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American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
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Platelet EVs contain an active proteasome involved in protein processing for antigen presentation via MHC-I molecules

Tracking no: BLD-2020-009957R1

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Abstract:
In addition to their hemostatic role, platelets play a significant role in immunity. Once activated, platelets release extracellular vesicles (EVs) formed by budding of their cytoplasmic membranes. Because of their heterogeneity, platelet EVs (PEVs) are thought to perform diverse functions. It is unknown, however, whether the proteasome is transferred from platelets to PEVs or whether its function is retained. We hypothesized that functional protein processing and antigen presentation machinery is transferred to PEVs by activated platelets. Using molecular and functional assays, we show that the active 20S proteasome is enriched in PEVs along with MHC-I and lymphocyte costimulatory molecules (CD40L and OX40L). Proteasome-containing PEVs were identified in healthy donor blood, but did not increase in platelet concentrates that caused adverse transfusion reactions. They were, however, augmented after immune complex injections in mice. The complete biodistribution of murine PEVs following injection into mice revealed that they could principally reach lymphoid organs such as spleen and lymph nodes, in addition to the bone marrow, and to a lesser extent liver and lungs. The PEV proteasome processed exogenous ovalbumin (OVA) and loaded its antigenic peptide onto MHC-I molecules which promoted OVA-specific $CD8^+$ T lymphocyte proliferation. These results suggest that PEVs contribute to adaptive immunity through cross-presentation of antigens and have privileged access to immune cells through the lymphatic system, a tissue location that is inaccessible to platelets.

Conflict of interest: No COI declared

COI notes: The authors have no conflict of interest to disclose.

Preprint server: No;

Author contributions and disclosures: Disclosures The authors have no conflict of interest to disclose. Authorship Contributions EB, BV, GM, AZ, conceived and designed the study and wrote the manuscript; GM, AL, SH, MB, MM, IA, MT and BV performed the experiments, analyzed the data, and participated in manuscript preparation. TL, JR, AKR, JT, HHC, FC, RK, JWS, MJH, SGB, FP, HSO, BIF and MD performed experiments, contributed critical biospecimens and analyzed data. All the authors read and approved the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: emails to the corresponding author

Clinical trial registration information (if any):

Platelet EVs contain an active proteasome involved in protein processing for antigen presentation via MHC-I molecules

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Word count: 3998

Keywords: platelet, extracellular vesicles, proteasome, MHC-I, antigen presentation, immunity

Short title: Active proteasome in extracellular vesicles

Key points:
Platelet-derived extracellular vesicles contain an active proteasome
Platelet-derived extracellular vesicles can process and present antigen

Abstract

In addition to their hemostatic role, platelets play a significant role in immunity. Once activated, platelets release extracellular vesicles (EVs) formed by budding of their cytoplasmic membranes. Because of their heterogeneity, platelet EVs (PEVs) are thought to perform diverse functions. It is unknown, however, whether the proteasome is transferred from platelets to PEVs or whether its function is retained. We hypothesized that functional protein processing and antigen presentation machinery is transferred to PEVs by activated platelets. Using molecular and functional assays, we show that the active 20S proteasome is enriched in PEVs along with MHC-I and lymphocyte costimulatory molecules (CD40L and OX40L). Proteasome-containing PEVs were identified in healthy donor blood, but did not increase in platelet concentrates that caused adverse transfusion reactions. They were, however, augmented after immune complex injections in mice. The complete biodistribution of murine PEVs following injection into mice revealed that they could principally reach lymphoid organs such as spleen and lymph nodes, in addition to the bone marrow, and to a lesser extent liver and lungs. The PEV proteasome processed exogenous ovalbumin (OVA) and loaded its antigenic peptide onto MHC-I molecules which promoted OVA-specific $CD8^+$ T lymphocyte proliferation. These results suggest that PEVs contribute to adaptive immunity through cross-presentation of antigens and have privileged access to immune cells through the lymphatic system, a tissue location that is inaccessible to platelets.

Introduction

Platelets are the second most abundant lineage in the blood and are best known for their role in hemostasis.[1] Platelets are small fragments produced by the large multinucleated megakaryocyte in the bone marrow. They bear receptors that permit recruitment of immune cells and carry an extensive set of immune and inflammatory molecules (*e.g.* cytokines/chemokines, lipid mediators, hormones) stored in their granules, cytoplasm, or synthesized by mRNA translation following platelet activation. Thus, while platelets may mount an innate immune response against injury, which is critical to combat pathogen invasion, organ and tissue damage may also favor platelet activation and inflammation in chronic inflammatory diseases.[2-8]

Albeit anucleate, the platelet cytoplasm includes numerous molecules comprising the proteasome, which are transferred from megakaryocytes to their progeny. The proteasome is a high molecular weight cylindrical protein complex through which unwanted or damaged proteins are degraded.[9,10] The central complex part, called the 20S proteasome, is made up of twenty-eight distinct subunits,[11] comprising the three catalytic subunits necessary for the degradation of proteins into peptides of three to fifteen amino acids in length.[11,12] Proteasome activity in megakaryocytes is required for platelet production[13,14] and in platelets, the proteasome regulates platelet lifespan,[15] activation[16-18] and the release of PEVs.[19,20] The platelet proteasome can hydrolyze proteins into smaller peptides,[11,21,22] thereby enabling peptide loading onto the platelet major histocompatibility complex (MHC) class I molecules (MHC-I).[23-25] Components of the peptide loading complex are also expressed in platelets and are found in close proximity with MHC-I during platelet activation.[26,27] As platelets can efficiently form an immunological synapse with T-lymphocytes to activate lymphocyte proliferation,[26,28,29] they are known to fulfill roles in cross-presentation of antigens in adaptive immunity. In a similar manner, megakaryocytes cross-present antigens to CD8 T-lymphocytes, thereby suggesting that they may also play a dual role in innate and adaptive immunity.[25,30-32]

Extracellular vesicles, produced in abundance by platelets, are small (up to 1µm in

diameter) membrane-bound vesicles released from the plasma membrane or endosomal compartments of activated cells. Platelet EVs are heterogeneous in terms of surface molecules and content (*e.g*. nucleic acids, lipids, transcription factors, enzymes, mitochondria) and as such, may play diverse functions beyond hemostasis.[33-35] For instance, PEVs convey mitochondrial components that are associated with inflammation and adverse transfusion reactions (ATRs).[36-38] Despite the fact that platelets are restricted to the blood circulation, PEVs can cross tissue barriers and enter synovial fluid,[39,40] lymph[41,42] and bone marrow[43] where they can deliver platelet-derived molecules and modulate target cells.[34] For instance, PEVs promote the formation of germinal centers and the production of IgG by B-cells.[44,45] They also interact with and modulate regulatory T cell differentiation and activity.[46,47] Thus, PEVs may be able to transport platelet-derived molecules relevant to adaptive immunity into lymphoid organs. However, it is unknown whether the proteasome and the molecules necessary for antigen presentation are also transferred during the budding of PEVs. In this study, we evaluated whether functional protein processing and antigen presentation machinery is transferred to PEVs by activated platelets.

Material and methods

More details are presented in supplemental methods.

Labelling of murine platelets, DCs and PEVs Platelets were isolated from C57BL/6J mice by retro-orbital or cardiac puncture in 200µL ACD, 350µL Tyrode's buffer pH 6.5. Whole blood was centrifuged at 600x*g* for 3min and then at 400x*g* for 2min to remove red blood cells. Supernatant was spun at 1,300x*g* for 5 min and the platelet-containing pellet was gently resuspended in 600µL Tyrode's buffer pH 7.4. Platelets were either left nonactivated or activated with thrombin (0.1U/mL) after addition of 5 mM of calcium for 90 min at RT (time based on kinetics of $CD41^+$ $Proteasome^+$ PEV release shown in **Supplementary figure 3C**). Platelet EVs were obtained by two rounds of centrifugation of stimulated platelets at 1,300x*g* for 5min at RT. Either activated platelets, EVs or DCs were pulsed with 100µg/mL OVA protein (Sigma-Aldrich), 200µg/mL of OVA peptide (SIINFEKL [Invivogen]) or left unpulsed for 4h at RT. These conditions were either left unlabelled for lymphoproliferation and intracellular staining experiments or labelled for Hs-FCM experiments.

Five µL of PEVs or platelet suspensions were labelled with 250nM LWA300 proteasome probe in a total volume of 100µL for 90min at 30°C. Samples were then incubated with the following antibodies for 30min at RT prior to dilution in Annexin V binding buffer and analysis by Hs-FCM: BUV395 anti-CD41, BV650 anti-CD62p, BUV395 anti-CD41, BV650 anti-CD62p, BV711 Annexin V, BV421 anti-OX40L, BUV737 anti-CD154 (all BD Biosciences), PeCy7 anti-CD40, PeCy7 anti-MHC-I (AF6-88.5) and PE anti MHC-I bound to OVA peptide (25D1.16) (all from Biolegend).

Results

PEVs contain functional proteasome

Following platelet activation by thrombin, remnant platelets were eliminated by centrifugation and larger EVs were isolated by a second high-speed centrifugation (18Kxg fraction). The supernatant obtained was further centrifuged at 100,000g and smaller EVs (likely exosomes) were obtained from this pellet. We found that 98.1±0.5 % of proteins were retrieved in the larger EV 18Kxg fraction. Immunoblotting confirmed that human PEVs from this fraction were enriched in proteasome 20S α subunit, in addition to mitochondria (indicated by TOM20 expression) and CD41, but lacked TSG101 (putative marker of exosomes) (**Figure 1A-E**). Using platelets as a positive control, we assessed proteasome function in these PEVs. Proteasome-associated trypsin-, caspase- and chymotrypsin-like activities were detectable in platelets and significatively increased in the PEV fraction, but were undetectable after treatment with epoxomicin, a proteasome inhibitor (**Figure 1F**). Visualization of immunogold-labelled proteasome 20S α subunit by transmission electron microscopy confirmed the presence of proteasomes in PEVs (**Figure 1G**). These data suggest the catalytically active proteasome was transferred to PEVs upon their release from platelets.

LWA300 is a conjugate between epoxomicin and BODIPY FL fluorophore that generates an activity-based, plasma membrane-permeable inhibitor that can identify the proteasome in cells.[48,49] Using LWA300, we detected and quantified active proteasome-containing PEVs directly in the platelet secretome.[48,49] High-sensitivity flow cytometry (Hs-FCM) confirmed PEV heterogeneity following platelet activation by thrombin (**Figure 1H**). Approximately 16.6±6.5% of the larger (*i.e.* ~500–900nm) PEVs[50] contained proteasome whereas smaller vesicles (*i.e.* less than 500nm) had no detectable proteasome (**Figure 1H and Supplementary figure 1**). The detection specificity of proteasome-containing PEVs by hs-FCM was confirmed using a combination of controls. We confirmed efficient competition of the LWA300 probe by unlabelled epoxomicin, and we determined the particulate nature and membrane moiety

of proteasome-containing PEVs, as they were respectively pelleted by ultracentrifugation and sensitive to detergent treatment (**Figure 1I-J**). Confocal microscopic visualization of platelets as positive controls, and PEVs from thrombin-activated platelets labelled with LWA300 revealed that both platelets and a subpopulation of PEVs contained active proteasome (**Figure 1K**).

Hs-FCM was further used to characterize proteasome-containing PEVs in terms of surface markers and mitochondrial content. Approximately half of the proteasome-containing PEVs exposed phosphatidylserine while the vast majority expressed surface P-selectin (**Supplementary figure 2A**). Furthermore, 68.3±7.8% of the proteasome-containing PEVs also contained mitochondria (**Supplementary figure 2A**). Investigation of the mechanisms underlying release of active proteasome-positive PEVs revealed that the total number of PEVs (with and without proteasomes) were significantly reduced in the presence of actin inhibitors (cytochalasins B, D, E and latrunculin A) but not by the tubulin polymerization inhibitor nocodazole (**Supplementary figure 2B**). Proteasome release in PEVs was not unique to thrombin stimulation as ADP, cross-linked collagen related peptide (CRP-XL) and heat-aggregated IgG (HA-IgG) also triggered release of proteasome-containing PEVs (**Supplementary figure 2C**).

Identification of proteasome-containing PEVs under physiological and pathological conditions

The presence of proteasome-containing PEVs was assessed under conditions conducive to platelet activation and PEV release. A mean of 1.82×10^6 (range:1.13×10^5 to 8.11×10^6, n=6) proteasome-containing PEVs/mL were detected by hs-FCM in the blood of healthy individuals, which corresponded to 2.6±1.8% of the total PEVs in blood. PEVs were quantified in platelet concentrates (PCs) known to have caused ATRs and compared with control PCs that did not induce ATRs. Given the reported increase in mitochondria-containing PEVs in ATRs,[36,37] we also determined their levels. High levels of proteasome-containing PEVs were found in all tested PCs (**Figure 2A**) but the concentrations of proteasome-containing PEVs (with or without mitochondria) were not

significantly elevated in PCs that induced ATR (**Figure 2A**). In contrast, compared with controls, the concentrations of mitochondria-containing PEVs were increased in ATR-associated PCs, consistent with prior findings.[36,37]

Transfusion-related acute lung injury (TRALI) is a potentially lethal adverse reaction that can result from transfusion of PCs.[51] Thus, we quantified proteasome-containing PEVs in murine bronchoalveolar lavages in an inducible TRALI model.[52,53] Proteasome-containing PEVs were detected in bronchoalveolar lavages from both TRALI and control mice (**Figure 2B**), however, no significant difference was observed between the two groups (**Figure 2B**). This suggests that proteasome-containing PEVs are not increased during lung inflammation in this model and therefore may not participate to acute inflammation that characterizes the pathogenesis.

Our *in vitro* investigations pointed to the high potency of immune complexes (HA-IgG) in generating proteasome-containing PEVs (**Supplementary figure 2C**). Although mice lack FcγRIIA, this is the only Fcγ receptor expressed by human platelets that is capable of responding to immune complexes.[54] Recent findings indicate that circulating immune complexes stimulate the release of mitochondria-containing PEVs in mice expressing the FcγRIIA transgene.[55,56] Compared with diluent injected control mice, there were significantly elevated levels of proteasome-containing PEVs in plasma of mice with immune-complexes challenge (**Figure 2C**). These findings confirmed that proteasome-containing PEVs are present under various physiological and pathological conditions.

Protein processing by proteasome-containing PEVs

In order to study proteasome function in PEVs, we investigated its ability to process proteins into smaller peptides by assessing their successful loading into the antigen-binding groove of MHC-I molecules. We confirmed the expression of MHC-I on resting and thrombin-activated murine platelets and verified whether MHC-I is maintained on PEVs present in the platelet secretome. We found that washed resting platelets did not express MHC-I on their surface (**Figure 3A-B**), however, thrombin activation led to a

significant increase in surface MHC-I expression (**Figure 3A-B**), consistent with the reported presence of this molecule in α-granules and its release upon activation.[26,27,57,58] A small proportion (0.93±0.13%) of the spontaneously released PEVs expressed MHC-I, but this proportion significantly increased upon platelet activation with thrombin (means of 4.64±0.98%).

To determine whether PEV MHC-I can indeed load small peptides, we pulsed PEVs present in the platelet secretome with the ovalbumin (OVA) peptide SIINFEKL and monitored its association with MHC-I molecules using the 25D1.16 monoclonal antibody, which specifically recognizes MHC-I/SIINFEKL complexes.[59] Similarly with platelets, PEVs loaded the SIINFEKL peptide onto their MHC-I molecules (**Figure 3C-D**). Native OVA was also efficiently processed by platelets and the SIINFEKL peptide was loaded in MHC-I (**Figure 3C-E**), consistent with prior work.[26] We found that an average of 2.53±0.74% of $CD41^+$ PEVs pulsed with the peptide and 1.83±0.24% of $CD41^+$ PEVs pulsed with OVA (n=18) were positive for 25D.1.16. Of interest, incubation of native OVA with PEVs resulted in proteolysis of the former and retrieval of the SIINFEKL peptide from MHC-I molecules expressed by the PEVs. Taken together, the data show that PEVs can process native proteins into smaller peptides thereby enabling antigen presentation through MHC-I.

Proteasome-containing PEVs can reach lymphoid organs and circulate through the lymphatic system

Intravenously injected PEVs have a limited circulation time in human blood, ranging from 10 min to hours depending on studies.[60,61] It is unclear, however, whether they can reach lymphoid organs. Fluorescently labelled PEVs generated from activated mouse platelets were intravenously injected into mice and their presence in blood and different organs was monitored. We could identify free PEVs (unbound to cells) for up to 2 minutes in blood (**Figure 4A and Supplementary figure 4A-C**). PEVs in blood were also mainly found bound to platelets and to leukocytes, mainly $Ly6G^+$ neutrophils, and to a lesser extent lymphocytes, but were mostly undetectable by 60min (**Figure 4A**).

Screening of individual PEVs in whole tissue sections in different organs identified spleen and lymph nodes (popliteal and inguinal) as primary targets, followed by liver, bone marrow, lungs, and kidneys, while none were found in brain (**Figure 4B-C and Supplementary figure 4D-E**). Moreover, aggregates of PEVs (i.e. larger than 1μm^2 and up to 541μm^2) were mainly observed in spleen (mean size 2.84±0.16μm^2), popliteal (4.16±0.40μm^2) and inguinal (4.08±0.30μm^2) lymph nodes, followed by bone marrow (2.75±0.15μm^2), lung (33.51±7.15μm^2) and liver (3.40±0.21μm^2). This may reflect their accumulation into smaller vessels or the internalization of numerous PEVs within single cellular recipients in these organs (**Figure 4B-C and Supplementary figure 4D-E**).

Platelet EVs can circulate through the lymphatic system and the levels of PEVs in lymph are increased in mouse models of atherosclerosis and autoimmune inflammatory arthritis.[34,41,42] Using the lymph from mice, we evaluated whether PEVs were associated with proteasome and MHC-I molecules. We found that a fraction of the PEVs in lymph expressed MHC-I (11.2±2.2%) and contained an active proteasome (12.0±3.9%). Remarkably, a detectable proportion (1.6±0.7%) of the lymph PEVs contained both proteasome and MHC-I molecules (**Figure 4D-E**) and this was significant given the substantial number of PEVs in lymph (mean of 2.5×10^7/mL in mice[41]).

Proteasome-containing PEVs express lymphocyte co-stimulatory molecules

Efficient stimulation of adaptive immunity requires both recognition of the antigen-MHC-I complexes by the T-cell receptor (TCR) and the activity of co-stimulatory molecules. We evaluated whether platelets or proteasome-containing PEVs loaded with SIINFEKL expressed co-stimulatory molecules in addition to other known PEV markers displayed by CD41$^+$ Proteasome$^+$ EVs. Compared with PEVs that had undetectable SIINFEKL loading, both platelets and PEVs present in the platelet secretome loaded with SIINFEKL (25D1.16-positive) expressed higher levels of proteasome (**Figure 5**). Moreover, in contrast to thrombin-activated platelets, where phosphatidylserine expression is increased when loaded with SIINFEKL, both PEVs bearing SIINFEKL and

those negative for SIINFEKL expressed similar levels of phosphatidylserine (**Figure 5**). Furthermore, both platelets and SIINFEKL-bearing PEVs expressed higher levels of P-selectin, and the co-stimulatory molecules CD40L, CD40 and OX40L (**Figure 5**). Thus, among the different subtypes of PEVs, those with a higher density of antigen–MHC-I complexes show more abundant expression of lymphocytes co-stimulatory molecules and bear a higher content of active proteasome.

Proteasome-containing PEVs can support antigen-specific T cell activation

T cells isolated from OT-1 mice[100] were co-incubated for 18h with PEVs present in the platelet secretome that were either pulsed or not with the SIINFEKL peptide or native OVA. Dendritic cells and platelets were treated similarly as positive controls and for comparison (**Figure 6A**). The T cells ($CD3^+CD8^+$) were then washed and the expression of CD40, OX40, IL-2 and IFN-γ was evaluated to assess T-cell activation.

Compared with DCs and platelets, PEVs could induce a significant release of IFN-γ when pulsed with the OVA peptide, whereas native OVA led to an increase in IFN-γ but did not reach statistical significance (**Figure 6B**). Moreover, DCs and, to a lesser extent, platelets and PEVs were only capable of inducing significant CD40 expression by T lymphocytes previously pulsed with the OVA peptide (**Figure 6C**). In contrast, OX40 and IL-2 expression were not induced by DCs, platelets or PEVs under these experimental conditions (**Figure 6D-6E**).

Whether PEVs could stimulate T cell proliferation, a hallmark response by the lymphocyte antigen-MHC-I complex was evaluated. T cells from OT-1 mice were labelled with CFSE to monitor cellular division and co-incubated for 5 days with either DCs, activated platelets or PEVs, which were either pulsed or not with either SIINFEKL or native OVA (**Figure 7A**). Lymphoproliferation would be represented by a decrease in the mean fluorescence intensity histogram, *i.e*. a dilution of CFSE fluorescence. (**Figure 7B**).

As expected, we found that the proportion of lymphoproliferative cells was significantly higher when OT-1 T lymphocytes were incubated with peptide- or native OVA-pulsed DCs or activated platelets (**Figure 7B-C).** Of particular note is that PEVs also supported T cell proliferation when pulsed with either the SIINFEKL or native OVA (**Figure 7B-C**). In addition, when PEVs present in the platelet secretome were removed from pulsed conditions by ultracentrifugation, no proliferation was observed, confirming that the pulsed proteins alone, or the platelet secretome devoid of PEVs, cannot support proliferation (**Figure 7D**). Furthermore, inhibition of PEV proteasome by epoxomicin before pulsing with native OVA inhibited the ability of PEV to induce T cell proliferation (**Figure 7E left panel**). The effect was directed toward PEV proteasome, as addition of epoxomicin prior to peptide pulsing at the same concentration used on DCs did not inhibit proliferation (**Figure 7E right panel**). Thus, PEVs are capable of proteosome-dependent processing of native proteins, thereby enabling peptide loading onto MHC-I. Platelet EVs express co-stimulatory molecules, and their interaction with T lymphocytes promotes lymphocyte cytokine production and proliferation.

Discussion

Megakaryocytes and platelets are emerging as active players in innate and adaptive immunity.[7,30,31] The platelet's role in immunity is mainly confined to the blood circulation, while megakaryocytes are localized in bone marrow and lungs. The latter location potentially provides the megakaryocyte with more direct access to airborne pathogens and allergens.[32,62,63] In contrast, PEVs can additionally disseminate into organs and tissues and this may be possibly due to their small dimensions and the presence of unique surface molecules. In this study, we found that the proteasome and the necessary machinery to process and present antigens to $CD8^+$ T cells are packaged into PEVs by platelets. Thus, PEVs may extend the immune functions played by platelets and megakaryocytes outside the confines of the blood.

Platelet EVs are heterogeneous in terms of surface molecules and their platelet-derived content. The presence of mitochondria within PEVs is well documented,[36,37,64] but it was unknown whether other organelles were also transferred from the platelet. The proteasome is much more abundant than mitochondria, at around 800,000 copies per cell[65] in contrast to approximately 3–7 mitochondria per platelet.[37] Further investigation will be necessary to determine if the presence of multiple organelles within a single vesicle is the result of a specific sorting mechanism, or because those vesicles are larger and may have more storage capacity. Nonetheless, we observed that the release of proteasome-containing PEVs requires cytoskeleton remodeling via intact actin microfilament dynamics and that a broad array of platelet agonists induce the release of proteasome-containing PEVs.[37]

The presence of an extracellular proteasome has already been documented in normal human blood, and elevated levels have been found in patients suffering from autoimmune diseases, sepsis or trauma.[66] Moreover, the 20S proteasome core is present and active within EVs derived from apoptotic endothelial cells and regulates tertiary lymphoid structure formation, autoantibody production and graft rejection following transplantation.[12] While some evidence supports that a circulating extracellular

proteasome may be transported by EVs, we show that EVs of platelet origin, among the most abundant EVs in blood, do contain the proteasome. We further suggest, based on our characterization of these EVs, that platelet microvesicles, not exosomes, contain the proteasome. Consistent with this, mass spectrometry analysis of the human PEV proteome identified numerous proteasomal subunits.[67-69] These include subunits of the 20S catalytic core and immunoproteasome subunit (PSMB8), subunits of the 11S and 19S regulator and the 26S proteasome.[67-69] Moreover, with calnexin, calreticulin, ERP57 and ERP29, other members of the ubiquitin-proteasome pathway were identified in PEVs such as members of the E1 and E2 ubiquitin-conjugating enzyme family.[67,69] Considering the presence of these proteins and the fact that intact ovalbumin needs to be ubiquitinated for degradation by the proteasome,[70] it points to the occurrence of functional ubiquitination in PEVs. To our knowledge, there is no evidence of protein TAP-1 and TAP-2 (related to TAP transporter) presence in PEVs. Further investigations are required to see if the TAP transporter is present in PEVs and/or if the processing pathway of the antigen differs in extracellular vesicles since there is no reported endoplasmic reticulum in PEV. Thus, while proteomic data points to ubiquitin-proteasome system proteins in PEVs, the present work unequivocally demonstrates its presence and documents that the extracellular proteasome in PEVs is functional and can contribute to antigen processing.

We used complementary approaches and developed a Hs-FCM-based assay to detect active proteasome at the single EV level, thereby permitting quantification and assessment of other molecules expressed by the EVs. In particular, the proteasome-containing PEVs also expressed MHC-I and co-stimulatory molecules, which enabled lymphocyte activation/proliferation and cytokine generation. These findings demonstrate a novel and potentially important role for PEVs in adaptive immunity. While our work suggests that PEVs may be involved in adaptive immunity through antigen presentation, it does not necessarily exclude that other cells may release proteasome-containing EVs capable of playing this role. Indeed, EVs derived from DCs, B and T lymphocytes, macrophages and NK cells can perform cross-presentation, suggesting that they also contain the necessary antigen processing machinery.[71-76] Further studies will be

necessary to determine the impact and the importance of PEVs as antigen presenting elements.

We identified proteasome-containing PEVs in the blood of healthy donors. As most PEVs in blood under healthy conditions are suggested to originate from megakaryocytes,[77,78] the latter may also constitutively release proteasome-containing EVs. Moreover, we found that numerous stimuli of human platelets, as well as *in vivo* stimulation of mouse platelets could induce release of proteasomes in PEVs, suggesting that proteasome release is at least conserved in both humans and mice and takes place via platelet activation. Furthermore, platelets can actively induce immunity against the *Plasmodium berghei* parasite[26] and megakaryocytes can be infected by Dengue virus[79] and can also phagocytose *E. coli.*[32] Given their small size, intact microorganisms may not necessarily be present inside PEVs, but PEVs might process cytosolic microbial proteins derived from intact platelets/megakaryocytes that lack the ability to enter the lymphatic system. Thus, PEVs may be implicated in immune surveillance and might contribute to presentation of microbial antigens within lymph tissues. Future studies are however needed to determine whether exposure to PEVs suffices to establish immunity *in vivo*, such as to less immunodominant antigens than OVA, or whether co-stimulation by inflammation or infection are needed to establish sustained immune response.

Self-antigens may also be presented by PEVs. Mitochondria were identified in a proportion of the proteasome-containing PEVs, and although prior studies showed that mitochondria-containing PEVs are rare in lymph (0.41±0.25% (n=4) of the PEVs in mouse lymph contain mitochondria)[41] in comparisons to proteasome-containing PEVs 13.61±4.27% (n=6), these proportions might be augmented in certain diseases. It would be interesting to determine whether PEVs contribute to the formation of mitochondrial autoantibodies that are described in autoimmune diseases, such as systemic lupus erythematosus.[80] Furthermore, the presence of proteasome-containing PEVs in platelet concentrates was not associated with increased risks of ATR or TRALI in a mouse model. It remains to be verified whether the presentation of platelet antigens (*e.g*. CD41 or CD61) by PEVs from PCs might contribute to generation of anti-platelet immunity in

transfused recipients, although this has been shown with megakaryocytes.[56] It is also not excluded that PEV might also participate in other immune responses such as autoantibody production or in tissue remodeling, or if they could be used a platform for cell-based vaccines.[81-83] The cross-presentation of PEVs presented here may allow for new therapeutic possibilities such as in anti-tumor or anti-viral immunity or to induce cytotoxic immunity by vaccination.[84] For example, PEVs have already been proposed as antigen carriers for vaccination,[85,86] and our results suggest that these types of PEVs are also endowed with cross-priming properties that offer new prophylactic or therapeutic vaccination.

Human platelets injected into WT mice circulate less than 2h, in contrast to mouse platelets transfused into mice that can circulate for several days.[87,88] We thus used mouse PEVs in our transfusion experiments, and yet most were undetectable from the blood circulation after 15min, pointing to their rapid uptake in surrounding tissues. In blood, the main absolute cellular target was the platelets, mostly because platelets outnumber leukocytes, which might suggest that PEVs might recycle molecules back to platelets. PEVs were also found in bone marrow, consistent with recent findings that pointed to their role in the stimulation of megakaryocyte biogenesis.[43] The main organs that were targeted were the lymphoid organs. Our findings in mouse lymph revealed that proteasome-containing PEVs can circulate in the lymphatic system, potentially explaining their accumulation in lymphoid organs following intravenous injection. This access to the lymphatic system by proteasome-containing PEVs may reveal a new immune route for PEVs to reach lymphoid organs or infected tissues. Our study highlights the diversity of PEVs and supports the concept that different subtypes of PEVs may play different roles depending on their cargo and tissue distribution.

Acknowledgments

We are grateful to the blood donors and patients who participated in this study. We acknowledge the generous technical help provided by Nicolas Tessandier and Carolanne Gélinas. We are thankful to Julie-Christine Lévesque from the Cytometry and Microscopy platform (CHU de Quebec) and Richard Janvier from the Microscopy platform (Université Laval).

Sources of Funding

This work was supported by the Natural Sciences and Engineering Research Council (awarded to EB); EB is recipient of senior award from the Fonds de Recherche du Québec - Santé (FRQS). This work was also supported by the Établissement Français du Sang (EFS), INSERM and Université Paris-Est (to BV) and by the Swedish Research Council (Vetenskapsrådet, #2017-01779) (to J.W.S). GM has an award from the Canadian Blood Services and from MITACS. AZ was recipient from a postdoctoral fellowship from Swiss National Science Foundation. AL has an award from the fonds Pierre Borgeat and from The Arthritis Society (TAS). MB is recipient of a postdoctoral fellowship from TAS.

Disclosures

The authors have no conflict of interest to disclose.

Authorship Contributions

EB, BV, GM, AZ, conceived and designed the study and wrote the manuscript; GM, AL, SH, MB, MM, IA, MT and BV performed the experiments, analyzed the data, and participated in manuscript preparation. TL, JR, AKR, JT, HHC, FC, RK, JWS, MJH, SGB, FP, HSO, BIF and MD performed experiments, contributed critical biospecimens and analyzed data. All the authors read and approved the manuscript.

The current affiliation for GM. is Division of Hematology and Transfusion Medicine, Lund University, Lund, Sweden under a postdoctoral fellowship from the FRQS.

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Figures Legends

Figure 1. Platelets and PEVs contain proteasome

(**A**) Proteasome 20S α subunit, CD41, TOM20, TSG101 and actin in human platelet extracellular vesicles (PEVs) (18Kxg fraction) and platelet (PLTs) preparations (20 µg protein per lane) were assessed by immunoblotting. Results are representative of five distinct preparations. (**B-E**) Protein quantifications were assessed by densitometry using image lab software (Biorad), results were normalized to actine and expressed as arbitrary units (AU). Mean ± SEM, n=5, paired t-test *P < 0.5. (**F**) Proteasome function was assessed by measuring trypsin-like, caspase-like or chymotrypsin-like activity of PEVs and platelets treated or not with epoxomicin using the Proteasome-Glo™ chymotrypsin-like, trypsin-like and caspase-like cell-based assays. Twenty and 10 µg of proteins were used for platelets and PEVs, respectively. Mean ± SEM, n = 6, * P < 0.05, ** P < 0.01, *** P < 0.001, Mann-Whitney. (**G**) TEM visualization of immunogold labelling of proteasome 20S α subunit in PEVs released from thrombin (0.5U/mL)-activated platelets. Data are representative of three independent experiments. (**H**) High-sensitivity flow cytometry (hs-FCM) analysis of resting platelets and thrombin (0.5U/mL)-activated platelets. Two distinct populations of PEVs, *i.e.* larger PEVs (approximately 17% of these PEVs contain active proteasome) and smaller PEVs, not containing active proteasome. (n = 20 data are presented as mean ± SEM, ** P < 0.01, *** P < 0.001 and **** P < 0.0001, Kruskal–Wallis). (**I-J**) Controls were performed to assess the specificity of PEV detection using hs-FCM. Sensitivity of $CD41^{+}Proteasome^{+}$ PEVs to competition by epoxomicin, ultracentrifugation (Ultracentri) or 0.05% Triton X-100 and unlabelled samples are presented as % of untreated (Control). Data are presented as mean ± SEM of 5 independent experiments, paired t-test ****P < 0.0001 compared with the control. (**K**) Confocal microscopy visualization of proteasome content associated with platelets (left panel) and PEVs (right panel). Visualization of CD41, wheat germ agglutinin (WGA) to determine plasma membrane surface, proteasome (LWA300) and merge is displayed in the region of interest (ROI). Populations originating from dashed lines squares and represented in ROI are triple positives (white arrowheads) or CD41- and WGA-positive but proteasome-negative (white arrows).

Figure 2. Identification of proteasome-containing PEVs under physiological and pathological conditions

(**A**) Proteasome-containing PEVs detected by hs-FCM are found in PFP from platelet concentrates that have caused adverse transfusion reaction (ATR) in recipients and in control concentrates that did not induce ATR. The total number of proteasome-containing PEVs (containing or not mitochondria (mito)), proteasome$^+$mito$^-$PEVs or proteasome$^+$mito$^+$PEVs does not significantly differ between control and ATR, while proteasome$^-$ mito$^+$ PEVs are increased in ATR (no adverse reaction group [n = 33] *vs*. adverse reaction group [n = 34] matched in terms of storage duration; data are presented as mean ± SEM, NS non-significant, **** $P < 0.0001$, Student's t-test). (**B**) Proteasome-containing PEVs detected by hs-FCM are found in bronchoalveolar lavages from mice after induction of transfusion related acute lung injury (TRALI) with 34-1-2s and AF6-88.5.5.3 antibody and in control mice (n = 5, data are presented as mean ± SEM, NS non-significant, Student's t-test). (**C**) Proteasome-containing PEVs are detected at significantly higher levels in mice 1-hour post *i.v*. injection of HA-IgG *vs*. control (diluent) mice. (n = 3, ** $P< 0.01$, data are presented as mean ± SEM, Student's t-test)

Figure 3. Platelets and PEVs load and process OVA onto MHC-I

(**A-B**) Thrombin (0.1 U/mL)-activated murine platelets and their PEVs express MHC-I (detected by hs-FCM). (n = 19, data are presented as mean ± SEM, **** $P < 0.0001$, Mann–Whitney). Activated platelets and their PEVs are able to load the SIINFEKL peptide (**C-D**) or to process and load ovalbumin (OVA) (**C-E**) onto MHC-I. (n = 19, **** $P < 0.0001$, data are presented as mean ± SEM, Kruskal-Wallis test comparisons between pulsed (+) to unpulsed (-))

Figure 4. PEVs in blood circulation can reach lymphoid organs and circulate in lymph

(**A-C**) Fluorescently labelled PEVs generated from activated mouse platelets were intravenously injected into mice and their presence in blood (**A**) and different organs (**B-**

C) was monitored after 2, 15 and 60 minutes. Free PEVs (unbound to cells) were identified by flow cytometry for up to 2 minutes in blood as well as PEVs bound to platelets and to leukocytes (mainly $Ly6G^+$ neutrophils, few lymphocytes), but were mostly undetectable by 60 minutes. Dashed lines represent mean of vehicle (n=9-13), n = 11 (2 min), n = 5 (15 min) and n = 6 (60 min), data are presented as mean ± SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, Kruskal-Wallis). (**B**) Representative images of CMFDA-labelled (Green) individual PEVs (White arrowhead) and PEV aggregates (* white asterisk) in whole tissue sections (Spleen, popliteal LN (PLN), inguinal LN (ILN), bone marrow, lungs and liver) at 15 and 60 min by confocal microscopy, nuclei (Hoeschst 3342) are in blue. Results are representative of observations made in 5-6 mice per group. (**C**) PEVs and aggregates were quantified using 5 different sections for lymph nodes (PLN and ILN) (representing a total surface of at least 1.5mm^2), 8 zones of 500,000 μm^2 each, randomly assigned on 2 different sections for femurs (total surface of 4 mm^2) and 10 zones of 500,000 μm^2 each, randomly assigned on 2 different sections for lungs, spleen, kidneys and brain and 1 section for liver (total surface of 5 mm^2) using Zen 3.3 software. (n=6 (PBS 60min), 5 (15 min) and 6 (60 min, data are presented as mean ± SEM, * P < 0.05, ** P < 0.01, Kruskal-Wallis). (**D-E)** PEVs in lymph were detected by hs-FCM. (**D**) Gating strategy to analyze expression of MHC-I and proteasome (LWA300) on CD41+ EVs in lymph and representative dot plot of labelled and unlabelled (CD41 only) lymph. (**E**) Expression of MHC-I and proteasome (LWA300) on CD41+ EVs in lymph was determined. +/+ double positive and −/− double negative for MHC-I and proteasome. (n = 6, data are presented as mean ± SEM).

Figure 5. Platelets and PEVs with loaded OVA peptide express activation and co-stimulatory molecules

(**A-B**) Activated platelets and (**C-D**) PEVs loaded with OVA peptide ($25D1.16^+$) express higher levels of proteasome (LWA300), and activation (Annexin V, P-selectin) and co-stimulatory molecules (CD40, CD40L, OX40L). (**A,C**) Mean fluorescence intensity (MFI) of the different markers assessed by hs-FCM (n = 7, data are mean ± SEM, NS non-significant, * P < 0.05, Student t test). (**B,D**) Representative MFI histogram of the

25D1.16 negative and positive populations for each marker shown on CD41+ Proteasome+ events.

Figure 6. PEVs can induce antigen-specific T cell activation and cytokine production through antigen presentation

(**A**) Schematic representation of the experimental plan. Cells and PEVs used for the stimulation of lymphocytes assessed by intracellular cytokines staining (ICS). DC: dendritic cells, NS: unpulsed, OVA: ovalbumin, O/N: overnight. (**B-E**) Expression of receptors or cytokines by $CD3^+$ $CD8^+$ T cells co-incubated with either DCs, activated platelets (PLTs) or PEVs, left unpulsed or pulsed with SIINFEKL (PP) or ovalbumin (OVA). (**B**) Interferon gamma (IFN-γ) production, (**C**) CD40 expression, (**D**) OX40 expression and (**E**) IL-2 production (n = 6, 7 or 9; data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ Wilcoxon *vs*. unpulsed). Dashed lines are unstimulated conditions.

Figure 7. PEVs loaded with native OVA process and present OVA peptide to induce antigen-specific T cell lymphoproliferation

(**A**) Schematic representation of the experimental plan. DC: dendritic cells, NS: unpulsed, OVA: ovalbumin, CFSE: Carboxyfluorescein succinimidyl ester. (**B)** Histogram showing CFSE fluorescence shift of $CD3^+$ $CD8^+$ T cells populations when co-incubated with either dendritic cells (DCs), activated platelets (PLTs) or PEVs left unpulsed or pulsed with SIINFEKL peptide (PP) or ovalbumin (OVA) for 7 days. (**C**) Percentage of $CD3^+$ $CD8^+$ lymphoproliferative cells after co-incubation with either DCs, PLTs or PEVs unpulsed or pulsed with PP or OVA for 7 days. (n = 14; data are presented as mean ± SEM. NS non-significant, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$, Friedman test followed by Dunn's post-test for multiple comparisons to unpulsed). (**D**) Percentage of $CD3^+$ $CD8^+$ lymphoproliferative cells after 7 days co-incubation with either PP pulsed DCs or supernatant (surn) depleted of PEVs by ultracentrifugation, left unpulsed or pulsed with PP or OVA. (n=5; data are presented as mean ± SEM, ** $P < 0.01$, Mann-Whitney *vs*. unpulsed). (**E**) Proportion of $CD3^+$ $CD8^+$ lymphoproliferative cells after 7 days co-incubation with OVA-pulsed PEVs treated or not with epoxomicin (epoxo) for 2 hours and PP-pulsed DCs (DC + PP) treated or not with epoxomicin (epoxo). (n = 9 for

PEVs and n = 3 for DC; data are mean ± SEM, NS non-significant, ** $P < 0.01$, Wilcoxon). Dashed lines are unstimulated conditions.

Figure 1

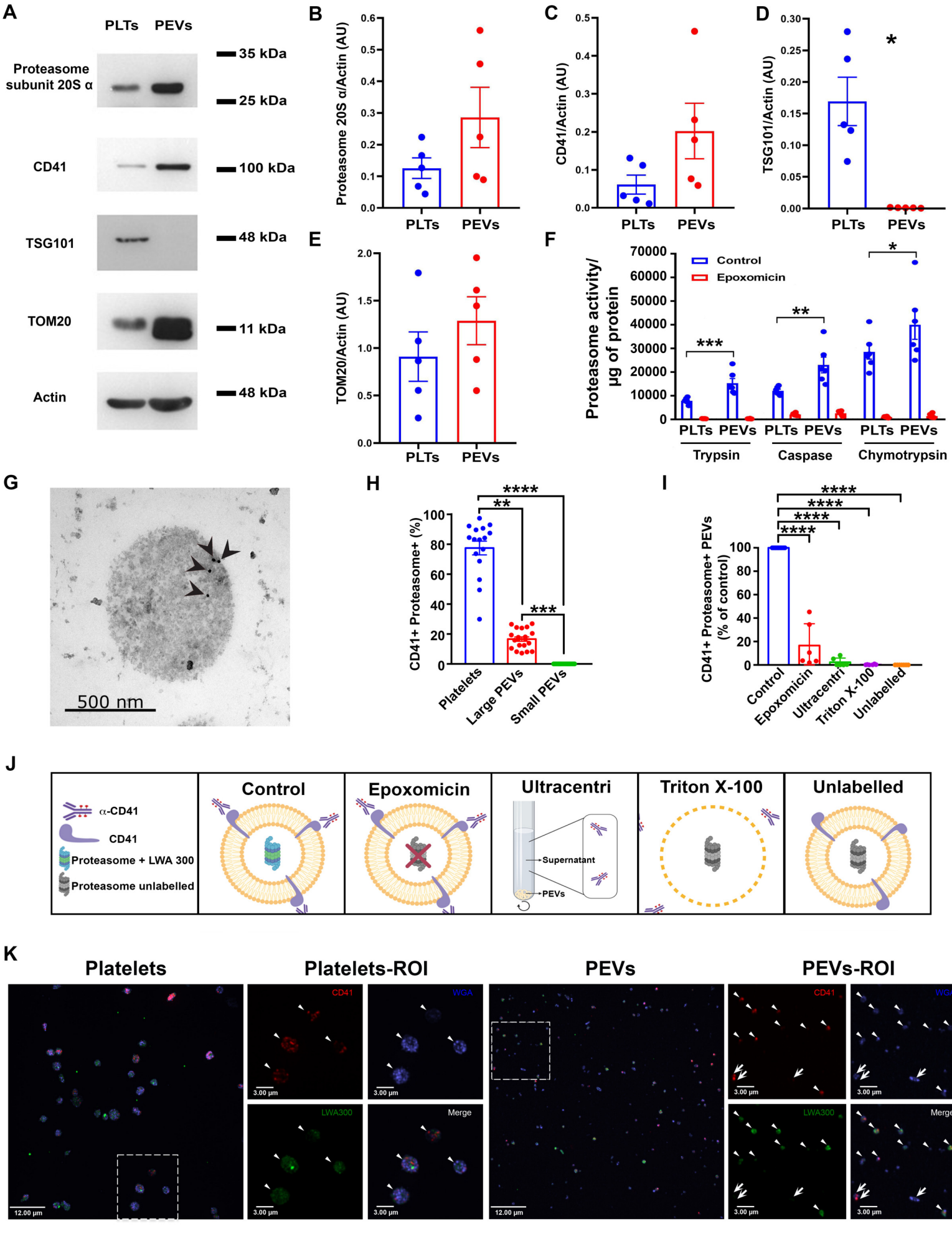

A
PLTs
PEVs
Proteasome subunit 20S α
35 kDa
25 kDa
CD41
100 kDa
TSG101
48 kDa
TOM20
11 kDa
Actin
48 kDa
B
Proteasome 20S α/Actin (AU)
PLTs
PEVs
C
CD41/Actin (AU)
PLTs
PEVs
D
TSG101/Actin (AU)
*
PLTs
PEVs
E
TOM20/Actin (AU)
PLTs
PEVs
F
Proteasome activity/ µg of protein
Control
Epoxomicin
***
**
*
PLTs PEVs PLTs PEVs PLTs PEVs
Trypsin
Caspase
Chymotrypsin
G
500 nm
H
CD41+ Proteasome+ (%)
****
**
***
Platelets
Large PEVs
Small PEVs
I
CD41+ Proteasome+ PEVs (% of control)
****
****
****
****
Control
Epoxomicin
Ultracentri
Triton X-100
Unlabelled
J
α-CD41
CD41
Proteasome + LWA 300
Proteasome unlabelled
Control
Epoxomicin
Ultracentri
Supernatant
PEVs
Triton X-100
Unlabelled
K
Platelets
Platelets-ROI
PEVs
PEVs-ROI
CD41
WGA
LWA300
Merge
12.00 µm
3.00 µm

Figure 2

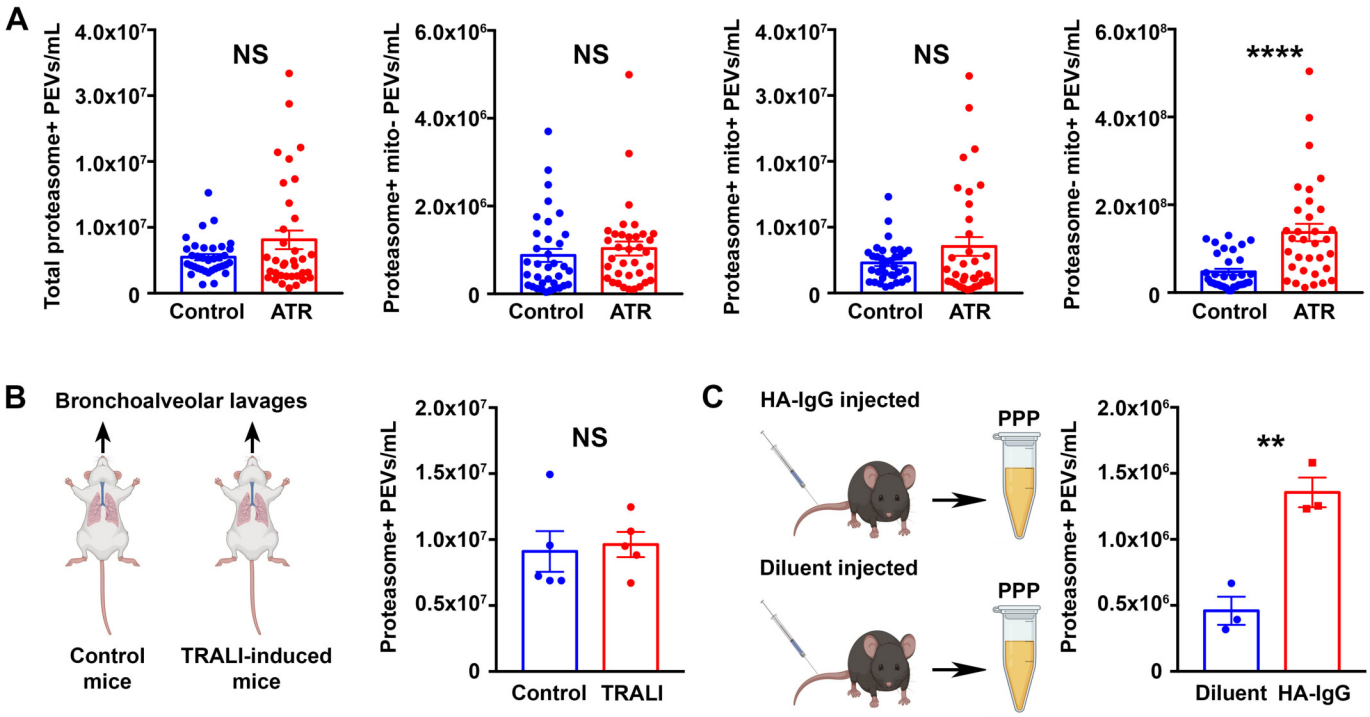

A
Total proteasome+ PEVs/mL
4.0x10^7
3.0x10^7
1.0x10^7
1.0x10^7
0
NS
Control
ATR
Proteasome+ mito- PEVs/mL
6.0x10^6
4.0x10^6
2.0x10^6
0
NS
Control
ATR
Proteasome+ mito+ PEVs/mL
4.0x10^7
3.0x10^7
1.0x10^7
1.0x10^7
0
NS
Control
ATR
Proteasome- mito+ PEVs/mL
6.0x10^8
4.0x10^8
2.0x10^8
0
****
Control
ATR
B
Bronchoalveolar lavages
Control mice
TRALI-induced mice
Proteasome+ PEVs/mL
2.0x10^7
1.5x10^7
1.0x10^7
0.5x10^7
0
NS
Control
TRALI
C
HA-IgG injected
PPP
Diluent injected
PPP
Proteasome+ PEVs/mL
2.0x10^6
1.5x10^6
1.0x10^6
0.5x10^6
0
**
Diluent
HA-IgG

Figure 3

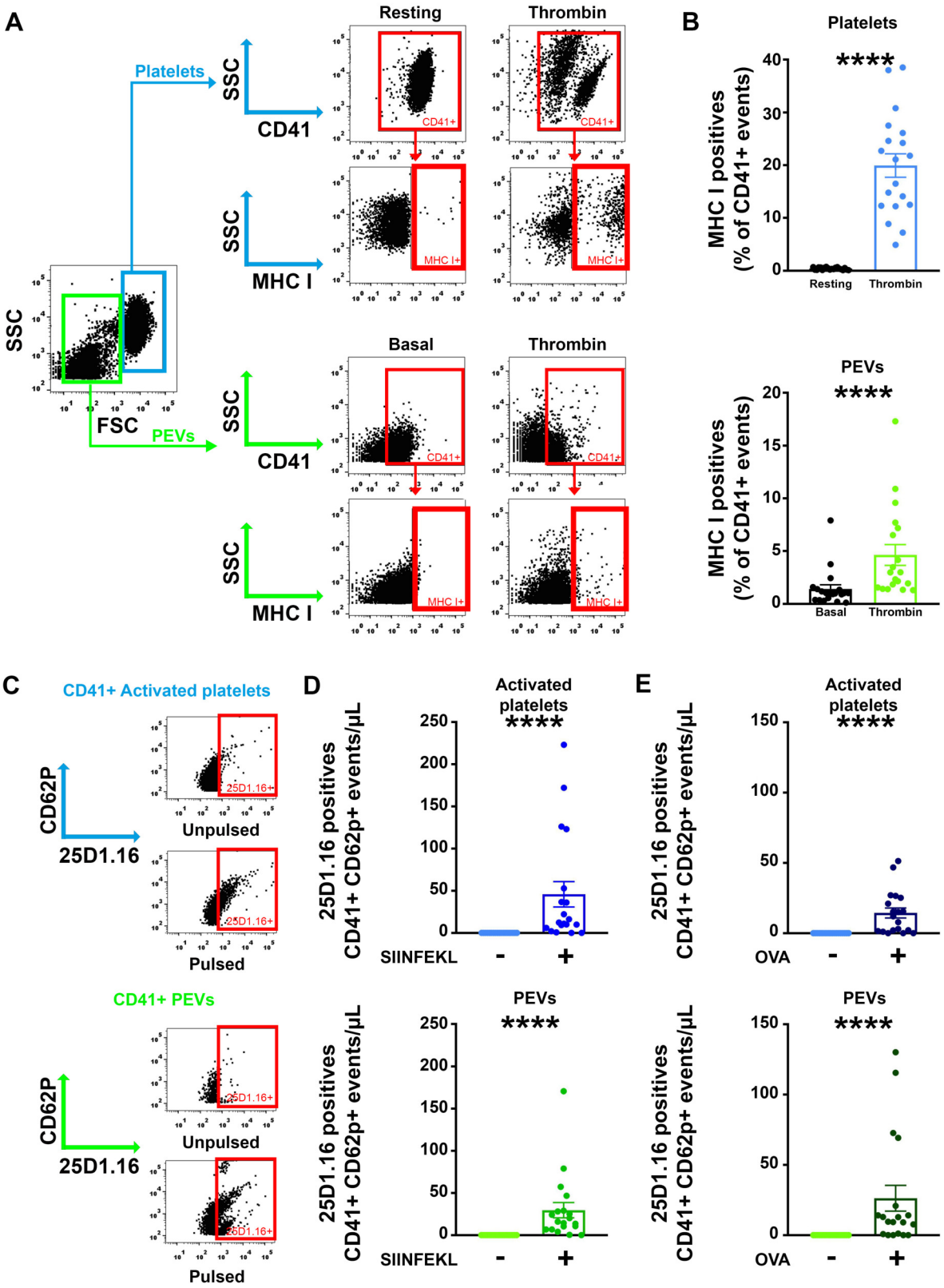

A
Platelets
SSC
FSC
PEVs
CD41
MHC I
Resting
Thrombin
Basal
CD41+
MHC I+
B
Platelets
MHC I positives (% of CD41+ events)
****
Resting
Thrombin
PEVs
Basal
C
CD41+ Activated platelets
CD62P
25D1.16
Unpulsed
Pulsed
25D1.16+
CD41+ PEVs
D
Activated platelets
PEVs
25D1.16 positives CD41+ CD62p+ events/µL
SIINFEKL
E
OVA

Figure 4

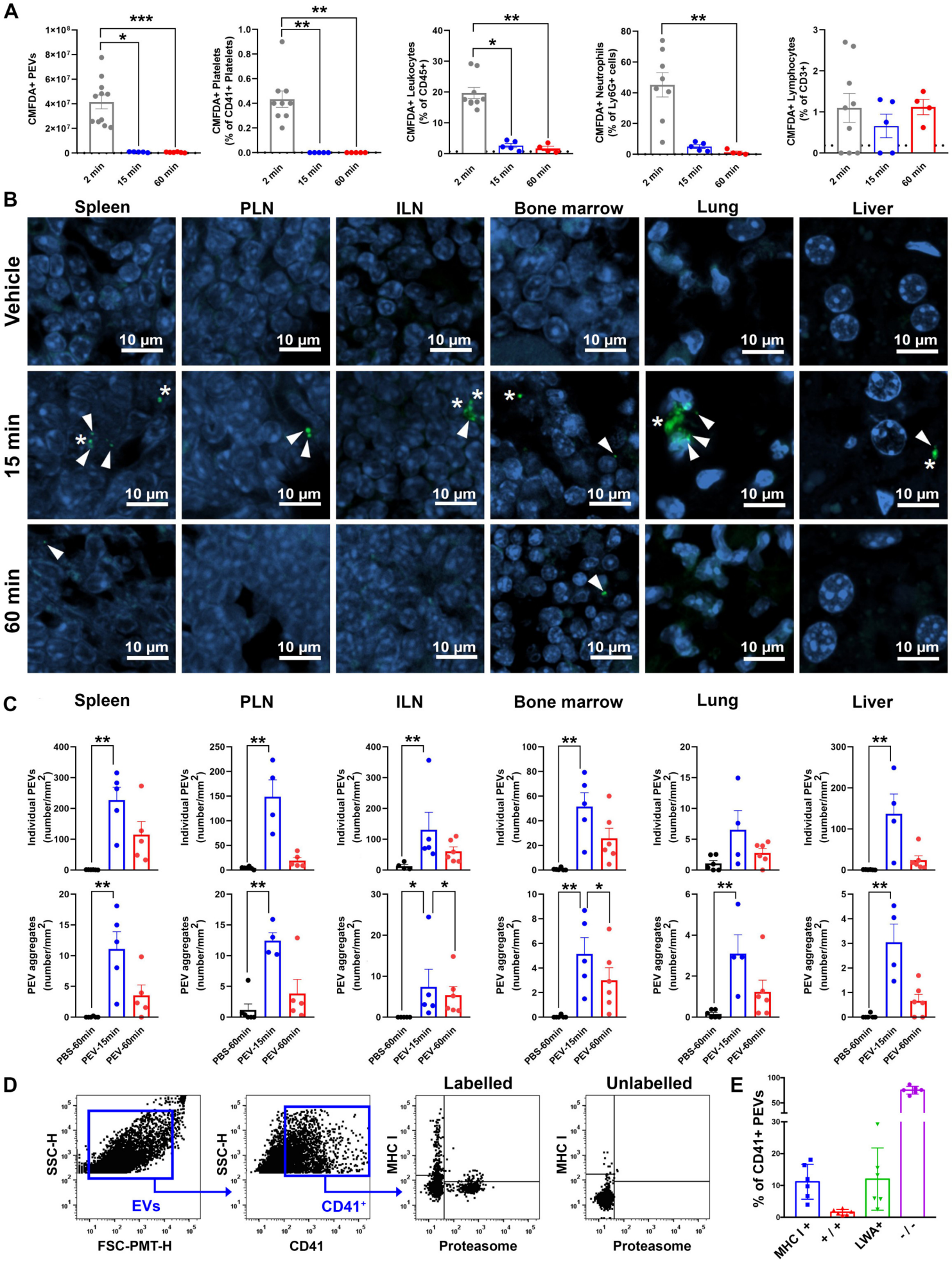

A
CMFDA+ PEVs
CMFDA+ Platelets (% of CD41+ Platelets)
CMFDA+ Leukocytes (% of CD45+)
CMFDA+ Neutrophils (% of Ly6G+ cells)
CMFDA+ Lymphocytes (% of CD3+)
2 min
15 min
60 min
B
Spleen
PLN
ILN
Bone marrow
Lung
Liver
Vehicle
15 min
60 min
10 µm
C
Individual PEVs (number/mm2)
PEV aggregates (number/mm2)
PBS-60min
PEV-15min
PEV-60min
D
Labelled
Unlabelled
SSC-H
FSC-PMT-H
EVs
CD41
CD41+
MHC I
Proteasome
E
% of CD41+ PEVs
MHC I +/+
+/+
LWA+
-/-

Figure 5

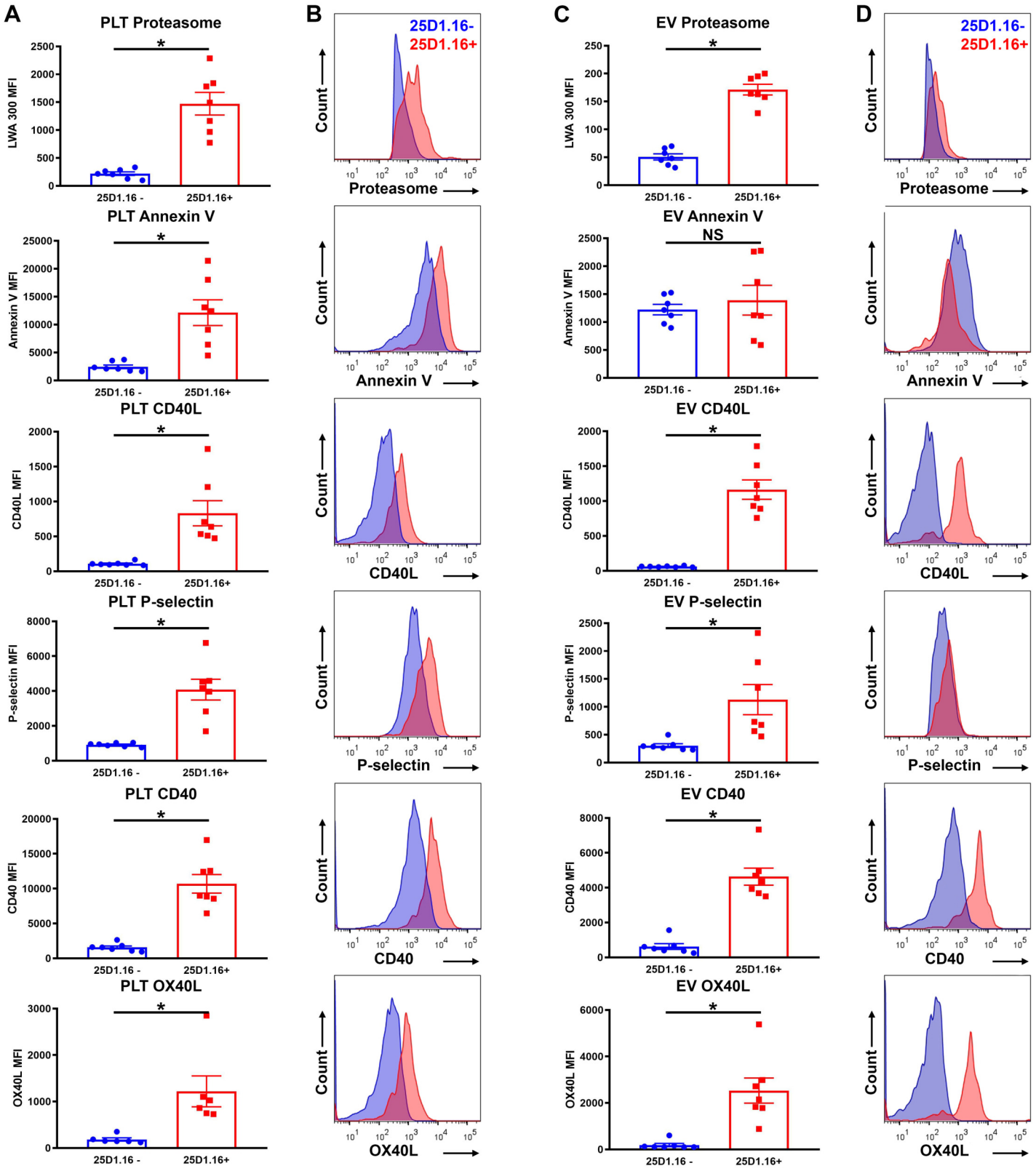
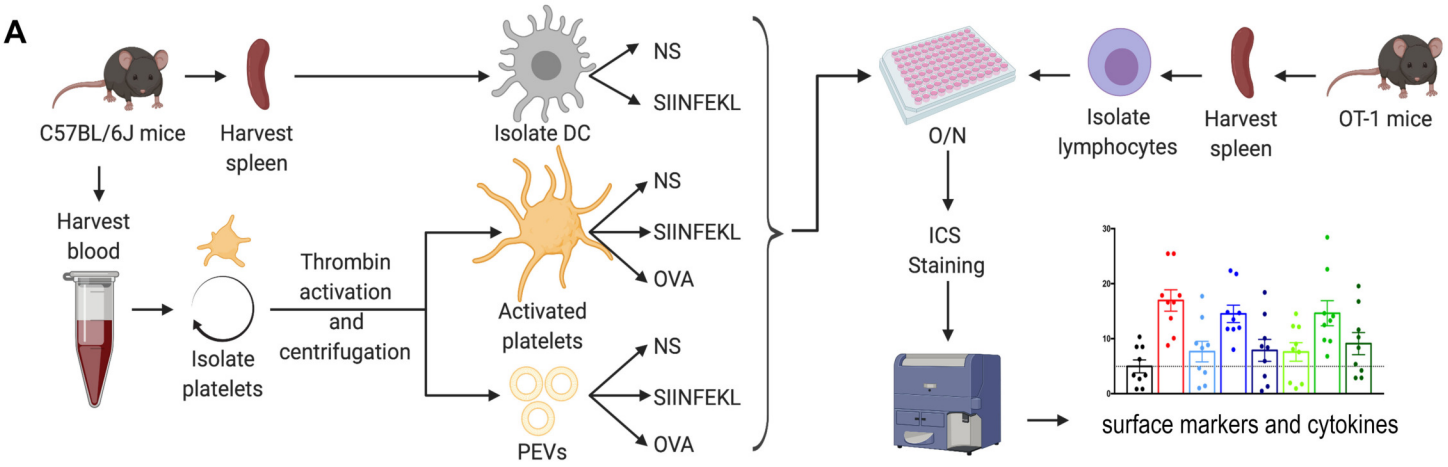
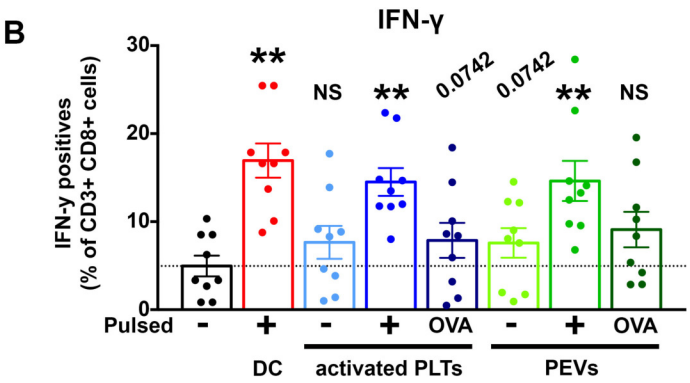
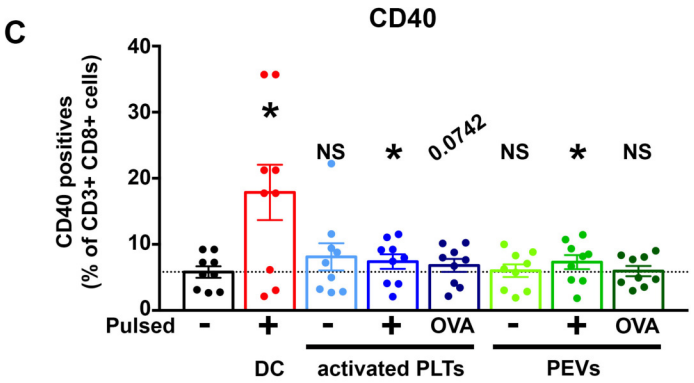
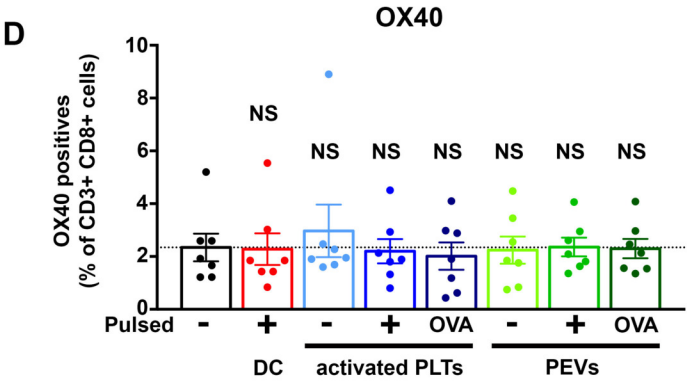

A
PLT Proteasome
LWA 300 MFI
25D1.16 -
25D1.16+
PLT Annexin V
Annexin V MFI
PLT CD40L
CD40L MFI
PLT P-selectin
P-selectin MFI
PLT CD40
CD40 MFI
PLT OX40L
OX40L MFI
B
25D1.16-
25D1.16+
Count
Proteasome
Annexin V
CD40L
P-selectin
CD40
OX40L
C
EV Proteasome
EV Annexin V
NS
EV CD40L
EV P-selectin
EV CD40
EV OX40L
D

Figure 6


A
C57BL/6J mice
Harvest spleen
Isolate DC
NS
SIINFEKL
Harvest blood
Isolate platelets
Thrombin activation and centrifugation
Activated platelets
NS
SIINFEKL
OVA
PEVs
NS
SIINFEKL
OVA
O/N
Isolate lymphocytes
Harvest spleen
OT-1 mice
ICS Staining
surface markers and cytokines


B
IFN-γ
IFN-γ positives (% of CD3+ CD8+ cells)
Pulsed
DC
activated PLTs
PEVs
OVA
NS
0.0742


C
CD40
CD40 positives (% of CD3+ CD8+ cells)
Pulsed
DC
activated PLTs
PEVs
OVA
NS
0.0742


D
OX40
OX40 positives (% of CD3+ CD8+ cells)
Pulsed
DC
activated PLTs
PEVs
OVA
NS

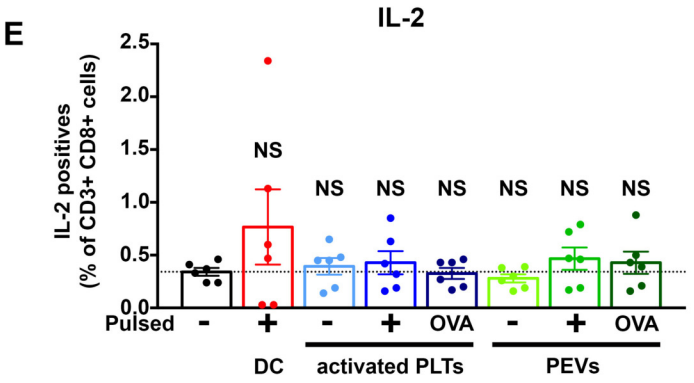
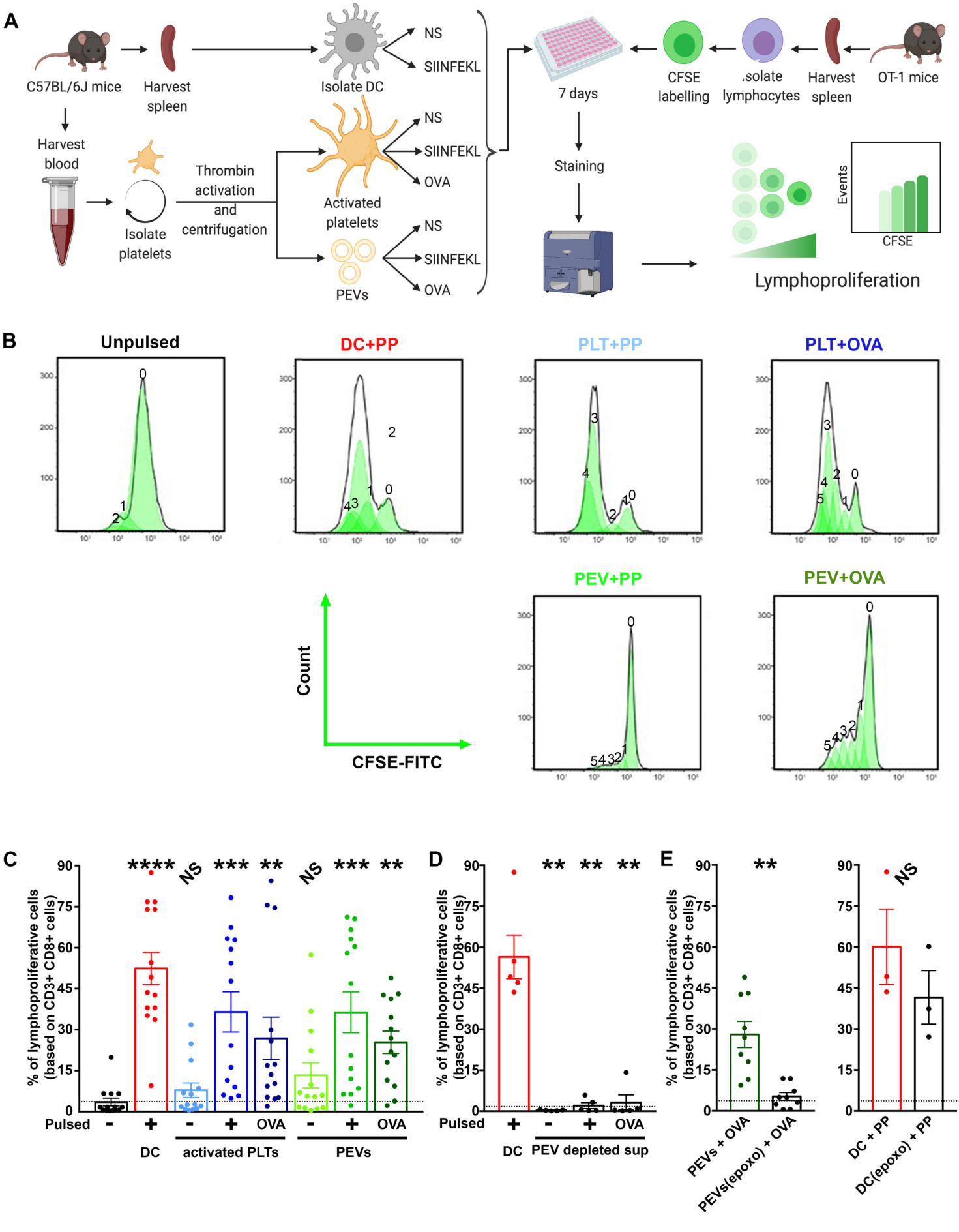

E
IL-2
IL-2 positives (% of CD3+ CD8+ cells)
Pulsed
DC
activated PLTs
PEVs
OVA
NS

Figure 7


A
C57BL/6J mice
Harvest spleen
Isolate DC
NS
SIINFEKL
Harvest blood
Isolate platelets
Thrombin activation and centrifugation
Activated platelets
NS
SIINFEKL
OVA
PEVs
NS
SIINFEKL
OVA
7 days
Staining
CFSE labelling
Isolate lymphocytes
Harvest spleen
OT-1 mice
Events
CFSE
Lymphoproliferation
B
Unpulsed
DC+PP
PLT+PP
PLT+OVA
PEV+PP
PEV+OVA
Count
CFSE-FITC
C
% of lymphoproliferative cells (based on CD3+ CD8+ cells)
Pulsed
DC
activated PLTs
PEVs
OVA
D
% of lymphoproliferative cells (based on CD3+ CD8+ cells)
Pulsed
DC
PEV depleted sup
OVA
E
% of lymphoproliferative cells (based on CD3+ CD8+ cells)
PEVs + OVA
PEVs(epoxo) + OVA
DC + PP
DC(epoxo) + PP