and normalized to untreated conditions. (Patient samples were collected on day 0 for all experiments above, unpaired t test and one-way ANOVA with Sidak's post hoc test, n=4-5, *P<0.05, ***P<0.001, ****P<0.0001, ns: not significant).

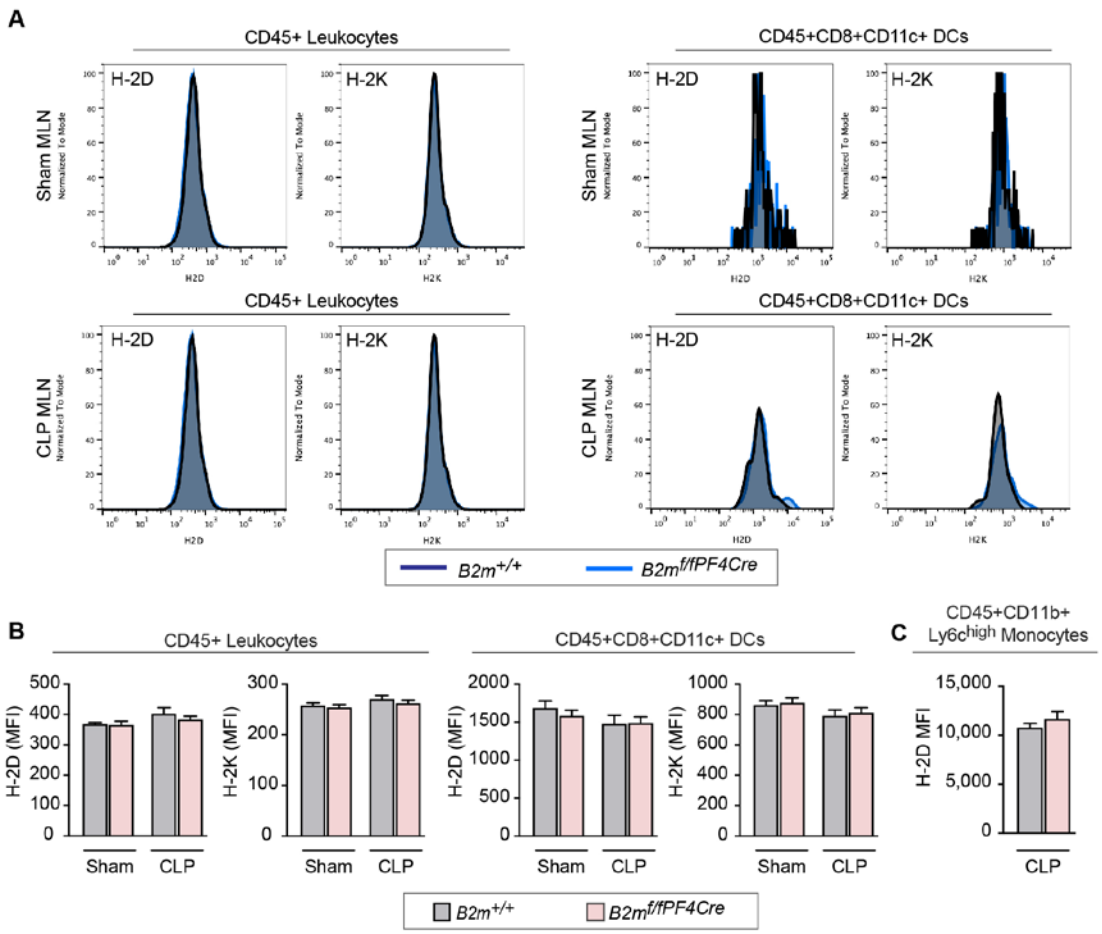


Supplemental Figure 3. MHC-I pathway genes are selectively upregulated in murine platelets during sepsis. (**A, left**) Representative IGV browser images illustrating the RNA expression of MHC-I allele H2-K1 in platelets from a sham treated and CLP septic mouse. (**A, right**) Bar graphs with average RNA expression of MHC-I allele H-2K in platelets from sham-treated and CLP septic mice (n=3-4). (**B**) Genes involved in the MHC-I pathway in platelets from sham treated and CLP septic mice (n=3-4). (**C**) The RNA expression of genes involved in the MHC-II pathway in platelets from sham treated and CLP septic mice. (**D**) MHC-I protein in

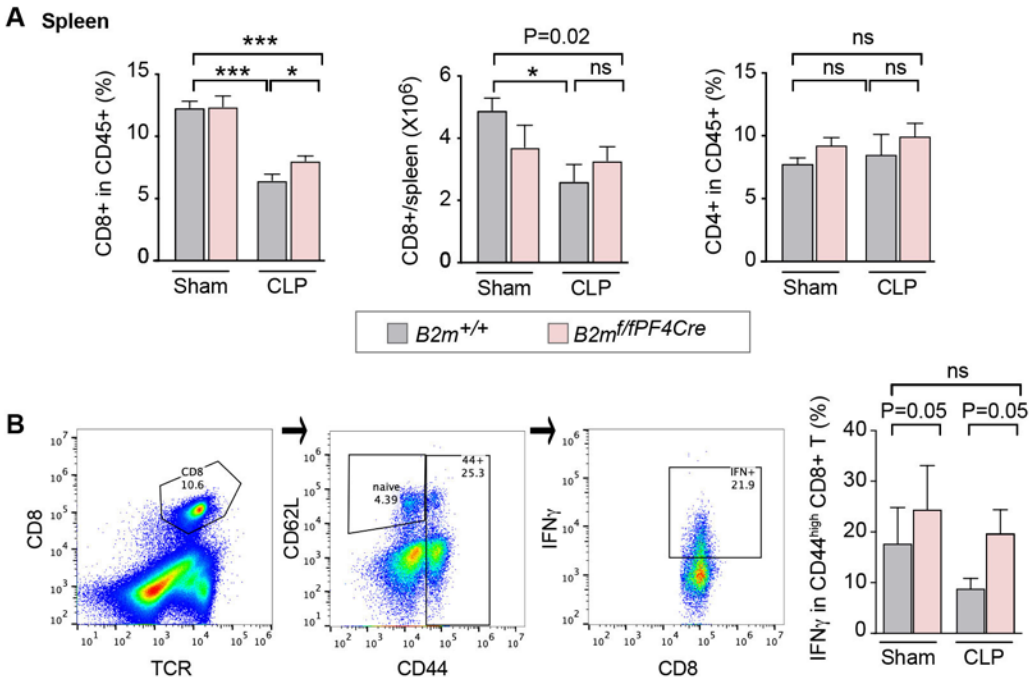
platelets from sham control mice or CLP septic mice was quantified by western blotting, with representative gels on the left (n=3, unpaired t test, *P<0.05).



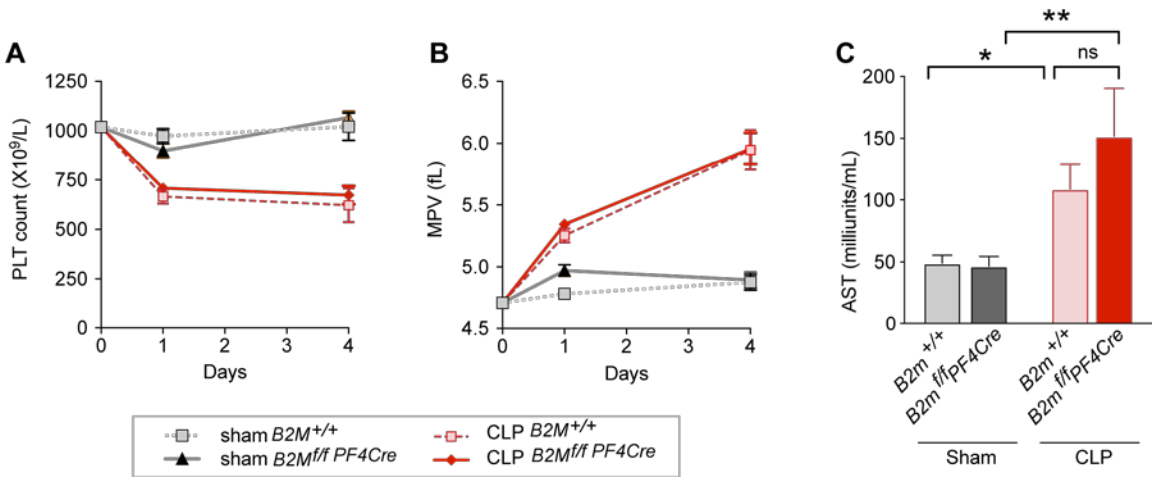
Supplemental Figure 4. During sepsis, exogenous antigen presented by platelet MHC-I mediates increased CD8$^+$ T cell binding. (**A**) Washed platelets isolated from either sham or CLP septic mice were seeded onto poly-L-lysine coated chamber wells, and co-cultured with SIIN:H-2K^b specific CD8$^+$ T cells from Rag2$^{-/-}$OT-I mice (t=1h). Unbound cells were then washed off. Attached CD8$^+$ T cells were fixed and stained for confocal immunofluorescent imaging. Representative max projection images taken at 60X are shown (yellow arrows illustrate CD8$^+$ T cells that are in close contact with platelets). (**B**) The number of CD8$^+$ T cells attached to poly-L-lysine were quantified (n=4, images at 40X, one-way ANOVA with Dunnett's post hoc test; *P<0.05, **P<0.01).



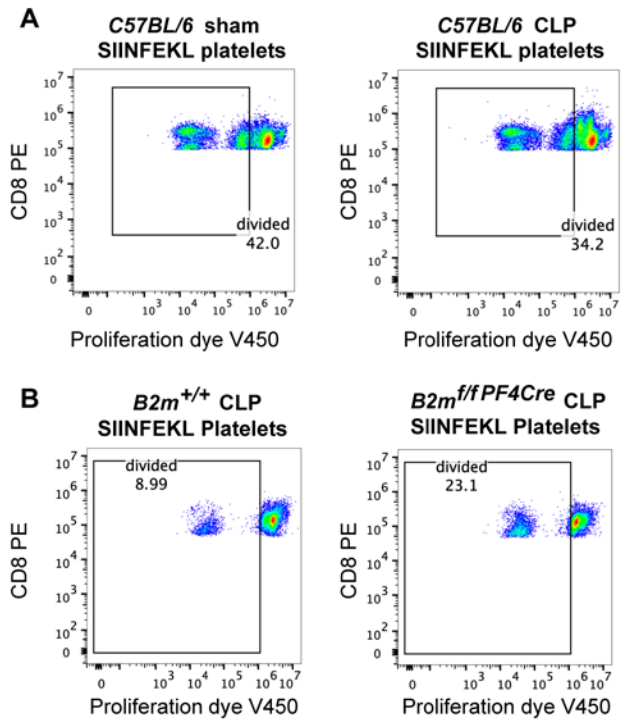
Supplemental Figure 5. MHC-I expression on total leukocytes, dendritic cells and monocytes is not altered in platelet lineage specific B2m deficient mice. (**A**) Representative flow plots showing the expression of MHC-I alleles H-2D and H-2K on $CD45^+$ leukocytes and $CD45^+CD8^+CD11c^+$ dendritic cells from $B2m^{+/+}$ and $B2m^{f/f}$-Pf4Cre mice subject to sham surgery or CLP sepsis. (**B**) The mean fluorescence intensity (MFI) of MHC-I alleles H-2D and H-2K on $CD45^+$ leukocytes and dendritic cells from $B2m^{+/+}$ and $B2m^{f/f}$-Pf4Cre mice subject to sham surgery or CLP sepsis. Panels A and B from n=4-5 mice/group. (**C**) The MFI of H-2K on $CD45^+CD11b^+Ly6c^{high}$ monocytes from $B2m^{+/+}$ and $B2m^{f/f}$-Pf4Cre mice subject to CLP, n=3 mice/group. One-way ANOVA with Sidak's post hoc test.



Supplemental Figure 6. Splenic CD8$^+$ T cells are higher in B2m$^{f/f}$-Pf4Cre mice during sepsis. (A) CD8$^+$ T cell frequency (far left) and absolute number (middle) were higher in B2m$^{f/f}$-Pf4Cre mice during sepsis. In contrast, CD4$^+$ T cells were not affected (far right). **(B)** Representative flow plots showing the gating strategy for intracellular IFN-γ staining in the CD44^{high}CD8$^+$ population and quantification of intracellular IFN-γ expression in the splenic CD44^{high}CD8$^+$ T cells. (n=11-14, One-way ANOVA with Sidak's post hoc test. *P<0.05, ***P<0.001, ns: not significant; upper significance bars in panels A and B show the p-value for the comparison between sham control condition in in B2m$^{+/+}$ mice and CLP condition in B2m$^{f/f}$-Pf4Cre mice).



Supplemental Figure 7. Evaluation of thrombocytopenia and liver injury in platelet specific MHC-I deficient mice. (**A-B**) Circulating platelet number and size are not affected by B2m deficiency. Platelet count **(A)** and mean platelet volume (MPV, panel **B)** showed comparable levels of thrombocytopenia in both $B2m^{+/+}$ and $B2m^{f/f}$-Pf4Cre mice after CLP (n=12-19 mice/group). (**C**) Sepsis induced significantly increased aspartate aminotransferase (AST) in both $B2m^{+/+}$ and $B2m^{f/f}$-Pf4Cre mice. AST is a marker of liver injury, in this case induced by sepsis. AST levels were quantified in the serum collected on day 4 after CLP (n=6-11, One-way ANOVA with Sidak's post hoc test, *P<0.05, **P<0.01).



Supplemental Figure 8. Platelets from septic mice reduce antigen specific CD8$^+$ T cell activation and proliferation *in vitro* in the presence of CD3/CD28 antibodies. (A-B) To evaluate the effect of platelet MHC-I on CD8$^+$ T cell proliferation in the presence of exogenous stimulatory signal, CD3/CD28 antibodies were added to Rag2$^{-/-}$ OT-I CD8$^+$ T cells in addition to fixed SIINFEKL pulsed CD45.1 cells. Platelets isolated either from C57BL/6J sham or CLP mice, or from B2m$^{+/+}$ CLP and B2m$^{f/f}$-Pf4Cre CLP mice on day 4 after CLP sepsis were added to the co-culture. CD8$^+$ T cell proliferation was measured by V450 dye after 60h. Data shown are from an n=1 experiment using n=3-5 pooled mice for each condition.

Supplemental Video legends

Supplemental Video 1. **Rag2$^{-/-}$ OT-I CD8^{+} T cells do not bind to unpulsed platelets isolated from WT sham mice.** In this 3D video, CD8^{+} T cells are labelled with DAPI (blue) and CD8 (magenta). Platelets are labelled with GPIb (Green) (4 Mb, mwv file).

Supplemental Video 2. **Rag2$^{-/-}$ OT-I CD8^{+} T cells do not bind to unpulsed platelets isolated from WT CLP mice.** In this 3D video, CD8^{+} T cells are labelled with DAPI (blue) and CD8 (magenta). Platelets are labelled with GPIb (Green) (4 Mb, mwv file).

Supplemental Video 3. **Rag2$^{-/-}$ OT-I CD8^{+} T cells bind to SIINFEKL pulsed platelets isolated from WT sham mice.** In this 3D video, CD8^{+} T cells are labelled with DAPI (blue) and CD8 (magenta). Platelets are labelled with GPIb (Green) (5 Mb, mwv file).

Supplemental Video 3. **Rag2$^{-/-}$ OT-I CD8^{+} T cells bind to SIINFEKL pulsed platelets isolated from WT CLP mice.** In this 3D video, CD8^{+} T cells are labelled with DAPI (blue) and CD8 (magenta). Platelets are labelled with GPIb (Green) (5 Mb, mwv file).

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