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Generation of PECAM-1 (CD31) Conditional Knockout Mice

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Abstract

Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) is an adhesion and signaling receptor that is expressed on endothelial and hematopoietic cells and plays important roles in angiogenesis, vascular permeability, and regulation of cellular responsiveness. To better understanding the tissue specificity of PECAM-1 functions, we generated mice in which *PECAM1*, the gene encoding PECAM-1, could be conditionally knocked out. A targeting construct was created that contains *loxP* sites flanking *PECAM1* exons 1 and 2 and a neomycin resistance gene flanked by Flippase Recognition Target (FRT) sites that was positioned upstream of the 3' *loxP* site. The targeting construct was electroporated into C57BL/6 embryonic stem (ES) cells, and correctly targeted ES cells were injected into C57BL/6 blastocysts, which were implanted into pseudo-pregnant females. Resulting chimeric animals were bred with transgenic mice expressing Flippase 1 (FLP1) to remove the FRT-flanked neomycin resistance gene and mice heterozygous for the floxed *PECAM1* allele were bred with each other to obtain homozygous *PECAM1^{flx/flx}* offspring, which expressed PECAM-1 at normal levels and had no overt phenotype. *PECAM1^{flx/flx}* mice were bred with mice expressing Cre recombinase under the control of the SRY-box containing gene 2 (*Sox2Cre*) promoter to delete the floxed *PECAM1* allele in offspring (*Sox2Cre; PECAM1^{del/WT}*), which were crossbred to generate *Sox2Cre; PECAM1^{del/del}* offspring. *Sox2Cre; PECAM1^{del/del}* mice recapitulated the phenotype of conventional global PECAM-1 knockout mice. *PECAM1^{flx/flx}* mice will be useful for studying distinct roles of PECAM-1 in tissue specific contexts and to gain insights into the roles that PECAM-1 plays in blood and vascular cell function.

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Author contributions:

T.K., H.Z., and P.J.N. designed the project. H.Z. and G.F. performed research, H.Z., G.F., D.N. and P.J.N. analyzed data. H.Z., D.K.N., and P.J.N. wrote the paper. All authors contributed to the manuscript preparation.

[#]These authors contributed equally to this study

Competing Interests

The authors declare that no competing interests exist.

Keywords

PECAM-1; CD31; conditional knockout; Cre-loxP; gene targeting

Introduction

Platelet/endothelial cell adhesion molecule-1 (PECAM-1; also known as CD31) is a 130 kDa type I transmembrane cellular adhesion and signaling receptor that has a tissue-restricted expression pattern. PECAM-1 is expressed on the surfaces of endothelial cells (Muller, Ratti, McDonnell, & Cohn, 1989; P. J. Newman et al., 1990), where it is concentrated at cell-cell borders (Albelda, Muller, Buck, & Newman, 1991; Sun et al., 2000) to which it can be delivered from a specialized lateral border recycling compartment (Mamdouh, Chen, Pierini, Maxfield, & Muller, 2003). PECAM-1 is also expressed early during hematopoiesis (Baumann et al., 2004; Watt et al., 1993) and continues to be expressed on circulating blood cells, including platelets, neutrophils, monocytes, mast cells, natural killer cells and subsets of T and B lymphocytes (Privratsky, Newman, & Newman, 2010; Woodfin, Voisin, & Nourshargh, 2007), some of which lose expression with maturation (D. K. Newman et al., 2018). Outside of the vasculature, PECAM-1 has been reported to be expressed on human cytotrophoblasts (Blankenship & Enders, 1997; Lyall et al., 2001), spermatozoa (Nixon et al., 2005) and brown adipose tissue (Rosso & Lucioni, 2006).

In vitro studies have revealed distinct functions of PECAM-1 in the different types of cells in which it is expressed. Endothelial cell PECAM-1 has been found to be important for mechanosensation (Conway & Schwartz, 2012; Dorland & Huveneers, 2017; Givens & Tzima, 2016; Komarova, Kruse, Mehta, & Malik, 2017), leukocyte transendothelial migration (Goswami & Vestweber, 2016; Muller, 2016; Reglero-Real, Colom, Bodkin, & Nourshargh, 2016), maintenance and restoration of the vascular permeability barrier (Lertkiatmongkol, Liao, Mei, Hu, & Newman, 2016; Privratsky & Newman, 2014), and angiogenesis (Cao et al., 2009). On circulating blood cells and platelets, PECAM-1 is important as a negative regulator of responsiveness (Coxon, Geer, & Senis, 2017; Marelli-Berg, Clement, Mauro, & Caligiuri, 2013; Stefanini & Bergmeier, 2018). In both endothelial and circulating blood cells, PECAM-1 has been reported to suppress apoptosis and enhance cell survival (Bergom, Gao, & Newman, 2005).

Since their description twenty years ago, studies of conventional PECAM-1-deficient mice, originally generated by Duncan, et al. (Duncan et al., 1999), have revealed multifaceted effects of PECAM-1 deficiency on multiple diseases *in vivo*. Thus, PECAM-1 was found to enhance hemostasis (Lishnevsky et al., 2014; Mahooti et al., 2000) and inhibit thrombosis (Falati et al., 2006; Kellermair et al., 2013). PECAM-1 was also found to play protective roles in autoimmunity (Cheung et al., 2015; Clement et al., 2014; Graesser et al., 2002), inflammatory diseases of the liver (Goel et al., 2007; G. Malik, Wilting, Hess, Ramadori, & Malik, 2019; I. A. Malik et al., 2015) and lung (Schenkel, Chew, Chlipala, Harbord, & Muller, 2006), and in sepsis (Carrithers et al., 2005; Maas et al., 2005; Privratsky, Tilkens, Newman, & Newman, 2012). In addition, PECAM-1 has been found to interfere with tumor

clearance (Ma et al., 2010; D. K. Newman et al., 2016), but support resistance to infection with certain strains of *Salmonella* (Lovelace et al., 2013; Ross et al., 2011). In the setting of atherosclerosis, PECAM-1 was shown to either enhance or inhibit atherosclerotic plaque formation depending on the site within the vasculature that was evaluated (Goel et al., 2008; Harrison et al., 2013; Harry et al., 2008; Stevens et al., 2008). It is possible that these disparate effects of PECAM-1 deficiency are attributable to loss of PECAM-1 from different cell types; however, the specific PECAM-1-expressing cell types that are responsible for disease modulation cannot be deduced with the use of conventional global PECAM-1-knockout mice.

To overcome limitations of the conventional PECAM-1-knockout mouse model, we generated a novel, conditional knockout mouse model in which the gene encoding PECAM-1 is flanked by loxP sites (*PECAM1^{fllox/flox}*). Herein, we confirm that deletion of the floxed *PECAM1* allele in all cells recapitulates the effects of global PECAM-1 deficiency that have been reported using conventional PECAM-1-knockout mice. *PECAM1^{fllox/flox}* mice provide a novel and promising conditional knockout model that will allow PECAM-1 to be ablated in a time- or cell type-specific manner with the help of inducible or tissue-specific expression of Cre recombinase. *PECAM1^{fllox/flox}* mice will be useful for studying distinct roles of PECAM-1 in different cells and tissues, and for gaining insight into the roles that PECAM-1 plays in the function of blood and vascular cells.

Materials and Methods

Construction of the targeting vector

A bacterial artificial chromosome (BAC RP23-120D9) that encompasses exons 1 and 2 of mouse *PECAM1* was obtained from Children's Hospital Oakland Research Institute (Oakland, CA). A targeting vector was constructed that contains a selection cassette comprised of a neomycin resistance gene (*Neo*) flanked by Flippase recognition target (FRT) sites, which was inserted in the intron between exons 2 and 3, and loxP sites that were inserted upstream of *PECAM1* exon 1 downstream of the selection cassette (see Figure 1A). The accuracy of the targeting vector was confirmed by DNA sequencing.

Generation of conditional PECAM-1 knockout mice

In collaboration with Cyagen Biosciences (Sunnyvale, CA), C57BL/6 embryonic stem (ES) cells were transfected with the targeting vector. Following electroporation, ES cells were grown in medium containing the neomycin sulfate analogue G418 to select for neomycin-resistant cells. Correctly targeted ES clones were microinjected into C57BL/6 blastocysts, which were implanted into pseudo-pregnant females, and chimeric pups were identified by PCR using genomic DNA isolated from tail tissue. Chimeric males that contained the floxed *PECAM1* allele were bred with female mice that expressed Flippase 1 (FLP1; B6.129S4-*Gt(ROSA)26Sor^{tm1(FLP1)}Dym*/RainJ, Stock 009086, The Jackson Laboratory) to excise *Neo*. Mice that were heterozygous for the *Neo*-deleted, floxed *PECAM1* allele (*PECAM1^{fllox/WT}*) were backcrossed with C57BL/6N mice to eliminate the *FLP1* transgene. *PECAM1^{fllox/WT}* mice that were missing the *FLP1* transgene were bred with each other to obtain offspring homozygous for the floxed allele (*PECAM1^{fllox/flox}*). Deletion of the floxed *PECAM1* allele

was accomplished by crossing male *PECAM1^{flox/flox}* mice with female mice that expressed Cre recombinase under the control of the *SRY-box containing gene 2 (Sox2)* promoter (B6.Cg-Tg(Sox2-cre)1Amc/J, Stock 8454, The Jackson Laboratory). Cre recombinase expression is active in the female germline of *Sox2Cre⁺* transgenic mice, which ensures rapid and efficient deletion in offspring that arise from the breeding of male mice bearing floxed alleles with *Sox2Cre⁺* transgenic females (Hayashi, Lewis, Pevny, & McMahon, 2002; Hayashi, Tenzen, & McMahon, 2003). Mice in which the floxed *PECAM1* allele had been deleted (*Sox2Cre⁺;PECAM1^{del/WT}*) were crossbred to generate *Sox2Cre⁺;PECAM1^{del/del}* mice.

The genotypes of ES cells and transgenic mice were evaluated by PCR and Southern Blot using primers and probes described in Table 1. All animal work was carried out in accordance with the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC). The *PECAM1^{flox/flox}* line is available to the research community (Stock no. 030758, The Jackson Laboratory).

Flow cytometric analysis of PECAM-1 expression

Washed platelets were isolated from peripheral blood and CD8⁺ T cells were isolated from the spleen using an EasySep Mouse CD8⁺ T Cell Isolation Kit (StemCell Technologies). Platelets were identified by staining with FITC-labeled rat anti-mouse CD41 (BD Biosciences). CD8⁺ T cells were identified by staining with FITC-labeled rat anti-mouse CD8 (eBioscience, Inc.). PECAM-1 expression was evaluated by staining with PE-Cyanine7-labeled rat anti-mouse CD31 (eBioscience, Inc.). Data were acquired on an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

Fluorescent immunohistochemical analysis of PECAM-1 expression

Fluorescent immunohistochemical analysis of PECAM-1 expression was performed using a Leica Bond Rx automated staining platform. Mouse tissues (kidney, liver, heart and testis) were embedded in Optimal Cutting Temperature (OCT) compound and snap frozen in isopentane-cooled liquid nitrogen. Samples (4 µm) were cryo-sectioned and placed onto charged slides. All sections were fixed in 4% paraformaldehyde for 20 min at room temperature and blocked with Background Sniper (Biocare Medical) for 30 min. The sections were stained with CD31 rat monoclonal antibody (Biocare Medical) at a dilution of 1:100 for 60 min at room temperature. Negative control tissues were incubated without primary antibody. The sections were subsequently incubated with an Alexa Fluor 488-labeled goat anti-rat secondary antibody (Thermo Fisher Scientific) at a dilution of 1:500 for 45 min at room temperature in the dark. Sections were washed and stained with DAPI (Sigma Aldrich) at 1:5000 dilution in DiH₂O for 3 min. The stained tissues were visualized using a Nikon TI2-E inverted motorized microscope. Representative images are shown.

CD8⁺ cell purification and TGFβ inhibition assay

CD8⁺ T cells were purified as described above. Purified cells were resuspended in T cell medium [RPMI 1640 complete medium containing penicillin (100 mg/ml), streptomycin (100 mg/ml), 2 mM L-glutamine, 10 mM Hepes (pH 7.0), 10 mM nonessential amino acids, 1 mM sodium pyruvate, 50 mM β-mercaptoethanol, 10% fetal bovine serum (FBS)] at 2 ×

10^6 cells/ml and divided into aliquots of 2×10^5 cells per well in a 96-well tissue culture plate (Midwest Scientific) that had been precoated with 1 mg/ml of anti-mouse CD3 ϵ antibody, clone 145-2C11 (BD Biosciences). Cells cultured in uncoated wells served as a control. Recombinant mouse TGF- β 1 protein (R&D Systems) was added at the indicated concentrations, and cells were cultured at 37°C for 72 hours. Intracellular staining of granzyme B with a PE-conjugated anti-granzyme B antibody (clone GB11, Invitrogen/Life Technologies) was performed according to the Fixation/Permeabilization solution kit protocol (BD Biosciences) and analyzed by flow cytometry as described above.

Analysis of platelet function

Whole blood drawn into 3.8% sodium citrate (1/10 volume) was collected from the inferior vena cava of mice and washed platelets (300 μ l, 2×10^8 /ml) were prepared in Tyrode-HEPES buffer containing 1 mM CaCl₂. Platelet aggregation in response to varying doses of collagen related peptide (CRP; Versiti Peptide Synthesis Core) was measured using a lumi-aggregometer (Chrono-Log). Light transmission was monitored over time, and aggregation was quantified as a function of light transmission, with 100% light transmission corresponding to 100% aggregation.

Results

Characterization of *PECAM1*^{flox/+} ES cells

PCR and Southern Blot analyses were performed to confirm the presence of the *PECAM1* targeting vector in transfected C57BL/6 ES cells that were resistant to G418. As depicted in Figure 1A, homologous recombination at the 3' end of the targeting vector was queried by PCR using a forward primer that hybridizes to *Neo* (p1f) and a reverse primer that hybridizes to an intronic sequence proximal to exon 3 (p1r). Primers p1f and p1r were expected to amplify a 3.3 kb fragment from the targeted *PECAM1* allele (Figure 1A). Clones that were positive for the 3.3 kb fragment (clones 2, 6, 9, 11 and 13-15, Figure 1B) were screened by PCR for homologous recombination at the 5' end of the targeting vector using a forward primer that hybridizes to an intronic sequence upstream of exon 1 (p2f) and a reverse primer that hybridizes to a sequence within exon 1 (p2r). Primers p2f and p2r were expected to amplify a 276 bp fragment from targeted allele and a 215 bp fragment from the wildtype (WT) allele (Figure 1A). Clones positive for the 276 bp fragment (clones 11 and 14, Figure 1C) were further screened for the correct genotype by Southern Blot analysis using a digoxigenin-based labeling and detection kit (Roche) according to the manufacturer's instructions. In brief, DNA was digested with BamHI or EcoRI, electrophoresed on agarose gels, transferred to nylon membranes and tested for hybridization with probes complementary to sequences adjacent to the 5'-BamHI restriction site (5' probe) or the 3'-BamHI restriction site (3' probe), or within *Neo* (Neo probe). Correct targeting of clones 11 and 14 was verified by the presence in BamHI digests of 8.4 kb (targeted allele) and 10.2 kb (WT allele) bands using the 5' probe (Figure 1D, left) and 3.8 kb (targeted allele) and 10.2 kb (wild type allele) bands using the 3' probe (Figure 1D, middle). Correct targeting of clones 11 and 14 was also verified by the presence in EcoRI digests of a 7.4 kb (targeted allele) using the Neo probe (Figure 1D, right).

Characterization of *PECAM1^{flox/flox}* transgenic mice

Correctly targeted ES clones were microinjected into C57BL/6 blastocysts, which were implanted into pseudo-pregnant females, and chimeric mice amongst live pups were identified by PCR using genomic DNA isolated from tail tissue. Chimeric mice, which were generated using clone 14, were identified by the presence, following amplification with primers p2f and p2r (Figure 2A), of a 276 bp fragment from the targeted (*PECAM1^{flox(neo)}*) allele and a 215 bp fragment from the WT allele (Figure 2B). Deletion of *Neo* in the progeny of chimeric mice was accomplished by breeding *PECAM1^{flox(neo)/WT}* male mice with FLP1 recombinase-expressing females. Mice that were heterozygous for the *Neo*-deleted, floxed *PECAM1* allele (*PECAM1^{flox/WT}*) were identified by the presence, following amplification with primers p3f and p3r (Figure 2A), of a 712 bp fragment from the *Neo*-deleted floxed *PECAM1* allele and a 572 bp fragment from the WT allele (Figure 2C). Deletion of the FLP1-encoding transgene was accomplished by breeding *PECAM1^{flox/WT}* mice with WT C57BL/6 mice.

Mice homozygous for the floxed *PECAM-1* allele (*PECAM1^{flox/flox}*) were generated by breeding *PECAM1^{flox/WT}* mice with each other. *PECAM1^{flox/flox}* mice exhibited normal fecundity and litter sizes, normal longevity, and exhibited no obvious phenotypic abnormality (data not shown). Cell surface expression of *PECAM-1* on platelets and CD8⁺ T cells of *PECAM1^{flox/flox}* mice was comparable to that of WT mice (Figure 4A and B). These results indicate that the floxed *PECAM1* allele functions normally in mice.

Characterization of global *PECAM-1* knockout mice generated by *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles

Male *PECAM1^{flox/flox}* mice were bred with *Sox2Cre/+* transgenic C57BL/6 female mice to accomplish deletion of the floxed *PECAM1* allele, and genotyping of progeny was performed using genomic DNA isolated from tail tissue. Mice that were heterozygous for the deleted floxed *PECAM1* allele (*Sox2Cre;PECAM1^{del/WT}*) were identified by the presence, following amplification with primers p4f and p2r (Figure 3A), of a 335 bp fragment from the deleted *PECAM1^{flox}* allele (*PECAM1^{del}*) and a 1356 bp fragment from the WT allele (Figure 3B). Germline deletion of the floxed *PECAM1* allele was confirmed by breeding of *Sox2Cre;PECAM1^{del/WT}* mice with each other to obtain *Sox2Cre;PECAM1^{del/del}* progeny, which were identified by the presence, following amplification with primers p4f and p2r, of only a 335 bp fragment from the *PECAM1^{del}* alleles (Figure 3B).

Defects in *Sox2Cre;PECAM1^{del/del}* mice recapitulate the phenotype of conventional *PECAM-1* knockout mice

PECAM-1 is normally expressed on endothelial cells and on circulating hematopoietic cells, including platelets and leukocytes. To evaluate the effect of *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles on *PECAM-1* expression by these cells, we analyzed expression of *PECAM-1* on the surfaces of platelets and CD8⁺ T cells by flow cytometry and of endothelial cells by fluorescence immunohistochemistry. High levels of *PECAM-1* expression were observed on platelets (Figure 4A) and CD8⁺ T cells (Figure 4B) obtained from wild-type and *PECAM1^{flox/flox}* but not *Sox2Cre;PECAM1^{del/del}* mice. Similarly,

PECAM-1 staining was strong in capillaries and arterioles of liver, heart, kidney and testis tissue isolated from *PECAM1^{flox/flox}* mice but was undetectable in the same tissues isolated from *Sox2Cre;PECAM1^{del/del}* mice (Figure 4C). These results confirm that *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles results in absence of PECAM-1 protein from cells that normally express it.

We have previously demonstrated that CD8⁺ T cells isolated from conventional PECAM-1 knockout mice are less sensitive to inhibition by TGFβ than are their PECAM-1-positive counterparts (D. K. Newman et al., 2016). To evaluate the effect of *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles on TGFβ-mediated inhibition of CD8⁺ T cell responsiveness, we assessed Granzyme B production by CD8⁺ T cells following stimulation through the T cell receptor (TCR), using an antibody specific for the TCR CD3ε subunit, in the absence vs. presence of TGFβ. We found that TGFβ dose-dependently inhibited Granzyme B production by both *PECAM1^{flox/flox}* and *Sox2Cre;PECAM1^{del/del}* CD8⁺ T cells, but that the magnitude of inhibition was greater for *PECAM1^{flox/flox}* than for *Sox2Cre;PECAM1^{del/del}* cells (Figure 5). These results confirm that *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles recapitulates the phenotype observed in conventional PECAM-1 knockout mice of relative insensitivity of CD8⁺ T cells to inhibition by TGFβ.

Previous studies have shown that platelets isolated from conventional PECAM-1 knockout mice are hyper-responsive to stimulation with low, but not high, doses of collagen related peptide (CRP), which activates platelets via the glycoprotein (GP) VI collagen receptor (Ming et al., 2011; Patil, Newman, & Newman, 2001). We therefore assessed the effect of *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles on CRP-mediated platelet aggregation. As shown in Figure 6, relative to those derived from *PECAM1^{flox/flox}* mice, platelets from *Sox2Cre;PECAM1^{del/del}* mice were hyper-responsive to a low dose, and equally responsive to a high dose, of CRP. These results confirm that *Sox2Cre*-mediated deletion of *PECAM1^{flox/flox}* alleles recapitulates the phenotype observed in conventional PECAM-1 knockout mice of hyper-responsiveness of platelets to activation via the GPVI collagen receptor.

Discussion

We describe herein the generation of *PECAM1^{flox/flox}* mice, in which the gene encoding PECAM-1 is floxed to enable its conditional deletion. Global deletion of the floxed *PECAM1* allele was accomplished by breeding *PECAM1^{flox/flox}* with *Sox2Cre/+* transgenic mice. Using PECAM-1-deficient CD8⁺ T cells and platelets from the progeny of *Sox2Cre;PECAM1^{del/del}* matings, we demonstrate that global deletion of the floxed *PECAM1* allele recapitulated two phenotypes characteristic of conventional PECAM-1-knockout mice, namely relative insensitivity of CD8⁺ T cells to inhibition by TGFβ and hyper-responsiveness of platelets to activation via the GPVI collagen receptor.

The *PECAM1^{flox/flox}* mice described herein provide a valuable model with which to characterize the distinct cell type-specific roles that PECAM-1 plays in disease modulation. Thus, studies of mixed bone marrow chimeras have led to the conclusion that endothelial PECAM-1 is necessary and sufficient to support hemostasis (Mahooti et al., 2000), inhibit

thrombosis (Falati et al., 2006), and control autoimmune disease (Graesser et al., 2002) and sepsis (Maas et al., 2005). Breeding of *PECAM1^{flox/flox}* mice with mice in which Cre-recombinase is expressed specifically in the endothelium (Payne, De Val, & Neal, 2018) can be used to confirm this conclusion. Mixed bone marrow chimera studies have also shown that endothelial PECAM-1 is necessary but *not* sufficient to suppress atherosclerosis in some regions of the vasculature (Goel et al., 2008; Harrison et al., 2013). Breeding of *PECAM1^{flox/flox}* mice with endothelial cell- or hematopoietic cell-specific (Kim, Kim, Im, & Fang, 2018) Cre recombinase-expressing mice can be used to confirm this conclusion, and mice that express Cre recombinase in specific populations of immune cells (Kim et al., 2018) or platelets (Nagy et al., 2019; Tiedt, Schomber, Hao-Shen, & Skoda, 2007) can be used to determine on which hematopoietic cells expression of PECAM-1 is important for modulation of this disease. Breeding of *PECAM1^{flox/flox}* mice with mice in which Cre-recombinase is expressed specifically in dendritic cells (Mattiuz et al., 2018; Salvermoser et al., 2018) can be used to determine the importance of dendritic cell expression of PECAM-1 for control of experimental autoimmune encephalomyelitis, as has been previously proposed (Clement et al., 2014). Similarly, breeding *PECAM1^{flox/flox}* mice with mice that express Cre-recombinase in CD4⁺ T cells (Kim et al., 2018) can help to verify their postulated role in resistance to bacterial infection (Ross et al., 2011). Finally, *PECAM1^{flox/flox}* and tissue-specific Cre-recombinase-expressing mice can be used to identify the specific PECAM-1-expressing cells that control inflammatory diseases of the liver (Goel et al., 2007; G. Malik et al., 2019; I. A. Malik et al., 2015) and lung (Schenkel et al., 2006), and that interfere with tumor clearance (Ma et al., 2010; D. K. Newman et al., 2016).

Overall, the *PECAM1^{flox/flox}* mice described in this study represent an exciting new tool for gaining insight into the roles that PECAM-1 plays in disease modulation by distinct cells in the blood and vasculature.

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Figure 1. Generation of ES cells containing the *PECAM1*^{lox(neo)} allele.

(A) Schematic diagram of the *PECAM1*^{lox(neo)} (top) and wild type (WT) alleles (bottom). The *PECAM1*^{lox(neo)} targeted allele contains a selection cassette comprised of a neomycin resistance gene (*Neo*) flanked by Flippase recognition target (FRT) sites that was inserted in the intron between exons 2 and 3, and two loxP sites that were inserted upstream of *PECAM1* exon 1 downstream of the selection cassette. Binding sites of PCR primers p1f, p1r, p2f and p2r and expected lengths of PCR products amplified from the targeted or WT allele with each primer pair are indicated. BamHI and EcoRI restriction sites are indicated with vertical lines and the 5', 3', and Neo probes used for Southern blot analysis are indicated by thick red lines. (B-D) Genotyping of ES clones generated following transfection of C57BL/6 ES cells with the *PECAM1*^{lox(neo)} targeting construct. (B) ES clones containing *Neo* (#2, 6, 9, 11, 13-15) were identified as those from which a PCR product of 3.3 kb was amplified using primers p1f and p1r. (C) *Neo*-containing ES clones that contained the 5' loxP site (#11 and 14) were identified as those from which a PCR product of 276 bp was amplified using primers p2f and p2r. Amplification of a 215 bp fragment from the WT allele using primers p2f and p2r served as a control. (D) The presence of the *PECAM1*^{lox(neo)} targeted allele in ES clones #11 and 14 was verified by Southern blot analysis of BamHI (left and middle) and EcoRI (right) digests of genomic DNA. The 5' probe detected an 8.4 kb band from the targeted allele and a 10.2 kb band from the WT allele in BamHI digests (left), the 3' probe detected a 3.8 kb band from the targeted allele and a 10.2 kb from the wild type allele in BamHI digests (middle), and the Neo probe detected a 7.4 kb from the targeted allele in EcoRI digests (right).

Figure 2. Generation of *PECAM1^{flox/WT}* mice.

(A) Schematic diagram of the *PECAM1^{flox(neo)}* (top), *PECAM1^{flox}* (middle) and WT alleles (bottom). Dashed lines between *PECAM1^{flox(neo)}* and *PECAM1^{flox}* indicate the result of Flp recombinase-mediated removal of *Neo*. Binding sites of PCR primers p2f, p2r, p3f and p3r and expected lengths of PCR products amplified from the targeted or WT allele with each primer pair are indicated. (B) Correctly targeted ES clones #11 and 14 were microinjected into C57BL/6 blastocysts, which were implanted into pseudo-pregnant females, and chimeric mice amongst live pups were identified by PCR using genomic DNA isolated from tail tissue. *PECAM1^{flox(neo)/WT}* mice were identified as those from which PCR products of 276 and 215 bp were amplified from the *PECAM1^{flox(neo)}* and WT alleles, respectively, using primers p2f and p2r. (C) *PECAM1^{flox(neo)}* male mice were bred with FLP1 recombinase-expressing females and progeny from which *Neo* was deleted were identified by PCR using genomic DNA isolated from tail tissue. *PECAM1^{flox/+}* mice were identified as those from which PCR products of 712 and 572 bp were amplified from the *PECAM1^{flox}* and WT alleles, respectively, using primers p3f and p3r.

Figure 3. Characterization of global PECAM-1 knockout mice generated by *Sox2Cre*-mediated deletion of *PECAM1*^{lox/flox} alleles.

(A) Schematic diagram of the *PECAM1*^{lox} (top), *PECAM1*-deleted (*Sox2Cre;PECAM1*^{del}; middle) and WT alleles (bottom). Dashed lines between *PECAM1*^{lox} and *Sox2;PECAM1*^{del} alleles indicate the result of Cre recombinase-mediated removal of *PECAM1* exons 1 and 2. Binding sites of PCR primers p4f and p2r and expected lengths of PCR products amplified from the targeted or WT allele with this primer pair are indicated. (B) PCR was performed using genomic DNA isolated from mice homozygous for the WT allele (*PECAM1*^{WT/WT}), homozygous for the floxed *PECAM1* allele (*PECAM1*^{lox/flox}), heterozygous for the floxed and WT *PECAM1* alleles (*PECAM1*^{lox/WT}), or from the progeny of mice that were heterozygous for the floxed and WT *PECAM1* alleles and bred with mice expressing Cre recombinase (*Sox2Cre;PECAM1*^{del/WT}), or homozygous for the floxed *PECAM1* allele and bred with mice expressing Cre recombinase (*Sox2Cre;PECAM1*^{del/del}). Mice that were heterozygous for the *Sox2Cre;PECAM1*^{del} and WT alleles (*Sox2Cre;PECAM1*^{del/WT}) were identified as those from which PCR products of 335 and 1356 bp were observed following amplification using primers p4f and p2r. Mice that were homozygous for the *PECAM1*-deleted allele (*Sox2Cre;PECAM1*^{del/del}) were identified as those from which only a PCR product of 335 bp was observed following amplification with primers p4f and p2r. As controls, mice that were homozygous for the WT allele (*PECAM1*^{WT/WT}), homozygous for the *PECAM1*^{lox} allele (*PECAM1*^{lox/flox}), or heterozygous for the *PECAM1*^{lox} and WT

alleles (*PECAM1^{fllox/WT}*) were identified as those from which PCR products of only 1356 bp, only 1549 bp, or both 1549 and 1356 bp, respectively, were observed following amplification using primers p4f and p2r.

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Figure 4. Characterization of PECAM-1 expression on platelets, T cells, and endothelial cells isolated from wild-type, *PECAM1*^{flox/flox} and *Sox2Cre;PECAM1*^{del/del} mice. Flow-cytometric analysis revealed high levels of PECAM-1 expression on (A) platelets and (B) CD8⁺ T cells obtained from wild type (green) and *PECAM1*^{flox/flox} (black) but not *Sox2Cre;PECAM1*^{del/del} (red) mice. (C) Fluorescent immunohistochemistry revealed strong PECAM-1 staining (green) in capillaries and arterioles of liver, heart, kidney and testis tissue isolated from *PECAM1*^{flox/flox} but not *Sox2Cre;PECAM1*^{del/del} mice. DAPI (blue) was used to stain cell nuclei. Scale bar = 100µm.

Figure 5. TGFβ more potently inhibits responses of CD8⁺ T cells isolated from *PECAM1*^{flox/flox} relative to *Sox2Cre;PECAM1*^{del/del} mice.

CD8⁺ T cells purified from *PECAM1*^{flox/flox} mice (black) and *Sox2Cre;PECAM1*^{del/del} mice (red) were left unstimulated or stimulated with plate-bound anti-CD3 in the presence of the indicated concentrations of TGFβ for 72 hours. Granzyme B synthesis was determined by flow cytometry. **(A)** Quantitative analysis of the percentages of cells that were positive for granzyme B. Data represent means ± SD calculated from seven independent experiments. Numbers directly over individual bars indicate the statistical significance of differences in the levels of granzyme B production observed in the presence of TGFβ relative to its absence for each genotype. Numbers across both bars indicate the statistical significance of differences in the extent of inhibition of granzyme B production by TGFβ in T cells from *PECAM1*^{flox/flox} relative to *Sox2Cre;PECAM1*^{del/del} mice. **(B)** Representative histograms from one experiment. Results are reported as cell number vs. fluorescence intensity (FI) of Granzyme B staining. The numbers in the histograms indicate the percentages of cells that fell within the granzyme B-positive gate, which is denoted by horizontal lines.

Figure 6. Platelets isolated from *Sox2Cre;PECAM1^{del/del}* mice are hyper-responsive to stimulation via the GPVI/FcR γ collagen receptor than are those isolated from *PECAM1^{flox/flox}* mice.

Washed platelets prepared from *PECAM1^{flox/flox}* (black) and *Sox2Cre;PECAM1^{del/del}* (red) mice were stimulated with a low (left) or high (right) dose of collagen-related peptide (CRP). Percent increases in light transmission, which are directly correlated with the extent of platelet aggregation, were measured by lumi-aggregometry. **(A)** Representative aggregation tracings from one experiment. **(B)** Quantitative analysis of percent platelet aggregation. Data represent means $\pm$ SD calculated from three independent experiments. Statistically significant differences are indicated by an asterisk (* $P < 0.05$); NS denotes differences that were not statistically significant.

Table 1.

Oligonucleotide primers used for PCR and Southern Blot

primer	Orientation	Primer sequence
Primer 1	Forward	5'-gtgaccgcttctctgtgcttta-3'
	Reverse	5'-cctctcccccttcttttgag-3'
Primer 2	Forward	5'-agccaccagtagcgttcctcaca-3'
	Reverse	5'-tcactgggttgaggactcttc-3'
Primer 3	Forward	5'-ggaatgggagagatgggttt-3'
	Reverse	5'-ggaggttccgaatcagctct-3'
Primer 4	Forward	5'-caccagtagcgttcctcaca-3'
	Reverse	5'-tgctccttcagcctccatag-3'
5' Probe	Forward	5'-taactgcagaagcatctctccagc-3'
	Reverse	5'-gcagaggtactgggtgatacaaatg-3'
3' Probe	Forward	5'-gtgaccctctgacccctgctat-3'
	Reverse	5'-aggcttggcatcaggtggtcttt-3'
Neo probe	Forward	5'-cctgaatgaactgcaggacgagg-3'
	Reverse	5'-agctcttcagcaatatcacggtagc-3'