Sepsis Alters the Megakaryocyte–Platelet Transcriptional Axis Resulting in Granzyme B–mediated Lymphotoxicity

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Rationale: Sepsis-related mortality results in part from immunodeficiency secondary to profound lymphoid apoptosis. The biological mechanisms responsible are not understood.

Objectives: Because recent evidence shows that platelets are involved in microvascular inflammation and that they accumulate in lymphoid microvasculature in sepsis, we hypothesized a direct role for platelets in sepsis-related lymphoid apoptosis.

Methods: We studied megakaryocytes and platelets from a murine-induced sepsis model, with validation in septic children, which showed induction of the cytotoxic serine protease granzyme B.

Measurements and Main Results: Platelets from septic mice induced marked apoptosis of healthy splenocytes *ex vivo*. Platelets from septic granzyme B null (–/–) mice showed no lymphotoxicity.

Conclusions: Our findings establish a conceptual advance in sepsis: Septic megakaryocytes produce platelets with acutely altered mRNA profiles, and these platelets mediate lymphotoxicity via granzyme B. Given the contribution of lymphoid apoptosis to sepsis-related mortality, modulation of platelet granzyme B becomes an important new target for investigation and therapy.

Keywords: blood platelets; sepsis; granzyme B; apoptosis

Despite several decades of advances in antimicrobials, critical care, and organ support modalities (1, 2), mortality rates from sepsis have remained largely unchanged at about 40% (3). Sepsis is responsible for 215,000 deaths annually in the United States, which is akin to mortality from acute myocardial infarction (3), making it the 10th leading cause of death (4). A recent paradigm shift indicates that sepsis-related mortality results in part from immunodeficiency secondary to profound lymphoid apoptosis (5). This apoptosis is considered a diagnostic hallmark of progressive sepsis and multiple organ dysfunction. However, the etiology of the apoptosis is unknown.

Studies of sepsis have demonstrated the accumulation of platelets in spleen and other end organs (6, 7). Furthermore, activated platelet-derived microparticles have cytotoxic activity toward vascular endothelium (8–10) and smooth muscle (10).

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

No studies have shown acute changes in platelet mRNA pools as a function of a systemic stimulus, such as experimental or clinical sepsis.

What This Study Adds to the Field

We determined that sepsis induces changes in the megakaryocyte–platelet transcriptional axis and show that the resulting platelets are strongly lymphotoxic. In addition, using platelets from a murine-induced sepsis model, we identified the potent cytotoxic serine protease, granzyme B, as the cause of this lymphotoxicity.

However, platelets are anucleate, having only cytoplasmic components imparted by megakaryocytes residing in the bone marrow, and are incapable of *de novo* gene transcription. Thus, these previous studies assumed that changes in platelet function were at the posttranscriptional level. Platelets do contain reservoirs of mRNA, and a number of studies have reported the transcriptome of human platelets using mRNA profiling (11–14). It has also been established that platelets regulate translation of their transcriptome in response to external stimuli (15–17). However, no studies have shown acute changes in platelet mRNA pools as a function of a systemic stimulus, such as experimental or clinical sepsis. Therefore, we hypothesized that responses of platelets to systemic perturbations in sepsis could lead to changes in mRNA expression of cell death–associated genes and that this *de novo* transcriptome would be imparted to platelets by megakaryocytes residing in bone marrow.

Our experiments to test this hypothesis led to two main findings. First, we characterized sepsis-induced changes in the megakaryocyte–platelet transcriptional axis and present a novel finding that the resulting platelets are strongly lymphotoxic. Second, using platelets from a murine-induced sepsis model, we identified the potent cytotoxic serine protease granzyme B as the cause of this lymphotoxicity. Some of the results of these studies have been previously reported in the form of abstracts (18, 19).

METHODS

Animals

Mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed and bred in a conventional animal facility. All experiments

were approved by our Institutional Animal Care and Use Committee. Cecal ligation and puncture was performed on male 8- to 12-week-old mice at time = 0 hours, as previously described (20). Briefly, under isoflurane anesthesia with spontaneous ventilation, the cecum was exposed through a 1-cm-long midline abdominal incision, ligated loosely with 4-0 silk ties (Ethicon, Cornelia, GA), and punctured twice proximally with an 18-gauge needle. Fecal material was expressed, and the bowel was replaced in the abdomen. The incision was closed with 4-0 nylon sutures. Mice were resuscitated with 4 ml/100 g of body weight of subcutaneous saline.

Platelet Isolation

Intracardiac blood was drawn directly into sodium citrate (Becton-Dickinson, Franklin Lakes, NJ) and immediately centrifuged at 770 rpm for 10 minutes at 25°C. Platelets were isolated from platelet-rich plasma by a single high-speed centrifugation over Ficoll-Paque Plus (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ). Microscopy of smears of platelet isolates showed >90% platelet purity. Platelets intended for mRNA studies were immediately placed in Trizol (Invitrogen, Carlsbad, CA). Platelets intended for functional studies were filtered through a 10-ml sepharose 2B gel column to remove extraneous proteins as described by Vollmar and colleagues (21). Platelet concentrations were measured using a manual hemocytometer, and concentrations were equalized between samples by diluting with PBS.

Megakaryocyte Isolation

Murine megakaryocytes were isolated from mouse tibial and femoral bone marrow by flushing with Iscove's modified Dulbecco's medium. The resulting marrow suspension was treated and passed through StemSep magnetic gravity columns (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol using biotin-labeled anti-CD42d antibodies for positive selection. Purity was confirmed by light microscopy with Wright's stain (Sigma-Aldrich, St. Louis, MO). mRNA was isolated as described for platelets.

Splenectomy

Healthy control spleens were removed and immediately ground through a 40- μ m mesh cell strainer. Splenocytes were centrifuged, washed, and layered over Ficoll-Paque Plus (GE Healthcare Bio-Sciences). CD4⁺ cells were isolated using StemSep magnetic gravity columns according to the manufacturer's protocol.

Expression Profiling

Expression values were calculated using the dChip difference model probe set algorithm (<http://biosun1.harvard.edu/complab/dchip/>) and Probe Logarithmic Intensity Error Estimation (PLIER) (Affymetrix, Santa Clara, CA) algorithm. dChip and PLIER signals were imported into Hierarchical Clustering Explorer (22), and the resulting unsupervised clusters were examined visually for appropriate grouping of profiles. The signals from the algorithm with the most appropriate profile grouping were used for all subsequent analyses within each species (i.e., murine = dChip, human = PLIER) and imported into GeneSpring GX (Agilent Technologies, Santa Clara, CA). The murine dataset (NCBI GEO Record #GSE10343) and human dataset (NCBI GEO Record #GSE10361) were normalized within each chip to the 50th percentile and per gene to control chips. Using the cross-gene error model without multiple testing corrections, one-way ANOVA ($P \leq 0.001$) generated a list of differentially expressed probe sets over time.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) per the manufacturer's protocol. DNA primers (Invitrogen) were designed according to known gene sequences as follows: granzyme A (forward) 5'-GAA CCA CTG CTA CTC GGC ATC TGG [FAM]TC-3'; granzyme A (reverse) 5'-CAG AAA TGT GGC TAT CCT TCA CC-3'; granzyme B (forward) 5'-GAC GAT CCT GCT CTG ATT ACC CAT CG[FAM] C-3'; granzyme B (reverse) 5'-TCA GAT CCT GCC ACC TGT CCT A-3'. GAPDH-containing wells served as positive controls, and polymerase-free wells served as negative controls. Reactions were run using an ABI

PRISM 7900HT polymerase chain reaction instrument (Applied Biosystems, Foster City, CA), and relative gene expression levels were calculated using Sequence Detection System 2.2 Software (Applied Biosystems). Expression values were normalized relative to sample GAPDH mRNA expression.

Detection of Apoptosis

CD4⁺ splenocytes from healthy control mice were coincubated with platelets isolated from control or septic mice for 90 minutes at 37°C and 5% CO₂ with or without platelet pretreatment with 10 ng/mL of recombinant tumor necrosis factor (TNF)- α (Sigma-Aldrich) for 90 minutes. Splenocyte apoptosis was evaluated by TiterTACS (Trevigen, Gaithersburg, MD), a quantitative colorimetric assay for *in situ* detection of DNA fragmentation. All samples were run in triplicate according to the manufacturer's protocol, with data normalized to negative and nuclease-induced positive controls.

Statistical Analysis

Data were maintained in Microsoft Excel 2007 (Microsoft, Redmond, WA). Statistical significance was tested with SPSS 15 (SPSS, Chicago, IL) using paired or unpaired *t* tests. Results are reported as mean $\pm$ SEM) unless otherwise specified.

RESULTS

Sepsis Induces Platelet Cell Death Gene Expression

All mice that underwent cecal ligation and puncture (CLP) developed signs and symptoms consistent with peritoneal sepsis, including decreased grooming, lethargy, and gross pathologic peritonitis at time of death. These mice developed significant weight loss over 48 hours (mean $\pm$ SEM_{0h} versus 48h: $-14.8 \pm 1.6\%$; $P < 0.0001$). Fourteen out of the 96 mice studied (14.6%) died between 6 and 48 hours status after CLP and were not included in the final analyses.

Expression profiles (Mouse 430 plus 2.0 GeneChips; Affymetrix) of platelet mRNA pooled from five mice at each time point (0-naive, 24-, and 48-h status after CLP) showed 59 probe sets, representing 56 unique genes, that were differentially regulated over the time interval studied (Table 1). These genes were primarily related to gene ontology biological process groups previously well described in the response to sepsis: cell adhesion, cell growth regulation, chemotaxis, inflammatory and immune responses, proteolysis, and signal transduction. Of these, six probe sets belonged to the gene ontology molecular function group for cell death (GO:0008219). In particular, between 0- and 48-hour granzymes A and B, potent cytotoxic serine proteases were > 100-fold up-regulated (fold change, 549.6 and 141.3, respectively).

We explored expression of these cell death genes in human sepsis in an Institutional Review Board–approved study of septic children ($n = 17$) between the ages of 1 and 18 years (8.8 ± 1.3 years). Nine participants (53%) were male. The diagnosis of sepsis was made using criteria adapted for pediatrics from the consensus definitions for sepsis (23–25). We collected clinical and laboratory data (i.e., the most extreme value in the previous 24 hours) over 72 hours. Relative clinical severity was determined by unsupervised clustering of all raw clinical and laboratory data in Hierarchical Clustering Explorer (<http://www.cs.umd.edu/hcil/hce/>) (Figure 1). The participants clearly clustered into two groups by clinical and laboratory variables. Group 1 ($n = 6$) was designated “severe” because it had significantly higher severity of illness scores (i.e., mean PRISM III (26) score [17.0 ± 2.7 vs. 4.5 ± 1.1 ; $P < 0.001$] and longer hospital length of stay [45.5 ± 10.6 vs. 13.7 ± 2.8 d; $P = 0.029$]). Group 2 ($n = 11$) was designated “moderate” and was not significantly different from the severe group for other analyzed outcome variables, including mortality and presence of shock.

TABLE 1. DIFFERENTIALLY REGULATED PROBE SETS (N = 59) BETWEEN 0-HOUR CONTROL MICE AND SEPTIC MICE AT 24- AND 48-HOUR STATUS AFTER CECAL LIGATION AND PUNCTURE

Affymetrix Probe Set ID	24-h Fold Change	48-h Fold Change	Genbank ID	Gene Symbol	Gene Name
1427747_a_at	1593.0	540.1	X14607	Lcn2	lipocalin 2
1440865_at	276.4	202.3	BB193024	Ifitm6	interferon-induced transmembrane protein 6
1419764_at	189.6	181.6	NM_009892	Chi3l3	chitinase 3-like 3
1442339_at	185.7	606.5	BB667930	MGI:3524944	stefin A2 like 1
1417898_a_at*	156.7	549.6	NM_010370	Gzma	granzyme A
1418809_at	153.0	530.7	NM_011087	Pira1	paired-Ig-like receptor A1
1449984_at	137.5	206.3	NM_009140	Cxcl2	chemokine (C-X-C motif) ligand 2
1451563_at	128.4	1831.0	AF396935	Emr4	EGF-like module containing, mucin-like, hormone receptor-like sequence 4
1456250_x_at	126.3	324.6	BB533460	Tgfb1	transforming growth factor, beta induced
1422013_at	120.3	759.8	NM_011999	Clec4a2	C-type lectin domain family 4, member a2
1436530_at	109.6	287.0	AA666504		CDNA clone MGC:107680 IMAGE:6766535
1450826_a_at	104.9	524.0	NM_011315	Saa3	serum amyloid A 3
1419394_s_at	104.6	48.6	NM_013650	S100a8	S100 calcium binding protein A8 (calgranulin A)
1424254_at	98.9	86.1	BC027285	Ifitm1	interferon-induced transmembrane protein 1
1442798_x_at	93.7	104.1	BB324660	Hk3	hexokinase 3
1456223_at	93.2	246.8	BF322016		Transcribed locus
1416635_at	83.0	683.2	NM_020561	Smpdl3a	sphingomyelin phosphodiesterase, acid-like 3A
1437478_s_at	81.2	200.3	AA409309	Efh2	EF hand domain containing 2
1422953_at	77.8	62.0	NM_008039	Fpr-rs2	formyl peptide receptor, related sequence 2
1436202_at	76.1	160.7	AI853644	Malat1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)
1419709_at	71.8	314.9	NM_025288	Stfa3	stefin A3
1450808_at	68.2	125.5	NM_013521	Fpr1	formyl peptide receptor 1
1430700_a_at	67.7	309.5	AK005158	Pla2g7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
1448756_at	67.5	23.5	NM_009114	S100a9	S100 calcium binding protein A9 (calgranulin B)
1420331_at	65.8	251.8	NM_019948	Clec4e	C-type lectin domain family 4, member e
1420330_at	64.9	220.2	NM_019948	Clec4e	C-type lectin domain family 4, member e
1423346_at	63.2	272.6	AV286991	Degs1	degenerative spermatocyte homolog 1 (Drosophila)
1418722_at	62.4	28.6	NM_008694	Ngp	neutrophilic granule protein
1429900_at	62.0	286.4	BM241296		RIKEN cDNA 5330406M23 gene
1434773_a_at	57.7	137.9	BM207588	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1
1420671_x_at	57.0	413.4	NM_029499	Ms4a4c	membrane-spanning 4-domains, subfamily A, member 4C
1419598_at	55.4	276.6	NM_026835	Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D
1421392_a_at	53.8	140.5	NM_007464	Birc3	baculoviral IAP repeat-containing 3
1418189_s_at	53.2	197.8	AF146523	Malat1	metastasis associated lung adenocarcinoma transcript 1 (noncoding RNA)
1435761_at	51.2	322.8	AW146083	Stfa3	stefin A3
1419599_s_at	49.6	362.9	NM_026835	Ms4a11	membrane-spanning 4-domains, subfamily A, member 11
1421408_at	49.3	246.7	NM_030691	Igsf6	immunoglobulin superfamily, member 6
1418204_s_at	46.1	282.2	NM_019467	Aif1	allograft inflammatory factor 1
1420394_s_at	40.3	89.0	U05264	Gp49a; Lilrb4	glycoprotein 49 A; leukocyte immunoglobulin-like receptor, subfamily B, member 4
1416530_a_at	39.0	168.2	BC003788	Pnp	purine-nucleoside phosphorylase
1437584_at	38.8	158.8	BE685667		Transcribed locus
1419647_a_at	38.6	109.6	NM_133662	Ier3	immediate early response 3
1419060_at	35.2	141.3	NM_013542	Gzmb	granzyme B
1448123_s_at	33.9	129.3	NM_009369	Tgfb1	transforming growth factor, beta induced
1429954_at	28.7	245.8	AK014135	Clec4a3	C-type lectin domain family 4, member a3
1448061_at	27.9	204.0	AA183642	Msr1	macrophage scavenger receptor 1
1438943_x_at	27.7	136.2	AV308148	Rpn1	ribophorin I
1439057_x_at	23.3	292.2	BB143557	Zdhhc6	zinc finger, DHHC domain containing 6
1448620_at	22.2	77.9	NM_010188	Fcgr3	Fc receptor, IgG, low affinity III
1455899_x_at	21.4	88.3	BB241535	Socs3	suppressor of cytokine signaling 3
1447277_s_at	20.9	630.1	BB785407	Pcyox1	prenylcysteine oxidase 1
1419209_at	20.5	407.7	NM_008176	Cxcl1	chemokine (C-X-C motif) ligand 1
1433699_at	17.7	58.8	BM241351	Tnfaip3	tumor necrosis factor, alpha-induced protein 3
1455908_a_at	16.3	212.3	AV102733	Scpep1	serine carboxypeptidase 1
1457666_s_at	14.8	67.8	AV229143	Ifi202b	interferon activated gene 202B
1427076_at	12.9	91.1	L20315	Mpeg1	macrophage expressed gene 1
1420249_s_at	8.8	94.7	AV084904	Ccl6	chemokine (C-C motif) ligand 6
1416382_at	6.1	101.0	NM_009982	Ctsc	cathepsin C
1449193_at	2.5	66.9	NM_009690	Cd5l	CD5 antigen-like

* Cell death (GO:0008219) genes (n = 6) are noted in bold.

ature; heart rate; respiratory rate; systolic, diastolic, and mean arterial blood pressure; Glasgow Coma Score; blood pH, P_{CO_2} , O_2 , and base excess; white blood cell count; absolute neutrophil, lymphocyte, and monocytes counts; blood hemoglobin and platelet count; prothrombin and activated partial thromboplastin times; serum sodium, potassium, chloride, glucose, creatinine; and blood urea nitrogen. Similarities between these phenotypes are reflected in the cluster shown with shorter bars representing more similarity. These results were used to classify the septic participants as severe ($n = 6$) and moderate ($n = 7$) as shown by the overlaid boxes.

As preliminary validation of the murine data, platelet mRNA from one exemplary severe and one exemplary moderate septic human subject was profiled using Human U133A GeneChips (Affymetrix) and compared with platelet gene expression in three healthy young adult control subjects. There was no intent to conduct a statistically robust genome-wide assessment on this small group of samples; rather, we focused on a cross-species screening for the six cell death genes identified in the murine study. Of those, only granzyme B was differentially regulated over 72 h (2.9-fold increase) in the severe subject. None of the other cell death genes studied showed differential expression in either group.

Validation of Sepsis-Induced Changes in the Megakaryocyte-Platelet Transcriptional Axis

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to validate the murine platelet granzyme A and B up-regulation detected by microarray. We studied only the first 24 hours after induction of sepsis because the bulk of granzyme up-regulation seen by microarray occurred during this period. In an independent cohort of septic mice ($n = 12$; three mice per time point, nonpooled), granzyme B mRNA expression significantly increased from 0 to 24 hours (mean $\pm$ SE_{0h versus 24h}: 0.77 ± 0.61 vs. 11.94 ± 3.65 ; $P = 0.04$) (Figure 2). The expression of granzyme A mRNA was not significantly increased over that same time (mean $\pm$ SE_{0h versus 24h}: 1.57 ± 2.73 vs. 2.61 ± 4.53 ; $P = 0.11$).

Because platelets are anucleate and lack transcriptional machinery, we hypothesized that increased platelet granzyme B mRNA expression in sepsis could be further validated by simultaneous measurement in autologous megakaryocytes. Using qRT-PCR, we measured platelet granzyme B mRNA expression in bone marrow megakaryocytes simultaneously acquired from the mice used in the platelet qRT-PCR validation step. Megakaryocyte granzyme B mRNA relative expression increased significantly by 24 hours (mean $\pm$ SE_{0h versus 24h}: 2.88 ± 0.27 vs. 8.25 ± 0.52 ; $P = 0.05$). Platelet granzyme B mRNA expression over time closely followed that of megakaryocytes (Figure 2). Megakaryocyte granzyme A mRNA expression did not change (mean $\pm$ SE_{0h versus 24h}: 3.18 ± 0.54 vs. 2.99 ± 0.12 ; $P = 0.42$).

Sepsis Induces Platelet Granzyme B Protein Expression

To determine if granzyme B mRNA up-regulation translates to increased granzyme B protein expression, additional citrated whole blood was collected from septic and control mice. The

blood was fixed with 1% paraformaldehyde, permeabilized, and intracellularly stained with anti-granzyme B (clone 16G6; eBioscience, San Diego, CA) using appropriate isotype and negative (unlabeled) control mice. Flow cytometry data were generated on a FACSCalibur System (BD Biosciences, San Jose, CA), gating on CD61⁺ (clone 2C9.G2; BD) platelets, and analyzed using FlowJo 7.2 (Tree Star, Inc., Ashland, OR). Platelets from septic mice ($n = 9$) showed an increase in intracellular granzyme B protein expression after 24 hours (mean $\pm$ SEM_{0h versus 24h}: 4.4 ± 1.3 vs. $19.6 \pm 6.3\%$; $P = 0.039$). Additional platelet activation with TNF- α did not alter intracellular granzyme B (data not shown).

In a cross-species validation step, citrated whole blood from septic and healthy children was studied in a similar manner. In this case, flow cytometry data were generated on CD61⁺ (clone VI-PL2; BD) platelets stained for intracellular granzyme B (clone

Figure 2. Platelet granzyme B mRNA expression reflects megakaryocyte expression in septic mice. Platelets do not have transcriptional machinery; therefore, changes in platelet granzyme B mRNA expression in septic mice ($n = 12$) were measured simultaneously in autologous megakaryocytes. Results of this quantitative reverse transcriptase polymerase chain reaction analysis show good correlation between increasing megakaryocyte and platelet granzyme B mRNA expression over time.

Figure 3. Flow cytometric measurement of intracellular granzyme B expression in platelets from septic and healthy children. Citrated whole blood was gated on CD61⁺ platelets. Intracellular granzyme B was measured in healthy children ($n = 10$) and septic children, whom we classified as severe ($n = 1$) and moderate ($n = 3$) 1 and 3 days after admission. Shown are results from the child with severe disease showing platelet granzyme B expression at Day 1 (49.7%) and Day 3 (44.3%) compared with the isotype control. Only one of the moderate septic subjects expressed granzyme B and only at Day 3 (24.0%). There was no measurable intracellular granzyme B in platelets from the control subjects.

GB11; BD). Granzyme B was measured in one “severe” and in three “moderate” subjects 1 and 3 days after subjects were admitted for sepsis and compared with similarly aged healthy control children ($n = 10$) having blood drawn for routine testing. Platelets from the severe subject expressed intracellular granzyme B at Day 1 (49.7%) and Day 3 (44.3%) (Figure 3). Only one of the moderate septic subjects expressed granzyme B and only at Day 3 (24.0%). There was no measurable intracellular granzyme B in platelets from the control subjects. Platelet activation state (i.e., CD62P⁺) did not affect granzyme B expression. Furthermore, we did not detect surface expression of other apoptosis inducing proteins (i.e., Fas ligand, IL1 β , TNF- α , and TNF-related apoptosis-inducing ligand) on platelets from the septic children.

Platelets Are Lymphotoxic Effectors in Sepsis via Granzyme B

Our finding of granzyme B in platelets from septic mice and humans led us to hypothesize that platelets could be lymphotoxic in this scenario. To study this question, platelets from mice 18 hours after CLP were coincubated with CD4⁺ splenocytes isolated from healthy control mice. Platelets from septic wild-type (i.e., C57BL6) mice induced marked splenocyte apoptosis compared with platelets from sham wild-type mice (rate of apoptosis, 26.0 ± 3.4 vs. $3.9 \pm 3.4\%$; $P = 0.007$) (Figure 4). This coincubation experiment was repeated with platelets from septic granzyme B null ($-/-$) mice (i.e., B6.129S2-*Gzmb*^{tm1Ley}). In this case, there was a complete lack of induced splenocyte apoptosis by septic platelets. Wild-type platelets further activated by TNF- α had no more lymphotoxicity ($4.5 \pm 1.3\%$; $P = 0.88$) than nonactivated control platelets (Figure 4).

DISCUSSION

Sepsis-related mortality results in part from immunodeficiency secondary to profound lymphoid apoptosis (1, 2, 27–30). The biological mechanisms responsible for this extensive lymphocyte cell death are not understood but have been attributed in part to direct pathogen signaling through toll-like receptors and MyD88 (31). In these studies, we explored the possibility that platelets play a direct role in this process by conducting time series studies in a murine experimental model of sepsis. Microarrays were used as an initial screening tool to hypothesize that responses of

platelets to systemic perturbations in sepsis could lead to changes in mRNA expression of cell death-associated genes. This model was then tested through a series of mouse and human studies. Our experiments led us to characterize sepsis-induced changes in the megakaryocyte–platelet transcriptional axis and present a novel finding that the resulting platelets are strongly lymphotoxic. Second, using platelets from a murine-induced sepsis model, we identified the serine protease, granzyme B, as the cause of this lymphotoxicity.

Figure 4. Platelets harvested from septic mice induce apoptosis in control CD4⁺ splenocytes except in the absence of granzyme B. Percent apoptosis was significantly higher in splenocytes coincubated with platelets harvested from septic wild-type (WT) (i.e., C57BL6) mice than with platelets from healthy wild-type mice and splenocytes without platelets. Repeat experiments with platelets from septic granzyme B null ($-/-$) mice (i.e., B6.129S2-*Gzmb*^{tm1Ley}) showed a lack of induced splenocyte apoptosis. Further platelet activation with recombinant tumor necrosis factor- α under any of these conditions did not alter lymphotoxic capacity.

The granzymes are a group of cytotoxic serine proteases that are most commonly secreted within cytotoxic granules by natural killer and cytotoxic T lymphocytes (32). Granzyme B is the most well characterized of these proteases (the other human granzymes include A, H, K, and M) and has multiple known caspase targets and a growing list of caspase-independent substrates, including poly(ADP-ribose) polymerase (33) and fibroblast growth factor receptor-1 (34). Granzyme B typically enters target cells through a channel of co-released perforin (35) but can also enter independently (36–38). Once in the target cell cytoplasm granzyme B cleaves several intracellular proapoptotic cysteine proteases, the most prominent and best-studied being caspase 3 (35). Alternatively, granzyme B has been shown to induce apoptosis via Bid-induced mitochondrial damage (39–41). Granzyme B has been shown to induce cell death by caspase- and noncaspase-mediated mechanisms simultaneously (34, 42). In addition, Wong and colleagues showed that granzyme B is among the transcripts up-regulated in whole blood from pediatric septic shock nonsurvivors compared with survivors (43).

Our experiments showed that platelets are strongly lymphotoxic due to granzyme B in sepsis. Our results build upon previous research demonstrating significant interregulatory interactions between platelets and lymphocytes in a variety of inflammatory disease states, particularly with respect to adaptive immunity. For instance, platelet CD40 has been shown to bind to T lymphocyte CD40 ligand, thereby inducing platelet release of CCL5, which further activates T lymphocytes and thus amplifies the immune response (44). In particular in sepsis, platelet-derived microparticles have been shown to be cytotoxic against vascular endothelium (8–10) and smooth muscle (10). To our knowledge, ours is the first study to examine acute changes in the platelet transcriptome in response to a disease insult. We found that megakaryocytes in the bone marrow respond to systemic sepsis and alter the transcriptome of platelets to include granzyme B.

The presence of granzyme B in platelets in sepsis raises intriguing questions, especially in light of the fact that platelet activation does not seem to affect its expression, implying there is no posttranscriptional regulation. First, it is possible that granzyme B serves a role in megakaryocyte caspase activation, which is critical for normal platelet formation (45). If so, it is possible that in the hyperthrombopoiesis of sepsis megakaryocyte up-regulation of granzyme B mRNA results in inclusion of this transcript in platelets. An alternative is that platelet granzyme B represented an evolutionary advantage at some point. Granzyme B's ability to induce apoptosis through a wide variety of mechanisms makes it a likely mechanism to circumvent the immune evasion strategies of intracellular pathogens. In fact, there is evidence that granzyme B from cytotoxic T cells may play a role in defense against *Toxoplasma gondii* and *Plasmodium* species (46, 47).

We acknowledge the following limitations to this study. First, the use of pooled platelets for the murine GeneChips and the use of a single GeneChip for each murine and human phenotype may be questioned. We argue that as a screening tool, these microarray experiments were useful in hypothesizing granzyme B's up-regulation, which then required extensive validation. Therefore, we conducted repeated validation steps for granzyme B, including platelet and megakaryocyte qRT-PCR and cross-species protein level measurement via flow cytometry. These validations make a false-positive microarray result for granzyme B extremely unlikely. Second, it is reasonable to question the effect of thrombocytopenia on platelet lymphotoxicity in sepsis. Thrombocytopenia is often a complication of sepsis and is associated with poor outcomes (48). Logically, fewer circulating platelets would lead to less induced apoptosis. However, sepsis-associated thrombocytopenia occurs in large part as a result of intense

platelet activation leading to increased platelet–endothelium interactions. Thus, platelet sequestration in the microvascular endothelium, although associated with circulating thrombocytopenia, results in increased inflammatory interactions with immune cells and endothelium (48). This places them in close proximity to splenic lymphoid tissue, in which the bulk of lymphoid apoptosis occurs.

In summary, we conclude that platelets up-regulate granzyme B in murine and human sepsis. We show that platelets from septic mice induced marked apoptosis of healthy splenocytes *ex vivo* via granzyme B action. Our findings establish a conceptual advance in sepsis: Septic megakaryocytes produce platelets with acutely altered mRNA profiles, and these platelets mediate lymphotoxicity via granzyme B. Given the contribution of lymphoid apoptosis to sepsis-related mortality, modulation of platelet granzyme B becomes an important new target for investigation and therapy.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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