Platelet CLEC-2 protects against lung injury via effects of its ligand podoplanin on inflammatory alveolar macrophages in the mouse

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Running title

Platelet CLEC-2 protects against LPS-induced lung injury

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Abstract

There is no therapeutic intervention proven to prevent the acute respiratory syndrome (ARDS).

Novel mechanistic insights into the pathophysiology of ARDS are therefore required. Platelets are implicated in regulating many of the pathogenic processes which occur during ARDS, however the mechanisms remain elusive. The platelet receptor C-type lectin-like 2 (CLEC-2) has been shown to regulate vascular integrity at sites of acute inflammation. Therefore, the purpose of this study was to establish the role of CLEC-2 and its ligand podoplanin in a mouse model of ARDS. Platelet-specific CLEC-2-deficient, as well as alveolar epithelial type I cell (AECI)-specific- or hematopoietic-specific podoplanin deficient mice were established using cre-loxP strategies. Combining these with intratracheal (IT) instillations of lipopolysaccharide (LPS), we demonstrate that arterial oxygen saturation decline in response to IT-LPS in platelet-specific CLEC-2-deficient mice is significantly augmented. An increase in bronchoalveolar lavage (BAL) neutrophils and protein was also observed 48h post IT-LPS, with significant increases in pro-inflammatory chemokines detected in BAL of platelet-specific CLEC-2 deficient animals. Deletion of podoplanin from hematopoietic cells but not AECIs also reduces lung function and increases pro-inflammatory chemokine expression following IT-LPS. Furthermore we demonstrate that following IT-LPS, platelets are present in BAL in aggregates with neutrophils which allows for CLEC-2 interaction with podoplanin expressed on BAL inflammatory alveolar macrophages. Taken together these data suggest that the platelet CLEC-2-podoplanin signaling axis regulates the severity of lung inflammation in mice and is a possible novel target for therapeutic intervention in patients at risk of developing ARDS.

Key words

Acute respiratory distress syndrome, platelets, alveolar macrophages, mouse models
Introduction

The acute respiratory distress syndrome (ARDS) is a devastating clinical syndrome of acute respiratory failure in the critically ill. It is the final common pathway of response to a variety of direct pulmonary insults such as bacterial/viral pneumonia and gastric aspiration, or indirect insults such as abdominal sepsis or battlefield trauma (10, 35, 45). Under the Berlin definition for ARDS, onset of diagnosis must be within 7 days, with bilateral opacities present on chest X-ray and severity defined as “mild” ($P_aO_2/FiO_2 = 200-300$), “moderate” ($P_aO_2/FiO_2 = 100-200$), or “severe” ($P_aO_2/FiO_2 < 100$) (10, 53). The incidence of ARDS has been reported to range from 7.2-78.9 cases per 100,000 worldwide, with mortality estimated at approximately 40% and pulmonary impairment persisting in up to 50% of survivors leading to enormous social and fiscal cost (45, 49, 55). There are no current readily available tests that can clearly identify those who are at high risk of ARDS, and no therapeutic interventions proven to prevent its occurrence. Clearly other mechanistic insights into the pathophysiology of ARDS are needed to identify new pathways for therapeutic manipulation.

Traditionally thought of as key regulators of physiological and pathogenic hemostasis, platelets are now recognized as essential mediators of both innate and adaptive immunity (22-24, 52). Platelets are implicated in regulating many of the pathogenic processes which occur during ARDS including, neutrophil recruitment, macrophage-dependent inflammation and alveolar-capillary permeability through complex mechanisms (37). Murine models suggest that thrombocytopenia is protective during sterile LPS-induced lung inflammation primarily by reducing neutrophil recruitment (16, 30). However, this would presumably be detrimental if the bactericidal activities of neutrophils are required. Indeed, thrombocytopenia in intensive care unit (ICU) patients is associated with an increased risk of ARDS (56). In addition, severe thrombocytopenia (<5x10^9 platelets/L) in a mouse model of pneumonia-induced sepsis enhanced pro-inflammatory cytokine release and significantly impaired survival (5).
Platelets are required to maintain endothelial barrier function under both homeostatic and inflammatory conditions. Looney et al. (30), originally demonstrated a pathogenic role for platelets in an anti-MHC I and LPS-induced murine transfusion-related acute lung injury (TRALI) model. A more recent publication, however, questioned the role of platelets in the initiation and development stages of the TRALI model, with only red blood cell accumulation/alveolar hemorrhage argued to be platelet-dependent due to their role in regulating vascular integrity (19). Maintenance of vascular integrity is primarily driven via activation of, and signaling through, the immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors (29). Murine platelets express two ITAMs; GPVI, and the hemITAM, C-type lectin-like 2 (CLEC-2), both of which are critical for securing vascular integrity within the lungs during inflammation (2). While GPVI is restricted to platelets, CLEC-2 expression has been shown to be present at a low level on a small subset of inflammatory cells (33, 39). To date, the only known endogenous ligand for CLEC-2 is podoplanin (also known as gp38 or T1α), a cell surface protein originally described on kidney podocytes and alveolar epithelial type I cells (AECIs) (3, 51), that is also expressed on lymphatic endothelial cells (43), inflammatory macrophages (26), CD4 T cell subsets (48) and stromal cells (11). Mice deficient in either CLEC-2 or podoplanin exhibit separation defects of the blood and lymphatic systems, and present with significantly altered lung function that may contribute to their peri-natal lethality (12, 50). Furthermore, platelet activation via CLEC-2 is independent of major hemostatic pathways, and thus is a candidate for the development of novel therapies in lung injury (40).

Based on the expression of podoplanin in the lung, the unusual role of CLEC-2 in platelet activation and the phenotype of CLEC-2 or podoplanin deficient mice, we hypothesized that the CLEC-2-podoplanin axis may play an as yet unrealized role in ARDS. In this study we investigate the in vivo consequences on acute lung injury of deleting CLEC-2 or podoplanin using a mouse model of ARDS. We show that LPS-induced lung inflammation leads to recruitment of platelets predominantly bound to neutrophils into alveoli. Furthermore, CLEC-2 expressed on platelets is required to limit lung function decline as assessed by arterial oxygen saturation, confirmed by increases in neutrophilia.
and BAL protein observed in CLEC-2 deficient animals. We further demonstrate that this action is mediated by interaction of CLEC-2 with its ligand podoplanin expressed on a macrophage population (CD11b^CD11c^F4/80^) only present in alveoli during inflammatory conditions. Taken together, these data suggest that the platelet CLEC-2-podoplanin signaling axis is protective during a model of mouse lung injury, and is therefore a possible novel target for future therapeutic intervention in patients at risk of developing ARDS.

Materials and methods

Mice

All mice were maintained in individually ventilated cages under a 12 h light/dark cycle at a constant temperature of 20 °C with food and water given ad libitum at BMSU, Birmingham University, UK. All experiments were performed in accordance with UK laws (Animal [Scientific Procedures] Act 1986) with approval of local ethics committee and UK Home Office approval under PPL 40/3741. Clec2^{fl/fl} mice with either PF4cre, Rosa26-ER^{T2}cre (ER^{T2}cre) or CD11c-cre along with Pdpn^{fl/fl}VAV1cre^ have been described previously (1, 12, 20, 33). Animals were fed with FormulaLab Diet 5008 (Lab-Diet, St-Louis, MO). When required, 4- to 6-week-old Clec2^{fl/fl}ER^{T2}cre and their Clec2^{fl/fl} control littermates were fed for two weeks with tamoxifen-supplemented diet TAM 400 (Envigo, UK), and then returned to the FormulaLab Diet 5008 chow. Mice expressing Aquaporin 5 cre (Aqp5cre) (13) were crossed with mice expressing the podoplanin floxed allele to generate the Pdpn^{fl/fl}Aqp5cre^ strain. Wildtype C57Bl/6 mice were purchased from Harlan Laboratories (Oxford, UK). Male mice, aged 9-16 weeks, were used.

Intratracheal LPS model

Instillations of 40μg LPS (E. coli O111:B4, InvivoGen, France) in a 50μl bolus of phosphate-buffered saline (PBS) or PBS alone were administered to each mouse. Mice were sacrificed at 48 hours post-
LPS instillation (peak of cellular recruitment), or following resolution of the inflammatory response, 9
days post-LPS instillation (28). Infrared pulse oximetry and pulse distention were assessed by
MouseOx Plus® (Starr Life Sciences Corp, USA) and bronchoalveolar lavage (BAL) collected as
previously described (28). The epithelial damage marker receptor for advanced glycation end-
products (RAGE), as well as cytokine and chemokine levels were analyzed using Fluorokine MAP
Multiplex (R&D Systems, UK). Whole blood was collected into ethylenediaminetetraacetic acid
(EDTA), analyzed on an ABX Pentra 60 (Horbia Ltd, UK) and plasma isolated by centrifugation at
2000g for 30 min.

Lung water and protein permeability

Protein permeability was calculated by measuring the concentration of total protein in BAL samples
per ml of fluid recovered. To calculate wet-to-dry weight ratios mice were culled by cervical
dislocation and lungs removed en bloc. Heart, thymus and connective tissue were removed and
“wet” lungs weighed. Lungs were dried in a 55°C oven for 48 hours. The resulting “dry” lungs were
weighed and the ratio between the two values determined.

Platelet depletion

Intra-peritoneal injections of 1.5μg/g anti-mouse GP1bα antibody (EMFRET analytics, Germany;
R300) were given 18 hours prior to IT-LPS. Complete and sustained platelet depletion was confirmed
in whole blood immediately prior to IT-LPS; 25 ±10 x10^3/mm^3 compared to 959 ±34 x10^3/mm^3
isotype controls (approximately 97%).

Platelet transfusion

Blood was collected from the inferior vena cava of wildtype C57Bl/6 mice under terminal anesthesia
and platelets prepared as previously described (46). Blood was pooled from a number of donor mice,
transfused into recipient Clec2^fl/fl or Clec2^fl/fl ER^T2 cre+ mice (2 x 10^8 in 200 μl of buffer via the tail
vein). Immediately after intravenous injections, recipient mice were subjected to IT-LPS. The
platelets to be transfused were confirmed to be inactive, with the potential to become fully activated, by analyzing P-selectin and fibrinogen binding via flow cytometry following incubation with or without 0.5U/ml thrombin (Sigma-Aldrich, UK) for 15 minutes at room temperature.

**Lung digestion and flow cytometry**

Lungs were perfused with 10ml of 200nM EDTA/PBS until white. BAL was extracted and the left lobe digested using Liberase TL (0.4Wunsch units/ml; Roche) with DNase (50μg/ml; Roche) and incubated at 37°C in a rotating shaker (200rpm) for 40 min. Single cell suspensions were ensured by passing through a 25G needle four times and washing the cells through a 70μm filter. Pelleted cells from BAL were first enumerated and then assessed by flow cytometry alongside lung tissue cells using fluorophore-conjugated antibodies (eBioscience). Only SyTOX (Invitrogen) negative cells - live cells - from digested tissue were analyzed. BAL neutrophil and red blood cell numbers are presented per ml of BAL. Cell populations from digested tissue were stained as previously described (18), or as stated in the text.

**Lung histology and podoplanin expression**

Lungs were inflated post-mortem with 1.5ml of a solution containing optimum cutting temperature (OCT) compound (Tissue-Tek, The Netherlands) in PBS (1:2) introduced via the trachea using a 19G needle. The trachea was then sutured and lungs removed en bloc, frozen over dry ice and stored at -80°C. 20μm frozen sections were processed for hematoxylin and eosin (H&E) or podoplanin staining (clone 8.1.1., eBioscience) Syrian hamster IgG controls were used to confirm specific staining (data not shown). Images were analyzed using a Zeiss Axio Scan.Z1 microscope and ZEN software.

**Statistical analysis**

All parameters were analyzed using Prism 6 (GraphPad Software, USA). Significance was assessed by ANOVA with relevant post-test analysis as indicated in the text. Data are presented as Box-Whisker plots with mean displayed and the range of minimum to maximum data points.
Results

Intratracheal LPS induces recruitment of platelet-neutrophil aggregates into lung alveoli

When assessed by flow cytometry, BAL from unchallenged mice contains predominantly alveolar macrophages (CD11b^−CD11c^+F4/80^+). As expected, following IT-LPS the percentage of neutrophils (CD11b^+CD11c^-Gr1^+) increases dramatically in BAL, along with an inflammatory macrophage population (CD11b^+CD11c^-F4/80^-) (Figure 1A and B). As a consequence resident alveolar macrophages are reduced in percentage. When enumerated, the numbers of tissue resident alveolar macrophages remain constant, with increases in neutrophils and inflammatory alveolar macrophages observed (Figure 1C). Total BAL protein and lung wet-to-dry weight ratio confirms that IT-LPS induces significant lung damage compared to PBS-treated or unchallenged controls (Figure 1D). No significant differences are observed between unchallenged and IT-PBS treated mice.

The critical role of platelets during LPS-induced lung injury has previously been demonstrated in platelet-depleted mice using administration of a rat anti-mouse GP1bα monoclonal antibody (7, 15). In our model we confirm these data, and observe that pre-treatment of wildtype mice with anti-GP1bα antibody significantly increases bleeding into the alveoli as assessed by RBC accumulation in BAL compared to isotype-treated controls following IT-LPS (53.9 fold; Figure 1E and F). Concomitantly, the number of neutrophils observed in BAL is significantly reduced in platelet-depleted animals following IT-LPS (4.5 fold; Figure 1G). As a consequence, anti-GP1bα-treated mice present with an irregular breathing pattern and reduced activity 6 hours after LPS administration compared to isotype-treated controls. One anti-GP1bα antibody treated animal had to be culled 4 hours post IT-LPS due to welfare concerns (* in Figure 1E). This animal was excluded from analysis.

In wildtype mice with normal platelet counts, IT-PBS induces a decrease in platelet count by 17% compared to unchallenged controls. Platelet count drops 13% further following IT-LPS (Figure 1G),
coinciding with the emergence of a CD41⁺ platelet population within the BAL, not seen in control or IT-PBS treated BAL (Figure 1H and I). Flow cytometry reveals that the majority of these platelets are within platelet-leucocyte aggregates (PLAs; 80.8 ±5.3%), primarily bound to Gr-1⁺ neutrophils (58.3 ±5.8%) (Figure 1J). These data demonstrate the location of a population of platelets, which express CLEC-2, within the alveoli during IT-LPS-induced inflammation.

Platelet-expressed CLEC-2 protects against LPS-induced lung injury

Constitutive deletion of CLEC-2 is peri-natally lethal (12). Therefore, we used two cre-loxP strategies to specifically delete CLEC-2 from platelets in mice. Firstly a rosa26-driven tamoxifen-inducible (ER<T2>) cre was used. When a tamoxifen diet is given for only two weeks recombination in the megakaryocyte lineage allows for newly synthesized “knockout” platelets to be produced due to their relatively short circulation time of 5 days ((33) and Figure 2). This is followed by a 4-5 week “wash out” period of normal diet to ensure the anti-inflammatory effects of tamoxifen do not alter responses to LPS. A PF4cre was used to delete CLEC-2 specifically in the megakaryocyte/platelet lineage (12).

To investigate the consequence of platelet-CLEC-2 deletion we first analyzed arterial oxygen saturation ($S_\text{a}O_2$) via pulse oximetry to assess overall lung function. Instillation of PBS in CLEC-2 deficient animals or littermate controls does not alter $S_\text{a}O_2$, which is maintained at approximately 96% (data not shown). Following IT-LPS deletion of CLEC-2 significantly reduces $S_\text{a}O_2$ in both genetically altered strains compared to floxed controls ($p<0.001$, Figure 3A and B). There is no significant difference between the CLEC-2 deficient strains ($p=0.34$) or between floxed only controls given a normal or tamoxifen diet ($p=0.54$). In addition, there are no significant differences in pulse distention, indicative of blood flow (41), between these strains (Figure 3C).
Immunohistochemistry demonstrates an increase in accumulation of both alveolar and interstitial neutrophils in CLEC-2 deficient animals 48 hours post IT-LPS compared to littermate controls (Figure 3D; arrowheads and arrows, respectively). To quantify this, we analyzed BAL neutrophil counts. A significant increase in alveolar neutrophilia 48 hours post IT-LPS in both models of CLEC-2 deficient mice is observed compared to littermate controls (2.3-2.9 fold; Figure 3E). In addition, quantification of interstitial neutrophilia suggests that CLEC-2 deletion driven by PF4cre leads to an increase in the number of neutrophils within lung tissue (Figure 3E).

A significant increase in BAL protein is observed in both CLEC-2 deficient strains compared to controls 48h post IT-LPS (1.5-1.7 fold, Figure 3E). However, no significant differences are observed in wet-to-dry weight ratio, expression of the epithelial cell damage marker, RAGE, or red blood cell (RBC) accumulation in BAL (alveolar hemorrhage) 48 hours post IT-LPS in CLEC-2 deficient strains compared to controls (Figure 3E). These data suggest that the increases in BAL protein observed may reflect the increase in protein derived from inflammatory cells rather than an increase in lung permeability in CLEC-2 deficient animals (36).

When using cre-loxP technology non-specific endonuclease activity of the cre recombinase is always a concern (31). However, we observe no cre-related effects on neutrophil recruitment or BAL protein using PF4cre only expressing mice 48h post IT-LPS (Figure 3E, striped plots). This suggests our data are specific to CLEC-2 deletion. Furthermore, as a cell type previously reported to express CLEC-2 and to have a significant role during inflammation, we used the previously characterized Clec2^fl/fl^CD11c-cre mice to assess the contribution of CLEC-2 expressed on dendritic cells (1). We again observe no effects on neutrophil recruitment or BAL protein using the dendritic cell-specific CLEC-2 deficient mice (Figure 3E, red plots).

Expression of the murine neutrophil recruitment chemokines, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2), in BAL are significantly increased in CLEC-2 deficient animals (2.6-3.0 and 8.8-14.8 fold, respectively) (Figure 3F). However, no significant
difference was observed in expression of the monocyte recruitment chemokine MCP-1 or pro-inflammatory cytokines, tumor necrosis factor (TNF)α and interleukin (IL)-6 (Figure 3G). Platelet numbers in whole blood from Clec2^{fl/fl}ER^{T2}\text{cre}^{+} mice, along with white blood cell (WBC) and RBC counts of all cre^{+} mice, were comparable to floxed-only controls (Figure 3H). Clec2^{fl/fl}PF4cre^{+} mice have a mild reduction in platelet numbers (~13-23%) due to blood-lymphatic mixing but this does not affect WBC or RBC, as previously reported (12). Together these data suggest that platelet CLEC-2 is protective during IT-LPS.

A recent publication utilizes a simple platelet transfusion to confirm the role of platelet CLEC-2 during a mouse model of deep vein thrombosis (DVT) (46). Therefore, using a similar strategy we sought to confirm that the dysregulation of lung inflammation in the CLEC-2 deficient strains is due to the lack of CLEC-2 on platelets. Purity, CLEC-2 expression and potential activation of the purified wildtype platelets used in these experiments were first confirmed by flow cytometry (Figure 4A).

While simple in concept, platelet transfusion within an inflammatory model such as IT-LPS however leads to a complex phenotype. Platelet transfusion immediately prior to IT-LPS into CLEC-2 deficient or sufficient controls has a negative impact on animal welfare, as evidenced by a significant decrease in total body weight loss and a pronounced systemic leukopenia 48 hours post IT-LPS compared to mice that did not receive a transfusion (Figure 4B). Transfusions did not alter systemic numbers of red blood cells or platelets in whole blood 48 hours after IT-LPS.

In wildtype, Clec2^{fl/fl} controls, platelet transfusion significantly increases neutrophil recruitment to the alveoli along with a significant increase in total BAL protein 48 hours post IT-LPS compared to mice that did not receive platelets (Figure 4C). When platelet transfusions were given to platelet-specific CLEC-2 deficient animals (Clec2^{fl/fl}ER^{T2}\text{cre}^{+}), although a trend for reduced neutrophilia was observed following IT-LPS, their response is not significantly different to Clec2^{fl/fl} mice that also received platelets (Figure 4C, grey plots). In addition, no difference in total BAL protein was observed comparing Clec2^{fl/fl} and Clec2^{fl/fl}ER^{T2}\text{cre}^{+} animals that had all received platelets.
The altered response observed in the control mice that received platelets makes subsequent analysis of CLEC-2 deficient animals problematic. Having observed that, without platelet transfusion, CLEC-2 deficient mice have increased BAL neutrophilia and protein, one may assume that this would also be upheld following the addition of a platelet transfusion. The fact that we do not observe any additive effects of lacking platelet-CLEC-2 in mice that receive CLEC-2-expressing platelets may suggest that the platelet transfusion has indeed “rescued” the defect in deficient mice. This supports our findings that it is CLEC-2, specifically expressed on platelets, that is required to limit pulmonary inflammation during IT-LPS. However this explanation may be too simplistic, with the possibility that platelet transfusion alters and/or overcomes any CLEC-2-dependent signaling.

Podoplanin expression in Aqp5cre and VAV1cre conditional knockout lungs

The only known endogenous ligand for CLEC-2 is podoplanin. Like CLEC-2, constitutive deletion of podoplanin is lethal in murine models (50). Therefore, we generated two conditional knockout strains using a floxed podoplanin allele (Pdpn^fl/fl; (32)). We first used previously characterized Aqp5cre mice (13), to generate a novel AECI-specific podoplanin-deficient strain. In addition, we used the Vav1cre strain to generate hematopoietic-specific podoplanin-deficient mice (20). Both strains are viable, without any overt phenotype (Table 1) and exhibit Mendelian inheritance (Table 2).

To characterize these novel mouse models we first used immunohistochemistry to evaluate podoplanin expression in unchallenged and IT-LPS-treated lungs. Deletion of podoplanin is observed only in the Pdpn^fl/fl/Aqp5cre` lungs (Figure 5A). To quantify podoplanin deletion in specific cell populations, lung tissue from unchallenged and IT-LPS-treated mice was perfused, lavage fluid isolated and tissue enzymatically digested. Each cell population was analyzed by flow cytometry using the gating strategy shown in Figure 6 and as previously reported (18). In digested lung tissue
From unchallenged (UC) mice, we first confirmed that podoplanin in controls is expressed on AECI, lymphatic endothelial cells (LECs), fibroblasts, tissue resident macrophages, dendritic cells (DCs) and CD4$^+$ T cells (Figure 5B, UC white plots). Furthermore, LPS-induced lung injury significantly increases podoplanin expression on the leucocyte populations (Figure 5B, 48h white plots).

As expected the Aqp5cre efficiently reduces podoplanin expression on AECIs (94.2±0.8%-to-7.5±0.4%), which is maintained following LPS-induced inflammation (Figure 5B, grey plots). $Pdpn^{fl/fl}Aqp5cre^+$ mice also have a significant reduction in podoplanin expression on LECs (83.9±2.5%-to-10.2±0.5%) and tissue resident macrophages (34.1±5.2%-to-8.9±2.8%). Podoplanin expression remains significantly reduced in these cell populations following IT-LPS. In contrast, expression of podoplanin is maintained in all lung cell populations analyzed in unchallenged $Pdpn^{fl/fl}VAV1cre^+$ lungs (Figure 5B – UC black plots). However, inflammation-induced increases in podoplanin expression are inhibited in lung leucocyte populations.

The two macrophage populations present in BAL (identified in Figure 1A) were also analyzed for podoplanin expression. Podoplanin is expressed at a very low level in alveolar macrophages, which does not change following IT-LPS (Figure 5C, white plots). In addition, there are no significant differences in expression in both conditional podoplanin knock out strains (Figure 5C, grey and black plots). This is in contrast to BAL inflammatory alveolar macrophages, which express a moderate level of podoplanin in controls (29.3%±3.8) and is significantly reduced only in $Pdpn^{fl/fl}VAV1cre^+$ animals (13.2%±2.2; Figure 5C, black plots).

Hematopoietic-expressed podoplanin protects against LPS-induced lung injury

To investigate the consequence of podoplanin deletion in our conditional strains, we first analyzed $S_aO_2$ following IT-LPS. Deletion of podoplanin significantly reduced $S_aO_2$ only in $Pdpn^{fl/fl}VAV1cre^+$ compared to controls and $Pdpn^{fl/fl}Aqp5cre^+$ animals (p=0.001, Figure 7A), without altering blood flow.
Immunohistochemistry analysis suggests that neutrophil recruitment following IT-LPS is unaltered in both podoplanin deficient strains (Figure 7C). This was supported by analysis of neutrophil counts in BAL and lung tissue 48 hours post IT-LPS which confirmed that LPS-induced neutrophilia is not significantly different in either strain (Figure 7D). However, in line with CLEC-2 deficient strains there is a significant increase in BAL protein in \(Pdpn^{fl/fl}\)\(VAV1cre^{+}\) animals (1.6 fold, Figure 7D). Again this is independent of changes in wet-to-dry weight ratio and epithelial cell damage as assessed by expression of RAGE in BAL fluid (Figure 7D). However, contrary to CLEC-2 deficient strains, the number of RBC in BAL is significantly increased only in \(Pdpn^{fl/fl}\)\(VAV1cre^{+}\) post IT-LPS (Figure 7D). Mice expressing VAV1cre only did not exhibit an increase in neutrophilia or BAL protein compared to floxed only controls 48h post IT-LPS suggesting our data are specific to podoplanin deletion (Figure 7D, striped plots).

Expression of the neutrophil recruitment chemokines KC and MIP-2 are both significantly increased in \(Pdpn^{fl/fl}\)\(VAV1cre^{+}\) BAL compared to floxed only controls and \(Pdpn^{fl/fl}\)\(Aqp5cre^{+}\) animals, mirroring the CLEC-2 deficient strains (Figure 7E). In addition, no significant difference is observed in MCP-1, TNF\(\alpha\) or IL-6 levels in BAL (Figure 7F). The increase in BAL RBCs is mirrored by a decrease in platelet counts observed in \(Pdpn^{fl/fl}\)\(VAV1cre^{+}\) mice following IT-LPS, while WBC and RBC counts are comparable to floxed only controls (Figure 7G).

**Discussion**

The data presented in this study (1) confirm that platelets are present in the alveolar space during inflammation, primarily in aggregates with neutrophils; (2) show that CLEC-2 expressed on platelets protects against excessive lung inflammation in a mouse model of ARDS; (3) are suggestive of a non-essential, or redundant, role for the CLEC-2 ligand, podoplanin, on AECI and LECs during lung development and function during inflammation; and, (4) implicate podoplanin expression on
Platelets have been implicated in regulating many processes dysregulated during ARDS, including neutrophil recruitment, macrophage-dependent inflammation and alveolar-capillary permeability; however, the mechanisms are incompletely understood (37, 52). Previously, Ortiz-Muñoz et al. (42), used 2-photon intravital microscopy to show neutrophil-platelet aggregates forming dynamically during LPS-induced inflammation, which migrate into the alveolar spaces. Here we first confirm that there is an increase in platelets present within alveoli following intratracheal LPS administration. In addition, we confirm that these platelets are primarily bound to neutrophils in BAL fluid.

Having confirmed the presence of platelet-neutrophil aggregates in the alveoli we investigated the role of the platelet receptor CLEC-2 in regulating lung inflammation. Using two complementary strategies of CLEC-2 deletion, our data suggest that platelet CLEC-2 is protective during LPS-induced lung injury. Our results show for the first time that in a mouse model of ARDS, expression of CLEC-2 on platelets limits neutrophil extravasation into the alveolar spaces, possibly via modulation of the chemokines KC and MIP-2, maintaining lung function. In the lung, MIP-2 is primarily produced by alveolar macrophages (8), whereas KC is expressed by epithelial cells, neutrophils and macrophages (21). Platelets also contain KC (14), so whether CLEC-2 deficiency only affects KC release from platelets or modulates expression of the inflammatory chemokines by other cell types is the focus of ongoing research.

This protective role for platelets in a mouse model of intra-tracheal LPS-induced lung injury contradicts recent studies which demonstrate that reduced platelet number and/or activation is protective during aerosolized LPS (16, 54), acid-induced (58) and TRALI (30, 42) mouse models. In line with these data a recent prospective study using a human model of ARDS suggests that reduced platelet activation via aspirin administration significantly reduces acute pulmonary neutrophilia (17). However, meta-analysis of clinical studies using aspirin in the management of ARDS concluded that
data are insufficient to justify the use of aspirin as yet (44). Interestingly, an injurious role for platelet
CLEC-2 during a mouse model of deep vein thrombosis was recently reported (46) with platelet
transfusion shown to be protective in mice that were first subjected to extracorporeal circulation
(34) or during mouse models of sepsis (57). Together with the data we present here that suggests
platelet transfusion prior to IT-LPS has a detrimental effect, these data emphasize the possible
model-dependent and/or receptor-dependent effects of platelets during inflammation.

The only known endogenous ligand for CLEC-2 is podoplanin, which we show is highly expressed in
the lung on multiple cell types and upregulated on leucocytes during inflammation in the lung, in line
with previously published data (11, 26, 43, 48, 51). We used cre-loxP technology to induce cell-
specific deletion of podoplanin. Mice generated using Aqp5cre or VAV1cre induced podoplanin
deletion, are viable and have no overt phenotype. In particular we show for the first time that
podoplanin expressed on AECIs is not required for lung development. This argues against a major
role of AECI-expressed podoplanin in contributing to the embryonic lethality observed in constitutive
podoplanin knockout mice (50). Furthermore, we observe that use of Aqp5cre has the ability to
reduce expression of the floxed gene in LECs and macrophages digested from lung tissue. Although
this was not reported in the original description of the Aqp5cre mouse, this may be due to
expression of Aqp5 on endothelium and leucocytes as published in other tissues (27, 38).

Furthermore, the Aqp5cre was first described on a 129S6/SvEvTac background. This newly
generated strain is on a C57Bl/6 background, which may account for the differences observed.

Using our podoplanin deficient mouse strains, the data presented here suggest that podoplanin
expressed on lung epithelial, endothelial and tissue leucocytes does not regulate lung injury or
inflammation. However, podoplanin expression on a distinct hematopoietic cell(s) is protective
during LPS-induced inflammation in the lung. Furthermore, VAV1+ podoplanin-expressing cells limit
BAL protein accumulation and alveolar hemorrhage, as well as maintain lung function, via
modulation of KC and MIP-2 expression. In particular, as the only cell population with significantly
lower podoplanin expression solely in $Pdpn^{fl/fl}VAV1cre^+$ mice following LPS (Figure 5C), loss of podoplanin from BAL CD11b$^+$CD11c$^+$F4/80$^+$ alveolar inflammatory macrophages may play a key role in limiting endotoxemia-induced inflammation in the lung. This subpopulation of alveolar macrophages has been reported to express high levels of metalloproteinase (MMP)-12 (9), which cleaves and inactivates CXC chemokines, including KC and MIP-2, contributing to the “off” signal during acute inflammation of the lung (6). Therefore, it is interesting to speculate that regulation by platelet-CLEC-2 may modulate MMP-12 release/activity via direct interaction with podoplanin on these inflammatory alveolar macrophages. This possible mechanism is summarized in Figure 8.

Differences between $Pdpn^{fl/fl}VAV1cre^+$ and CLEC-2 deficient animals were also observed. Most notably the lack of increased BAL neutrophilia and conversely an increase in BAL RBC accumulation in the $Pdpn^{fl/fl}VAV1cre^+$ strain post IT-LPS. There is growing evidence that podoplanin may interact with other proteins, including CCL21 and galectin 8 (4, 25). This may suggest that in $Pdpn^{fl/fl}VAV1cre^+$ animals both CLEC-2-dependent and CLEC-2-independent mechanisms are active, both of which contribute to the phenotype observed post IT-LPS. We also cannot rule out the possibility that a stromal/fibroblast podoplanin-expressing cell contributes to the phenotype of CLEC-2 deficient mice.

Future mechanistic studies are required to fully understand the role of CLEC-2 and podoplanin during lung inflammation.

Both cre-loxP strategies we employed to delete CLEC-2 have their limitations; (1) use of tamoxifen-may also delete CLEC-2 in cells besides platelets, although the use of the CD11c-cre does not support this; (2) tamoxifen is anti-inflammatory, however parameters from our floxed-only controls of tamoxifen- and non-tamoxifen treated controls are comparable; and, (3) previous reports suggest there are limitations in specificity using cre-loxP technology; specifically, expression of the PF4cre has recently been shown in low numbers of non-megakaryocytic lineages (47). However, the close correlation of data generated using both strategies strongly support the validity of our findings.
A further limitation of this study is the use of only one model of ARDS, which on its own does not completely reproduce all the features of the clinical syndrome. The main feature of the IT-LPS model is alveolar neutrophilia. As a role for platelets in regulating neutrophil recruitment has already been suggested, we reasoned that IT-LPS would be the best to initially investigate the role of platelet-expressed CLEC-2. It will now be essential to confirm these findings in other mouse models of ARDS.

In conclusion, our data support the finding that CLEC-2 expressed on platelets is required to limit neutrophil recruitment, which in turn, limits lung function decline in a mouse model of ARDS. In addition, expression of the CLEC-2 ligand, podoplanin, is required on hematopoietic cells to limit neutrophil chemokine expression and consequently arterial oxygen saturation decline. Therefore, we (1) demonstrate that the platelet CLEC-2-podoplanin signaling axis is a novel regulator of lung inflammation in mouse; (2) emphasize the complex role platelets play during mouse ARDS; and, (3) identify the platelet CLEC-2-podoplanin pathway as a possible novel target for therapeutic intervention in patients at risk of developing ARDS.

Acknowledgements

Grants

This work was supported by grants from the British Heart Foundation awarded to S.P.W. (CH/03/003 and RG/13/18/30563) and a Wellcome Trust-funded grant awarded to S.L. and D.R.T. (091864/Z/10/Z). Z.B. and E.D.C. are supported by the Hastings Foundation, Whittier Foundation and NIH research grants HL126877 (Z.B.), HL112638 (Z.B.), HL114094 (Z.B.) and HL108634 (E.D.C.). E.D.C. is Hastings Professor and Norris Chair of Medicine. Z.B. is Edgington Chair of Medicine.

Disclosures
The authors declare no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
References


**Figure legends**

**FIGURE 1:** Platelets are present in BAL during a mouse model of ARDS. Unchallenged bronchoalveolar lavage (BAL) and BAL isolated 48h after IT-PBS or LPS were analyzed from WT mice by flow cytometry. (A) Representative histograms for the identification of leucocyte (CD45+) cell populations in mouse BAL prior to, and 48h after IT-PBS or LPS (adapted from (9)). Podoplanin expression was analyzed by setting the gate at ≤1% using a Syrian hamster IgG isotype. The major leucocyte populations observed in BAL displayed as percentage of total CD45+BAL cells (B) and absolute cell counts (C). n=8; (D) Alveolar-capillary damage assessed in unchallenged mice and 48h after IT-PBS or LPS. n=6-8; (E) BAL from wildtype mice pre-treated with isotype (control) or anti-GP1bα antibody (platelet depleted) mice 6 hours after IT-LPS. * = excluded animal. (F) The numbers of red blood cells (RBCs) and neutrophils in BAL of wildtype mice pre-treated with isotype (control) or anti-GP1bα antibody (platelet depleted) mice 6 hours after IT-LPS. Isotype control n=6, anti-GP1bα n=5; . Student’s T test. * p<0.05, ** p<0.01. (G) Systemic platelet counts from WT mice where analyzed in whole blood on an ABX Pentra 60 (Horbia Ltd, UK) in unchallenged mice or 48h after IT-PBS or LPS. n=8; . (H) Representative histograms for the identification of CD41+ platelets in BAL and characterization of platelet-leucocyte aggregates (PLA) following IT-LPS. (I) CD41+ platelets observed in BAL in WT mice 48h following IT-LPS. (J) Identification of CD41+CD45+, PLAs in BAL 48h following IT-LPS. n=8; . One-way ANOVA performed with Tukey’s multiple comparison post-tests. * p<0.05, ** p<0.01, *** p<0.001. UC = unchallenged.

**FIGURE 2:** Two week tamoxifen-induced deletion of CLEC-2 on platelets. (A) Representative histograms using 17D9-FITC to detect CLEC-2 expression on platelets in whole blood analyzed 1 week after starting tamoxifen diet and after 2 weeks tamoxifen diet followed by 5 weeks of normal diet in Clec2fl/flER12cre mice compared to floxed only controls. An IgG2b-FITC isotype control was also included. (B) Percentage of CLEC-2 expression in CD41 positive platelets was analyzed by flow cytometry. n=4. One-way ANOVA with Tukey’s multiple post-tests. *** p<0.001. Tam = tamoxifen.
FIGURE 3: Effect of CLEC-2 deletion in platelets during a mouse model of ARDS. Arterial oxygen saturation analyzed by non-invasive pulse oximetry following IT-LPS in tamoxifen (ER\textsuperscript{T2}cre- or PF4cre-induced CLEC-2 deficient mice compared to cre negative controls (A and B, respectively).

Tamoxifen treated \textit{Clec2}\textsuperscript{fl/fl} versus \textit{Clec2}\textsuperscript{fl/fl}ER\textsuperscript{T2}cre \textit{p}<0.001; \textit{Clec2}\textsuperscript{fl/fl} versus \textit{Clec2}\textsuperscript{fl/fl}PF4cre; \textit{p}<0.001.

(C) Pulse distention analyzed by MouseOx Plus\textsuperscript® following IT-LPS in tamoxifen (ER\textsuperscript{T2}- or PF4cre-induced CLEC-2 deficient mice compared to cre negative controls. Tamoxifen treated \textit{Clec2}\textsuperscript{fl/fl} n=8, \textit{Clec2}\textsuperscript{fl/fl}ER\textsuperscript{T2}cre, n=7; \textit{Clec2}\textsuperscript{fl/fl} n=8, \textit{Clec2}\textsuperscript{fl/fl}PF4cre n=8; mean \pm SEM. Two-way ANOVA performed with Tukey’s multiple comparisons test. (D) Representative images of lung sections stained for H&E in unchallenged and 48h after IT-LPS treated ER\textsuperscript{T2}cre- or PF4cre-induced CLEC-2 deficient mice compared to cre negative controls. Alveolar and interstitial neutrophils are highlighted by arrows and arrowheads, respectively. Scale bar: 100\textmu m. n=3-5. (E) Neutrophil recruitment and pulmonary endothelial and epithelial damage parameters in unchallenged and IT-LPS treated ER\textsuperscript{T2}cre- or PF4cre-induced CLEC-2 deficient mice compared to cre negative controls; n=7-13. PF4cre only, n=4; \textit{Clec2}\textsuperscript{fl/fl}CD11c-cre, n=8. (F) Bronchoalveolar lavage (BAL) cyto- and chemokine levels in unchallenged and IT-LPS treated ER\textsuperscript{T2}cre- or PF4cre-induced CLEC-2 deficient mice compared to cre negative controls. n=6. (G) Systemic platelet, white blood cell (WBC) and red blood cell (RBC) counts were analyzed in whole blood on an ABX Pentra 60 (Horibia Ltd, UK). n=6-11. One-way ANOVA performed with Tukey’s multiple comparison post-tests. * \textit{p}<0.05; ** \textit{p}<0.01; *** \textit{p}<0.001. UC = unchallenged.

N.D. = Not detected. 48h = 48h post IT-LPS.

FIGURE 4: Effect of platelet transfusion during a mouse model of ARDS. (A) Purity, CLEC-2 expression and activity were confirmed in platelets prepared from wildtype mice. These platelets were given via intra-venous injection (2 x 10\textsuperscript{8} per mouse) immediately prior to LPS instillations into CLEC-2 sufficient (\textit{Clec2}\textsuperscript{fl/fl}) and platelet-specific CLEC-2 deficient (\textit{Clec2}\textsuperscript{fl/fl}ER\textsuperscript{T2}cre+) mice. Control, CLEC-2 sufficient, mice that were given IT-LPS on the same day but did not receive platelets were also included. (B) Total weight loss along with systemic white blood cell (WBC), red blood cell (RBC) and platelet counts in whole blood were analyzed 48h hours post IT-LPS. (C) Bronchoalveolar (BAL)
neutrophilia and total BAL protein were also analyzed 48 hours post IT-LPS. One-way ANOVA performed with Tukey’s multiple comparison post-tests. Clec2\textsuperscript{fl/fl} (no transfusion) n=4, Clec2\textsuperscript{fl/fl} + platelets n=5, Clec2\textsuperscript{fl/fl}ER\textsuperscript{12}cre + platelets n=7. * p<0.05; ** p<0.01.

FIGURE 5: Podoplanin expression in unchallenged and IT-LPS treated Pdpn\textsuperscript{fl/fl} Aqp5cre and VAV1cre mice. (A) Representative images of lung sections stained using 8.1.1 to detect podoplanin (brown) and hematoxylin (blue) in unchallenged and 48h after IT-LPS treated Aqp5cre- or VAV1cre-induced podoplanin deficient mice compared to cre negative controls. Scale bar: 100μm. n=4. (B) Flow cytometry analysis of podoplanin-expressing cell populations isolated from perfused lung tissue following enzymatic digestion. n=7-14. (C) Flow cytometry analysis of podoplanin expression in alveolar macrophage subpopulations isolated from bronchoalveolar lavage (BAL). n=7-14. One-way ANOVA performed with Tukey’s multiple comparison post-tests. * p<0.05; ** p<0.01; *** p<0.001.

UC = unchallenged. EpCAM = epithelial cell adhesion molecule. LEC = lymphatic endothelial cell. DC = dendritic cell. AM = alveolar macrophage.

FIGURE 6: Gating strategy used in digested lung tissue. Representative histograms for the identification of podoplanin-expressing cell populations following Liberase/DNase digestion (adapted from (18)). Only alive (SyTOX Red negative) cells were analyzed using a stromal (A) or leucocyte (B) antibody panel in perfused and digested mouse lungs. Podoplanin expression was analyzed by setting the gate at ≤1% using a Syrian hamster IgG isotype as shown. Blood endothelial cells (BECs) are defined within the stromal gate as EpCAM\textsuperscript{−}CD31\textsuperscript{−}LYVE\textsuperscript{−}. AECI = alveolar epithelial type I cell. AECII = alveolar epithelial type II cell. LEC = lymphatic endothelial cell. BEC = blood endothelial cell. AM = alveolar macrophage. DC = dendritic cell.

FIGURE 7: Effect of podoplanin deletion from Aqp5\textsuperscript{+} or VAV1\textsuperscript{+} cells on lung function in a mouse model of ARDS. (A) Arterial oxygen saturation analyzed by non-invasive pulse oximetry following IT-LPS in Aqp5cre- or VAV1cre-induced podoplanin deficient mice compared to cre negative controls (p=0.001). (B) Pulse distention analyzed by MouseOx Plus\textsuperscript{®} following IT-LPS in Aqp5cre- or VAV1cre-
induced podoplanin deficient mice compared to cre negative controls. $Pdpn^{fl/fl}$ n=8, $Pdpn^{fl/fl}$ Aqp5cre n=7, $Pdpn^{fl/fl}$ VAV1cre n=7, mean ±SEM. Two-way ANOVA performed with Tukey’s multiple comparisons test. *** p<0.001. (C) Representative images of lung sections stained for H&E in unchallenged and 48h after IT-LPS treatment in Aqp5cre or VAV1cre-induced podoplanin deficient mice compared to cre negative controls. Alveolar and interstitial neutrophils are highlighted by arrows and arrowheads, respectively. Scale bar: 100μm. n=3. (D) Neutrophil recruitment and pulmonary endothelial and epithelial damage parameters in unchallenged and IT-LPS treated Aqp5cre- or VAV1cre-induced podoplanin deficient mice compared to cre negative controls. n=7-14. (E) BAL cyto- and chemokine levels in unchallenged and IT-LPS treated Aqp5cre- or VAV1cre-induced podoplanin deficient mice compared to cre negative controls. n=6. (F) Systemic platelet, white blood cell (WBC) and red blood cell counts (RBC) were analyzed in whole blood on an ABX Pentra 60 (Horbia Ltd, UK). n=6-11. One-way ANOVA performed with Tukey’s multiple comparison post-tests. * p<0.05; ** p<0.01; *** p<0.001. UC = unchallenged. N.D. = Not detected. 48h = 48h post IT-LPS.

**FIGURE 8: Proposed model for the mechanism of CLEC-2-podoplanin dependent regulation of acute lung injury.** During acute lung inflammation, the CXC chemokines, KC and MIP-2 are produced by alveolar macrophages (AM) and epithelial cells (1). Platelets in aggregates with neutrophils (N) are recruited into the alveoli (2). At the same time, tissue resident AM and monocytes (m) from the blood contribute to the appearance of CD11b+ inflammatory AM (iAM) in the alveoli, which express podoplanin (3). Together in the alveolar space, CLEC-2 expressed on platelets interacts with podoplanin on the iAMs regulating MMP-12 release from iAMs (4). MMP-12 is able to cleave and inactivate the CXC chemokines contributing to the “off” signal during acute inflammation in the lung and limiting lung injury.
Figure 1

A

Red blood cells

CD45 Log

48h post IT-LPS

Neutrophils

CD11c Log

CD11b Log

F4/80 Log

Podoplanin Log

SS Lin

Alveolar Macrophages

Control

Platelet depleted

6h IT-LPS

F

Bal cell populations

Counts per mi BAL

RBC

Neutrophils

G

Platelet number

(10^7/mm^3)

H

IT-PBS

IT-LPS

Count

CD41 Log

SSC Lin K10^3

CD45 Log

Gr-1 Log

CD11c Log

I

BAL CD4+ cells (%)

J

BAL PLNG (%)
Figure 3
Figure 6

A

CD45 Log
TER119 Log
Podoplanin Log

EpCAM Log

Lympathic Endothelial Cells

CD31 Log
SS Lin
LYVE-1 Log

FS Lin

Alveolar Epithelial Type I Cells
Isotype control

Podoplanin Log

Podoplanin Log

B

F4/80 Log
CD11b Log

CD45 Log
FS Lin

CD11c Log

Macrophages
Isotype control

CD11c Log

Dendritic Cells
Isotype control

CD19 Log
IgM Log

CD4 Log
CD8 Log

CD4^+ T Cells

CD8^+ T Cells

Podoplanin Log

Podoplanin Log
Figure 8
## Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pdpn&lt;sup&gt;fl/fl&lt;/sup&gt;</th>
<th>Pdpn&lt;sup&gt;fl/fl&lt;/sup&gt;Aqp5cre</th>
<th>Pdpn&lt;sup&gt;fl/fl&lt;/sup&gt;VAV1cre</th>
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<tr>
<td>Body weight (g)</td>
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<td>27.8 ±0.7</td>
<td>27.1 ±2.8</td>
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<td>Normalized organ weight</td>
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<td></td>
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<tr>
<td>Lungs</td>
<td>11.5 ±1.0</td>
<td>11.2 ±0.4</td>
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<td>Heart</td>
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<td>5.5 ±0.5</td>
<td>5.4 ±0.4</td>
<td>0.829</td>
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<tr>
<td>Spleen</td>
<td>2.9 ±0.4</td>
<td>3.0 ±0.2</td>
<td>2.7 ±0.2</td>
<td>0.206</td>
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<tr>
<td>Thymus</td>
<td>1.8 ±0.3</td>
<td>2.0 ±0.2</td>
<td>1.9 ±0.5</td>
<td>0.787</td>
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<tr>
<td>Systemic platelet count (10&lt;sup&gt;3&lt;/sup&gt;/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>858 ±82</td>
<td>822 ±87</td>
<td>847 ±66</td>
<td>0.700</td>
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<tr>
<td>CLEC-2 expression on platelets (%)</td>
<td>99.1 ±0.5</td>
<td>99.0 ±0.7</td>
<td>98.7 ±0.8</td>
<td>0.599</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
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</table>

**TABLE 1.** Phenotype of Pdpn<sup>fl/fl</sup>Aqp5cre and Pdpn<sup>fl/fl</sup>VAV1cre mouse strains. 10.5-12 week old male mice were analyzed and compared to Pdpn<sup>fl/fl</sup> littermate controls. Organ weights were normalized to body weight. Mean ±SD with one-way ANOVA.
<table>
<thead>
<tr>
<th>Breeding pair</th>
<th>Sex</th>
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<th>Total</th>
<th>Expected</th>
<th>Observed</th>
<th>P</th>
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<tr>
<td>Pdpn&lt;sup&gt;fl/fl&lt;/sup&gt; X Pdpn&lt;sup&gt;fl/flAqp5cre&lt;/sup&gt;</td>
<td>M</td>
<td>fl/fl</td>
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<td>22</td>
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<td>0.162</td>
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<td>M</td>
<td>fl/fl</td>
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<td>27.25</td>
<td>32</td>
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<tr>
<td></td>
<td>F</td>
<td>fl/fl</td>
<td>-</td>
<td>27.25</td>
<td>25</td>
<td>0.579</td>
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<tr>
<td></td>
<td>F</td>
<td>fl/fl</td>
<td>Aqp5</td>
<td>27.25</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Pdpn&lt;sup&gt;fl/fl&lt;/sup&gt; X Pdpn&lt;sup&gt;fl/flVAV1cre&lt;/sup&gt;</td>
<td>M</td>
<td>fl/fl</td>
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<tr>
<td></td>
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<td>VAV1</td>
<td>61.75</td>
<td>53</td>
<td></td>
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<tr>
<td></td>
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<td>-</td>
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<td>63</td>
<td>0.747</td>
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<tr>
<td></td>
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<td>fl/fl</td>
<td>VAV1</td>
<td>61.75</td>
<td>64</td>
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</tbody>
</table>

**TABLE 2.** Genetic inheritance in *Pdpn<sup>fl/flAqp5cre</sup>* and *Pdpn<sup>fl/flVAV1cre</sup>* mouse strains. Chi-square test did not detect any significant differences in terms of inheritance in either strain or within sexes.