PLATELET-BOUND LIPOPOLYSACCHARIDE ENHANCES Fc RECEPTOR-
MEDIATED PHAGOCYTOSIS OF IgG OPSONIZED PLATELETS.\textsuperscript{a}

John W. Semple,\textsuperscript{1-6} Rukhsana Aslam,\textsuperscript{5,6} Michael Kim,\textsuperscript{6} Edwin R. Speck\textsuperscript{5,6} and John Freedman.\textsuperscript{1,3-6}

Department of Laboratory Medicine, St Michael's Hospital,\textsuperscript{1} Departments of Pharmacology,\textsuperscript{2} Medicine\textsuperscript{3} and Laboratory Medicine and Pathobiology,\textsuperscript{4} University of Toronto, Canadian Blood Services\textsuperscript{5} and The Toronto Platelet Immunobiology Group,\textsuperscript{6} Toronto, Ontario, Canada.

Running Title: LPS increases opsonized-platelet phagocytosis.

Correspondence to: Dr. John W. Semple,

St. Michael's Hospital,

30 Bond Street,

Toronto, Ontario, Canada, M5B 1W8.

Phone: 416-864-5534

FAX: 416-864-3053

a. Supported by a grant from the Canadian Blood Services (XT00027).

Editorial note. Author’s contributions: Semple, designed research, wrote first draft; Aslam, designed and performed research, analyzed data, corrected draft; Kim, performed research, analyzed data; Speck, performed research, analyzed data; Freedman, designed research, contributed human samples, analyzed data, corrected draft.
Platelets express toll-like receptor (TLR) 4 and this has been shown to be responsible for the thrombocytopenia induced by lipopolysaccharide (LPS) administration in vivo. We studied the role of LPS in mediating platelet phagocytosis by THP-1 cells in vitro by flow cytometry. Opsonization of platelets with an IgG monoclonal (W6/32) antibody or with IgG autoantibody-positive sera from patients with autoimmune thrombocytopenia (AITP) significantly enhanced platelet phagocytosis (p=0.0001). In contrast, platelet phagocytosis did not occur if platelets were bound with only LPS. If, however, the LPS-bound platelets were also opsonized with either W6/32 or autoantibody-positive sera with titres >4, there was a significant and synergistic increase in Fc-dependent platelet phagocytosis (p<0.0001, p=0.0031, p=0.0481 and p=0.0471). These results suggest that, in the presence of anti-platelet antibodies, bacterial products can significantly alter platelet phagocytosis and this may have relevance to how gram negative infections enhance platelet destruction in some patients with AITP.
Introduction

Autoimmune thrombocytopenic purpura (AITP) is a bleeding disorder caused by autoantibodies that opsonize platelets and enhance their destruction by FcR-mediated phagocytosis within the spleen.\(^1,2\) Although both acute and chronic forms can be distinguished, acute AITP primarily affects children and often occurs after a viral or bacterial infection.\(^1,2\) Furthermore, viral-specific antibodies with cross-reactivity against platelets have been identified in children with acute AITP\(^3,4\) and in patients with HIV-related AITP\(^4,5\) suggesting infections may play a role in AITP pathogenesis. On the other hand, in some patients with chronic AITP, infections are associated with an exacerbation of thrombocytopenia and this has also been demonstrated in a mouse model.\(^6\) Alternatively, eradication of the gram negative bacterium H. pylori in patients with AITP increases platelet counts although this has not been observed in some studies.\(^7-10\) It is possible that, in susceptible individuals, infectious agents in the presence of anti-platelet antibodies affect platelet-monocyte interactions and alter platelet destruction.

Pathogens are first encountered by Toll-like receptors (TLR) on professional phagocytes.\(^11,12\) TLR are germline encoded proteins and bind a variety of infectious molecular structures and are critical for stimulating innate immune mechanisms.\(^11,12\) TLR are also expressed on many cell types including platelets\(^13-17\) and platelet TLR4 expression, at least, appears responsible for mediating lipopolysaccharide (LPS)-induced thrombocytopenia in vivo.\(^15,16\) To determine how bacterial agents modulate platelet destruction, we used flow cytometry to investigate whether LPS affects opsonized-platelet phagocytosis by mononuclear cells. The results demonstrate that LPS can synergize with IgG autoantibodies and significantly enhance platelet phagocytosis suggesting, at least, one mechanism of how bacterial products may enhance platelet destruction in vivo.
Methods

This study had no direct contact with human patients. Approval was obtained from the St. Michael’s Hospital Institutional Review Board.

Antibodies: Sera prepared from the blood of 8 patients with AITP previously screened for platelet-associated autoantibodies were obtained from the laboratory of Dr. John Freedman (Dept. of Laboratory Medicine, St. Michael's Hospital, Toronto, ON, Canada). Serum IgG autoantibodies were detected and titred by flow cytometry and then blinded (samples 1-8) for laboratory personnel performing the phagocytosis assays. The murine IgG2a anti-human MHC class I monoclonal antibody (W6/32) was produced in vitro by hybridoma HB-95 (ATTC#HB-95). F(ab’)_2 fragments of W6/32 were produced as previous described; purity by HPLC analysis was >96%.

Platelets: Blood was obtained by venipuncture into trisodium citrate from healthy laboratory volunteers under an REB-approved protocol. PRP was prepared, platelets were counted and adjusted to 10^9 cells/ml. Platelets were labeled with 20uM CellTracker Green CMFDA (CM-G; Molecular Probes, Eugene, OR), washed and resuspended in PBS. Where indicated, CM-G-labeled platelets were either incubated with titrations of LPS and/or 5 ug W6/32 and/or a 1:2 dilution of human serum for 30 minutes at RT in the dark. Cell were washed once and used in the phagocytosis assay.

Phagocytosis Assay: Human monocytic THP-1 cells (ATCC# TIB-202) were counted and 1 x 10^7 cells/ml were activated with 50ng/ml phorbol 12-myristate 13-acetate for 15 minutes and washed. The phagocytic reaction was started by incubating 5x10^6 THP-1 cells with 250x10^6 of platelets in 0.1 ml duplicate tubes for 60 minutes on ice or at 37°C. Extracellular fluorescence was then quenched by addition of 0.1% trypan blue. The mixture was centrifuged at 200xg for 10 min at 4°C, the supernatant discarded and 200 ul of LDS DNA stain (FL3; Molecular Probes, Eugene, OR) was added. Flow cytometry was performed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA) and
acquisition was through a live electronic FL3 gate. Intracellular FL1 CM-G platelet fluorescence in the nucleated events was determined. Phagocytic Index was calculated by the formula: median FL1 fluorescence at 37°C / median FL1 fluorescence at 0°C.

**Statistical analysis:** A one-way ANOVA was used to compare the means of W6/32 phagocytosis and a two-tailed paired t test for comparison between means was used for the patient sera.

**Results and Discussion**

Compared with non-opsonized platelets, when W6/32-opsonized platelets were incubated with THP-1 cells, a significant (p=0.0001) increase in intracellular platelet fluorescence occurred (0 value from Figure 1A compared with 0 value from Figure 1B). In contrast, LPS-bound but non-opsonized platelets did not increases THP-1 intracellular platelet fluorescence (Figure 1B). If the LPS-bound platelets were, however, opsonized with W6/32, a significant (p<0.0001) and synergistic enhancement of opsonized platelet phagocytosis occurred (Figure 1A). The synergistic LPS/antibody enhancement of phagocytosis was Fc-specific because THP-1 phagocytosis was abrogated when the platelets were bound with W6/32 F(ab')2 fragments (Figure 1C). This suggests that LPS bound to platelets in the presence of anti-platelet antibody significantly increases platelet phagocytosis by human mononuclear cells. The binding of bacterial products such as LPS to platelets has been demonstrated to mediate several physiological processes including platelet aggregation and platelet-monocyte interactions and may also play a role in atherogenesis. To our knowledge, however, this is the first demonstration of a synergistic effect between platelet-bound LPS and anti-platelet antibodies in enhancing platelet phagocytosis.

We then attempted to reproduce the W6/32 results with anti-platelet autoantibodies from the sera of patients with AITP. By flow cytometry, 4 of 8 sera were negative for IgG anti-platelet autoantibodies and 4 contained autoantibodies with titres ranging from 4 to 128. None of
the autoantibody-negative sera could mediate THP-1 phagocytosis of platelets (open bars, Figure 2A), even when LPS was also bound to the platelets (solid bars, Figure 2A). In contrast, however, 3 of the 4 autoantibody-positive sera (those with the higher titres: 64, 64 and 128) opsonized human platelets and enhanced their phagocytosis by the THP-1 cells (open bars, Figure 2B). As with W6/32, when the autoantibody-opsonized platelets were also bound with LPS, their phagocytosis was significantly (p=0.0310, p=0.0481, p=0.0471) and synergistically enhanced (solid bars, Figure 2B) when compared to only opsonized platelets (open bars, Figure 2B). Thus, LPS in conjunction with IgG anti-platelet autoantibodies from patients with AITP can significantly enhance platelet phagocytosis.

These observations with LPS-bound platelets may be related to the recent reports demonstrating that platelets express toll-like receptors (TLR) and can bind LPS via TLR4.13-17 The mechanism of how platelet-bound LPS together with autoantibody-opsonization synergizes to enhance platelet phagocytosis is unknown, but since the increase was Fc-dependent (Figure 1C), it may suggest that the interaction of TLR- and FcR-mediated signaling pathways could be responsible. For example, TLR signaling and phagocytosis are hallmarks of macrophage-mediated innate immune responses to bacterial infections21 and genes involved in Fc-dependent phagocytosis (e.g. Lyn and Syk) have been found to be up-regulated by TLRs.22,23 Furthermore, both FcR- and TLR-mediated phagocytosis appear coupled as several TLR family members are known to localize to phagosomes where they can recognize molecules specific to pathogens and mediate inflammatory signaling.24,25 Perhaps the combination of LPS and autoantibody presented by the platelets to the THP-1 cells utilize shared components that synergistically increase signaling events and maximally stimulate macrophage phagocytosis.
In summary, LPS together with IgG bound to platelets significantly enhances Fc-mediated platelet phagocytosis by mononuclear phagocytes. These results suggest infectious agents in combination with anti-platelet antibodies could affect platelet destruction in vivo and may be at least one explanation of why thrombocytopenia worsens in some patients with AITP during infections and, alternatively, resolves in other patients with AITP who are treated with bacterial eradication therapy.

Acknowledgement

We would like to thank Dr. Peter Gross (Dept. of Medicine, St. Michael's Hospital) for his helpful discussions and critical review of this manuscript.
References:


Figure Legends.

Figure 1.

Effect of the indicated additions of LPS on the phagocytosis of A) W6/32-opsonized, B) non-opsonized and C) W6/32 F(ab’)2 fragment-bound CM-Green labeled human platelets by THP-1 cells. The data are presented as the mean phagocytic index (+SD) from 10 (A and B) and 5 (C) independent experiments and was calculated by the formula: median channel FL1 fluorescence at 37°C / median channel FL1 fluorescence at 4°C. Statistical significance in A by a one-way ANOVA was p<0.0001.

Figure 2.

THP-1 phagocytosis of CM-Green labelled platelets incubated with either A) autoantibody-negative sera samples 1-4 or B) autoantibody positive sera samples 5-8 and either no LPS (open bars) or 0.1 ug LPS (solid bars). The data is presented as the mean phagocytic index (+SD) from 4 independent experiments and was calculated as in Figure 1. The x-axes numbers refer to the human serum sample number and IgG anti-platelet autoantibody titres in B are shown above the bars for each sera sample. Statistical significance (p values) by a paired t test are shown.
Figure 2

A. Phagocytic Index

B. Sample No.

Sample No. 1 2 3 4

Phagocytic Index 0 10 20 30 40

Sample No. 5 6 7 8

Phagocytic Index 0 10 20 30 40

p = 0.0310

p = 0.0481

p = 0.0471

Sample No. 5 6 7 8

Phagocytic Index 64 64 4 128

p = 0.0310

p = 0.0481

p = 0.0471
Platelet-bound lipopolysaccharide enhances Fc receptor-mediated phagocytosis of IgG opsonized platelets

John W. Semple, Rukhsana Aslam, Michael Kim, Edwin R. Speck and John Freedman