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Platelet Endothelial Cell Adhesion Molecule Deficiency or Blockade Significantly Reduces Leukocyte Emigration in a Majority of Mouse Strains

Alan R. Schenkel,[1]* Tina W. Chew,[†] and William A. Muller[†]

PECAM is a molecule used specifically during the diapedesis step when neutrophils and monocytes leave the blood compartment. Anti-PECAM reagents, such as Abs and soluble fusion proteins, block diapedesis both in vivo and in vitro. However, the PECAM knockout mouse in C57BL/6 strain has no serious defects in most models of inflammation. We show in this study that the same PECAM knockout backcrossed into the FVB/n strain clearly has reduced leukocyte emigration in two models of inflammation. Furthermore, we show that anti-PECAM reagents can block leukocyte emigration in several other wild-type strains of mice like FVB/n, SJL, and the outbred strain Swiss Webster. This clearly shows that the C57BL/6 strain is uniquely able to compensate for the loss of PECAM function. Murine models of inflammatory disease that have been studied using C57BL/6 mice should be re-evaluated using FVB/n or other mouse strains to determine whether PECAM plays a role in those models. ***The Journal of Immunology,*** **2004, 173: 6403–6408.**

The molecule PECAM is a member of the Ig superfamily of molecules. It has six Ig-like domains and a molecular mass of ~130 kDa (1). It is expressed on endothelial cell borders and diffusely on the surface of many leukocytes. Homophilic interactions for diapedesis require the first domain; domain 6 interacts heterophilically with components of the extracellular matrix (2). PECAM also inhabits a surface-connected vesicle-like compartment adjacent to endothelial cell junctions (3). PECAM has two ITIM domains on the cytoplasmic tail (4, 5).

Blockade of PECAM using mAbs and chimeric soluble PECAM (sPECAM)[2] fused to human IgG Fc block at least 75% of monocyte and neutrophil emigration in vitro and in vivo (2, 6–13). Therefore, it was expected that PECAM knockout mice might have a compromised inflammatory response. Surprisingly, they had very little discernible phenotype in response to a variety of inflammatory models when produced in the C57BL/6 background (14). Other groups have described some hematological and immunological alterations, such as increased bleeding time (15), increased vascular permeability (16), decreased vasculogenesis (17), earlier onset of autoimmune CNS disease due to vascular permeability (13), and decreased neutrophil emigration after peritoneal IL-1β injection in the C57BL/6 strain (12).

The C57BL/6 strain is the most commonly used strain of laboratory mouse. However, this strain has many unique sensitivities to viral and bacterial agents and a Y chromosome from an Asiatic mouse (18). Additionally, this strain does not have large litters and frequently cannibalizes its own pups. The FVB/n strain has much larger litters and was used to generate a PECAM knockout by nine successive backcrossing from the C57BL/6 strain. We found that leukocyte emigration was substantially blocked in PECAM knockout mice bred into the FVB/n strain, transgenic FVB/n mice expressing sPECAM (11), and wild-type FVB/n strain mice treated with anti-PECAM Abs or sPECAM. We also found that leukocyte emigration can be blocked by anti-PECAM reagents in many other strains, including outbred Swiss Webster mice. In contrast, even wild-type C57BL/6 mice are insensitive to anti-PECAM treatment and have normal leukocyte emigration in response to peritoneal thioglycolate broth injection. Thus, the C57BL/6 strain appears to be unique among mouse strains in their ability to compensate for the loss or blockade of PECAM.

Materials and Methods

Mouse strains

C57BL/6 or FVB/n wild-type and PECAM knockout mice were raised and housed at Weill Medical College of Cornell University. PECAM-deficient mice in the C57BL/6 background have been previously described (14). PECAM-deficient mice in the FVB/n background were generated by nine successive backcrosses. SJL and Swiss Webster were purchased from Charles River (Wilmington, MA)/The Jackson Laboratory (Bar Harbor, ME) and housed for at least 2 wk to allow the animals to regain comfort and to control for environmental effects on experiments.

Thioglycolate-induced aseptic peritonitis

C57BL/6 or FVB/n PECAM knockout mice and wild-type controls were injected i.p. with 1 ml of 4% thioglycolate broth (Difco Laboratories, Sparks, MD). Additionally, wild-type mice from all four strains were injected i.v. with 100 μg of anti-PECAM Abs (clone 2H8) or soluble mouse PECAM-Fc (11) 1 h before thioglycolate injection to block CD31 function. We also simultaneously injected age- and sex-matched controls with PBS as control for trauma. Experiments were conducted as described previously (8). Briefly, at 18 h after thioglycolate injection, mice were sacrificed. The peritoneal cavity was washed with 5 ml of HBSS and 10 mM EDTA. Cytospin preparation and cell counts were done immediately. Blood was drawn by cardiac puncture into heparinized syringes for white blood cell and differential counts. All internal organs were harvested and fixed in 10% buffered formalin for later analysis.

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[2] Abbreviation used in this paper: sPECAM, soluble PECAM.

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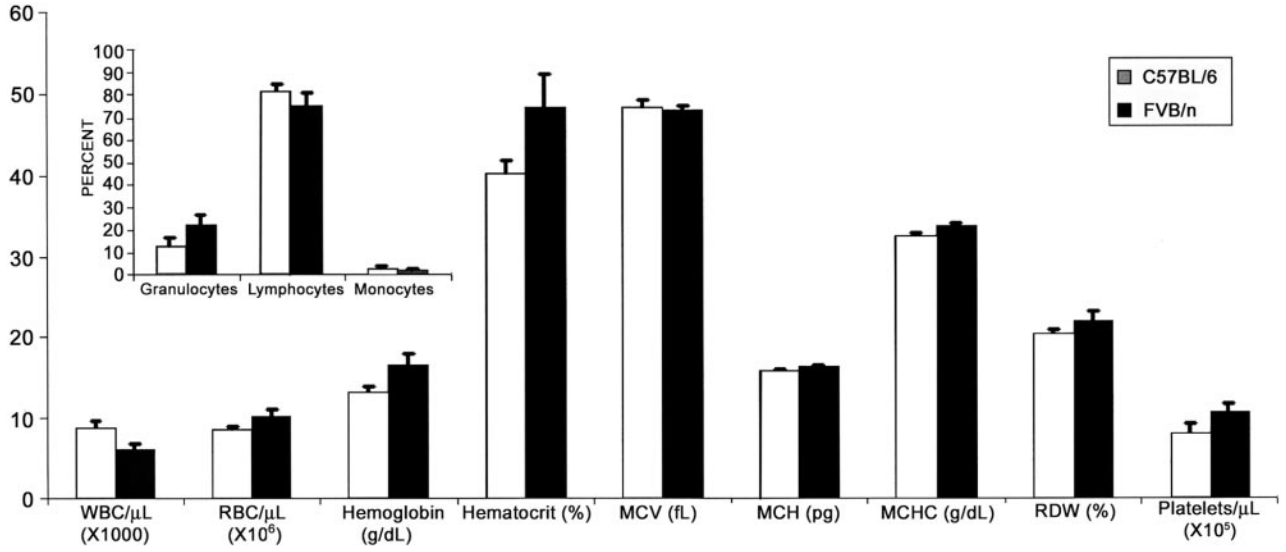

FIGURE 1. Complete blood cell counts on C57BL/6 and FVB/n PECAM knockout mice. Eight age- and sex-matched mice from both strains were sacrificed for complete blood cell counts. Mice were from 2 to 10 mo old. WBC, White blood cells. MCV, Mean corpuscular volume. MCH, Mean corpuscular hemoglobin. MCHC, Mean corpuscular hemoglobin concentration. RDW, RBC distribution width. Almost all measurements were similar, total WBC were slightly lower in FVB/n ($p < 0.05$), but counts multiplied by the differential yielded no differences in absolute numbers.

Croton oil-induced topical dermatitis

Croton oil (Sigma-Aldrich, St. Louis, MO) is prepared as a 2% solution in a 4:1 mixture of acetone-olive oil. A volume of 10 μl is applied to one ear, and the contralateral ear is used as a control (acetone/olive oil only). Age- and sex-matched FVB/n and C57BL/6 PECAM knockout mice were tested along with wild-type controls. At 6 h after application, the mice were

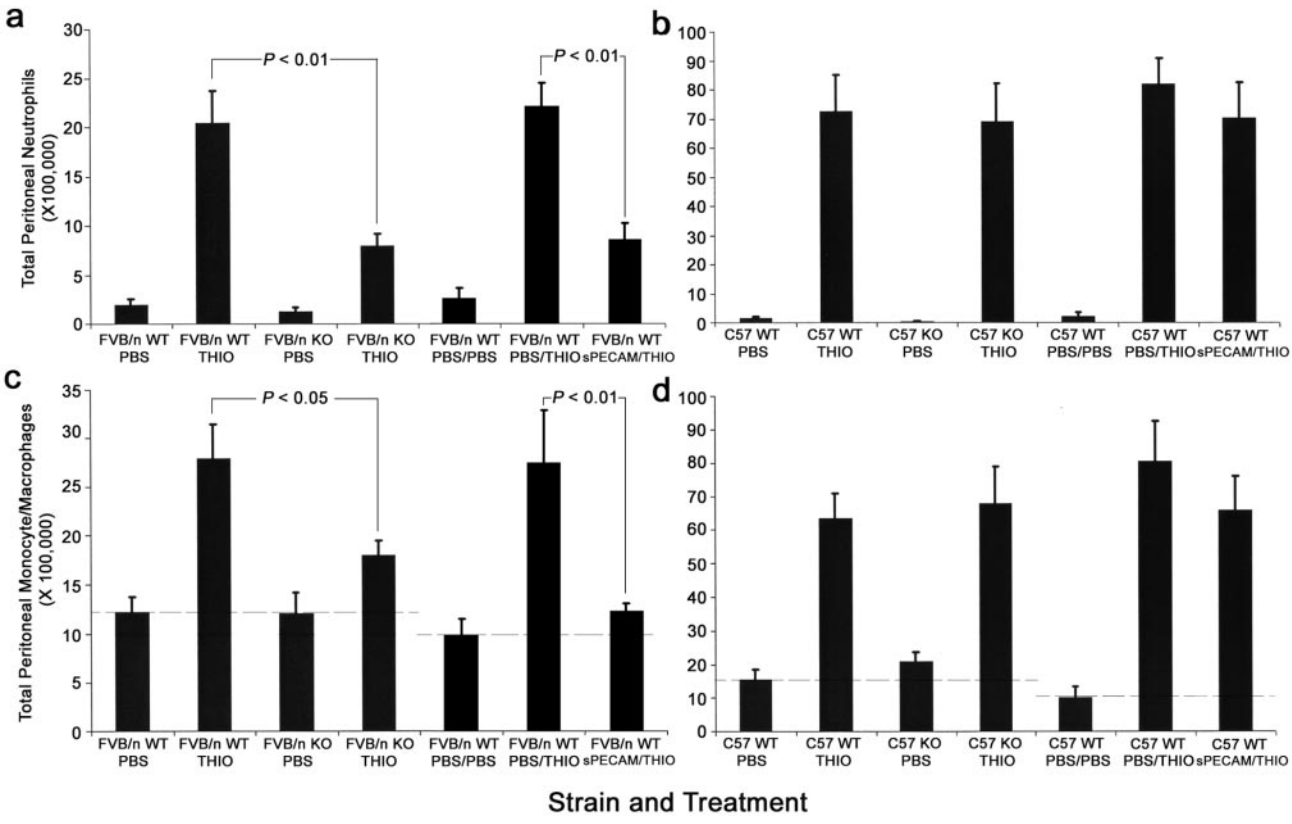

FIGURE 2. Thioglycolate treatment in C57BL/6 and FVB/n PECAM knockout and wild-type mice. Mice were injected i.p. with PBS or thioglycolate (THIO) broth and sacrificed at 18 h for peritoneal lavage followed by neutrophil (*a* and *b*) and monocyte/macrophage (*c* and *d*) cell counts. In addition to comparing knockouts with wild-type mice, groups of wild-type mice from each strain were also pretreated with i.v. PBS (controls) or sPECAM-Fc (sPECAM) as indicated. Data from both C57BL/6 and FVB/n mice were collected from the simultaneous experiments but are shown in separate graphs. PECAM-deficient FVB/n mice had markedly reduced responses to thioglycolate ($p < 0.01$), as did wild-type FVB/n mice pretreated with sPECAM. C57BL/6 knockout mice had essentially the same response as wild-type mice with or without sPECAM. WT, Wild type. KO, PECAM deficient. THIO, thioglycolate broth i.p. Data shown are means from 8 to 10 mice per group in three combined experiments. Error bar is SEM. Dotted lines in *c* and *d* represent baseline peritoneal monocyte/macrophage counts in untreated mice.

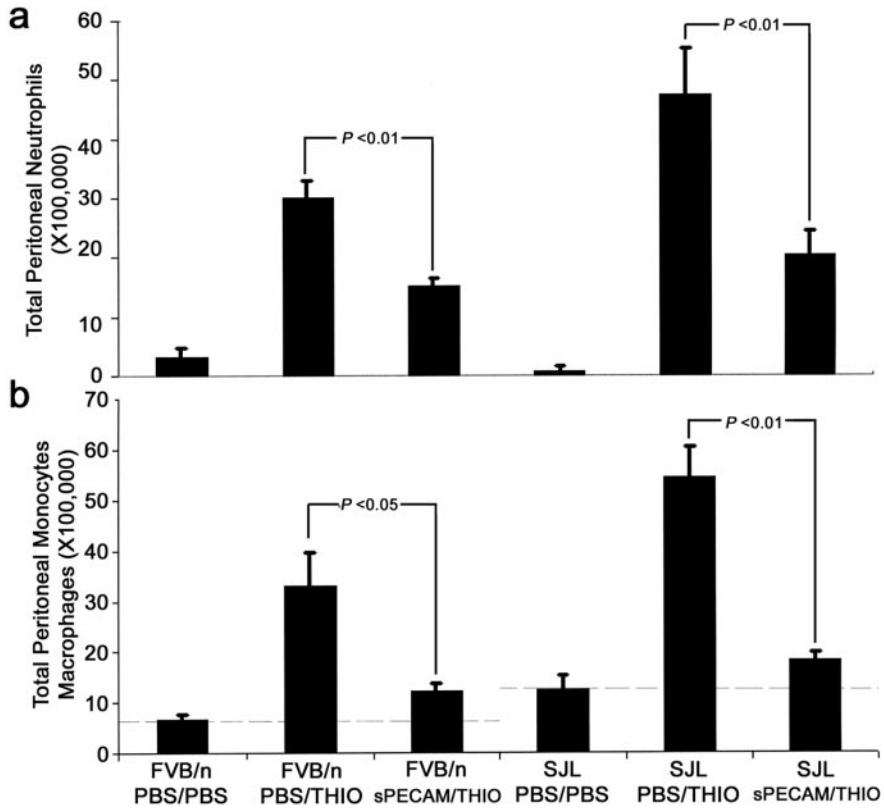

FIGURE 3. Thioglycolate treatment in SJL mice. Wild-type SJL strain mice were tested because they have a similar magnitude response to C57BL/6 mice. SJL mice were pretreated with i.v. PBS (controls) or sPECAM-Fc (sPECAM) as indicated, 1 h before i.p. PBS or thioglycolate both (THIO). Like FVB/n mice, sPECAM severely blunts the response by neutrophils (*a*) and monocyte/macrophages (*b*). Data shown are means from four or five mice per group in one experiment. Error bar is SEM. Dotted line in *b* represent baseline peritoneal monocyte/macrophage counts in untreated mice. THIO, Thioglycolate broth i.p.

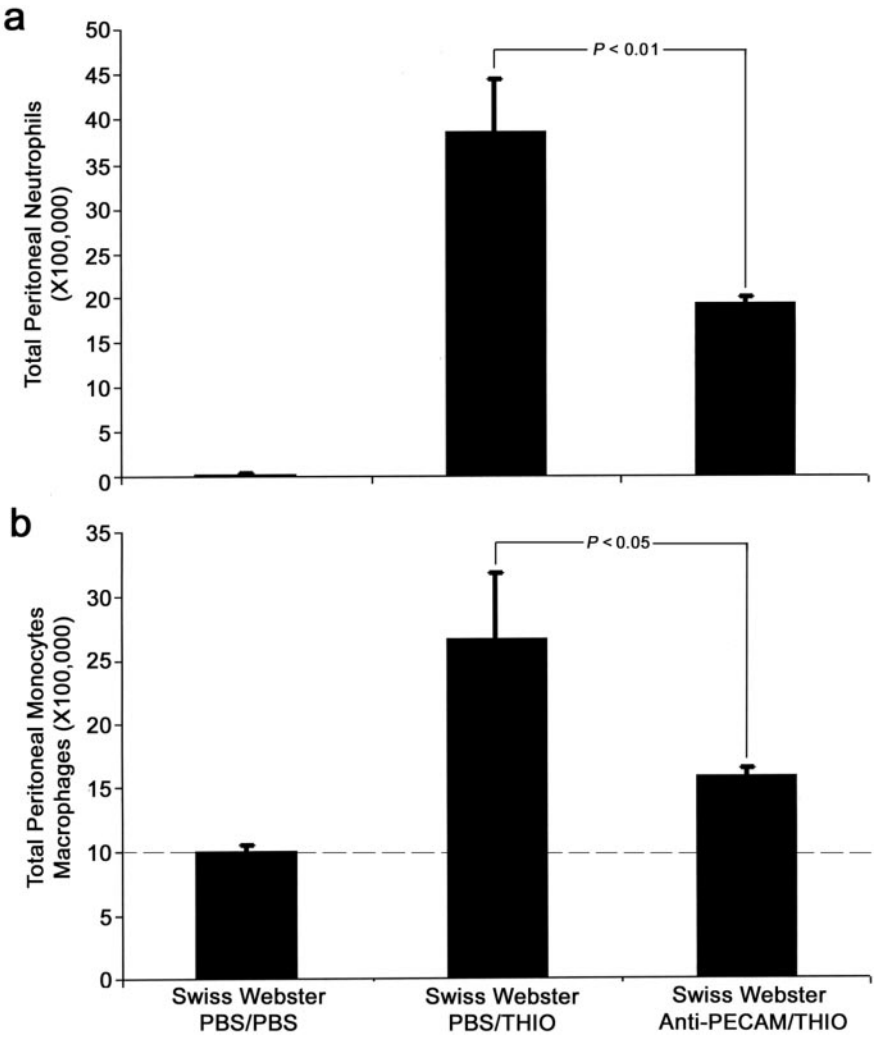

FIGURE 4. Thioglycolate treatment in Swiss Webster mice. To test the effects of anti-PECAM reagents on an outbred strain of mice, Swiss Webster mice were used in the thioglycolate studies. In this experiment, 100 μg of rat anti-mouse PECAM mAb, clone 2H8, was injected i.v. 1 h before thioglycolate injection (anti-PECAM). Again, like FVB/n mice, anti-PECAM blocks leukocyte emigration. Data shown are means from four mice per group in this experiment. Error bar is SEM.

sacrificed. The ears were measured for swelling by calipers. Both ears were harvested, fixed in 10% buffered formalin, embedded in paraffin, and sectioned longitudinally. Total leukocyte counts and localization was done by microscopy after staining in H&E.

Statistical analysis

Tukey's honestly significant difference test was used to do multiple comparisons across groups (JMPIn; SAS Institute, Cary, NC).

Results

PECAM knockout mice in both strains had similar complete blood cell counts (Fig. 1). Total white blood cell counts were slightly lower in FVB/n ($p < 0.05$), but the differential counts were similar, and the absolute number of circulating granulocytes was not significantly different between the two strains. In general, the two strains were healthy, although the FVB/n PECAM knockout strain occasionally succumbed to a chronic pneumonia characterized by mononuclear cell exudates and accumulation of crystals of the antifungal Ym1 protein (A. R. Schenkel, T. Chew, M. Harbord, and W. A. Muller, manuscript in preparation). Older C57BL/6 PECAM knockout mice occasionally also had evidence of lung inflammation; however, this was marked by expansion of pulmonary perivascular lymphocytic vessels. The lungs of all experimental animals were examined histologically. In the experiments described in this study, asymptomatic mice showing early evidence of pneumonia did not have any significant difference in response from unaffected controls. Symptomatically ill mice were not used for experiments.

Two models of acute inflammation were used in this study, thioglycolate-induced aseptic peritonitis and croton oil-induced topical dermatitis. We compared PECAM knockout mice in both strains using both models. It was known that the C57BL/6 knockout would respond like wild-type mice (14), but we had not tested the FVB/n knockout mice. In the peritonitis model, we also included matched wild-type C57BL/6 or FVB/n mice treated with PBS or with 100 μg of sPECAM-Fc, a soluble inhibitor of PECAM-mediated transendothelial migration (11).

We first tested wild-type and PECAM-deficient mice from both strains using the peritonitis model (Fig. 2). Comparing both strains head to head, C57BL/6 strain knockout and wild-type C57BL/6 treated with sPECAM-Fc had high responses to thioglycolate similar to untreated controls. FVB/n mice mobilize lower numbers of neutrophils and monocytes overall; however, PECAM knockout and wild-type FVB/n treated with sPECAM-Fc mice had markedly fewer inflammatory cells entering the peritoneal cavity compared with strain-matched controls. Neutrophil emigration was reduced by 60–75%, and monocyte emigration was nearly reduced to background levels.

The blunted leukocyte emigration seen in the FVB/n mice was not unique to that strain. We also tested SJL (Fig. 3) and outbred strain Swiss Webster (Fig. 4) mice in the peritonitis model with sPECAM-Fc or Abs against PECAM. Even though these strains have higher overall leukocyte emigration than FVB/n mice, these strains were also susceptible to anti-PECAM reagents, as were several other mouse strains and rats used in other studies (7, 8, 13, 19, 20). Thus, the C57BL/6 strain seems uniquely resistant to anti-PECAM reagents.

To test the PECAM-deficient mice in another model of acute inflammation, we used croton oil dermatitis. In this model, croton oil, acting as a local irritant, is applied to the surface of one ear, while the same volume of vehicle is applied to the contralateral ear as a control. Croton oil causes marked edema of the affected ear, due to histamine release from local mast cells. This and other mediators recruit acute inflammatory cells into the ear. Six hours later, mice are sacrificed, and the ears are removed and examined histologically for the presence of extravasated neutrophils.

When we compared wild-type and PECAM-deficient mice in the FVB/n and C57BL/6 strains, the results were consistent with the peritonitis experiments. In the wild-type mice of both strains and the PECAM-deficient C57BL/6 mice, the soft tissue of the dermis of the ear was filled with neutrophils that had extravasated at the site of the acute inflammation (Fig. 5, *b*, *f*, and *h*). No leukocyte infiltration was seen in any of the contralateral ears in any strain (Fig. 5, *a*, *c*, *e*, and *g*). In contrast, leukocytes in the PECAM-deficient FVB/n mice mostly remained within the vessels (Fig. 5*d*). Only ~30% of the leukocytes extravasated compared with over 80% in the other mice. In many profiles, neutrophils appeared to be in contact with the vessel wall (Fig. 5*d*, arrows), as previously seen when PECAM function was inhibited with mAb (8) or sPECAM-Fc (11).

The number and location of leukocytes in these sections was manually counted (Fig. 6). Only in PECAM-deficient FVB/n mice were more leukocytes located in the blood vessels compared with

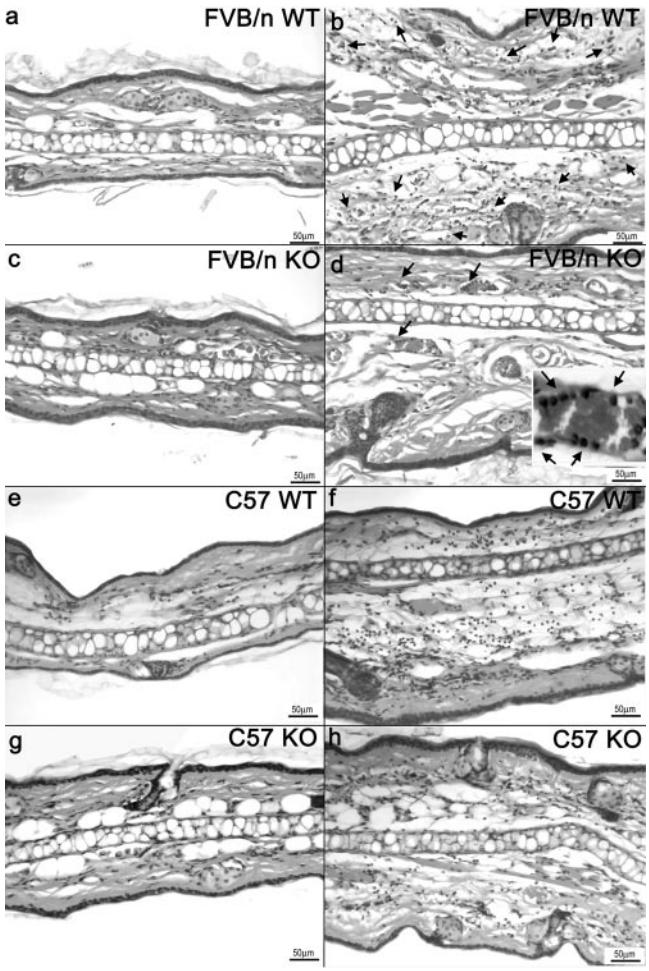

FIGURE 5. Croton oil treatment in FVB/n PECAM knockout and wild-type mice. Representative images using croton oil induced acute inflammation. The contralateral ear of each mouse was not inflamed by the carrier (*a*, *c*, *e*, *g*). Edema and swelling were noted in both wild-type and PECAM knockout mice (*b*, *d*, *f*, and *h*). However, neutrophils extravasated from vessels into tissues in wild-type FVB/n mice (*b*, *arrows*) wild-type C57BL/6 (*f*), and C57BL/6 PECAM knockout (*h*) mice. FVB/n PECAM knockout mice had many fewer neutrophils in the extravascular ear tissue, and leukocytes are seen accumulating on endothelial surface of vessels in the PECAM knockout mice (*d*, *inset*, *arrows*). *a* and *b*, FVB/n wild type. *c* and *d*, FVB/n PECAM knockout. *e* and *f*, C57BL/6 wild type. *g* and *h*, C57BL/6 PECAM knockout

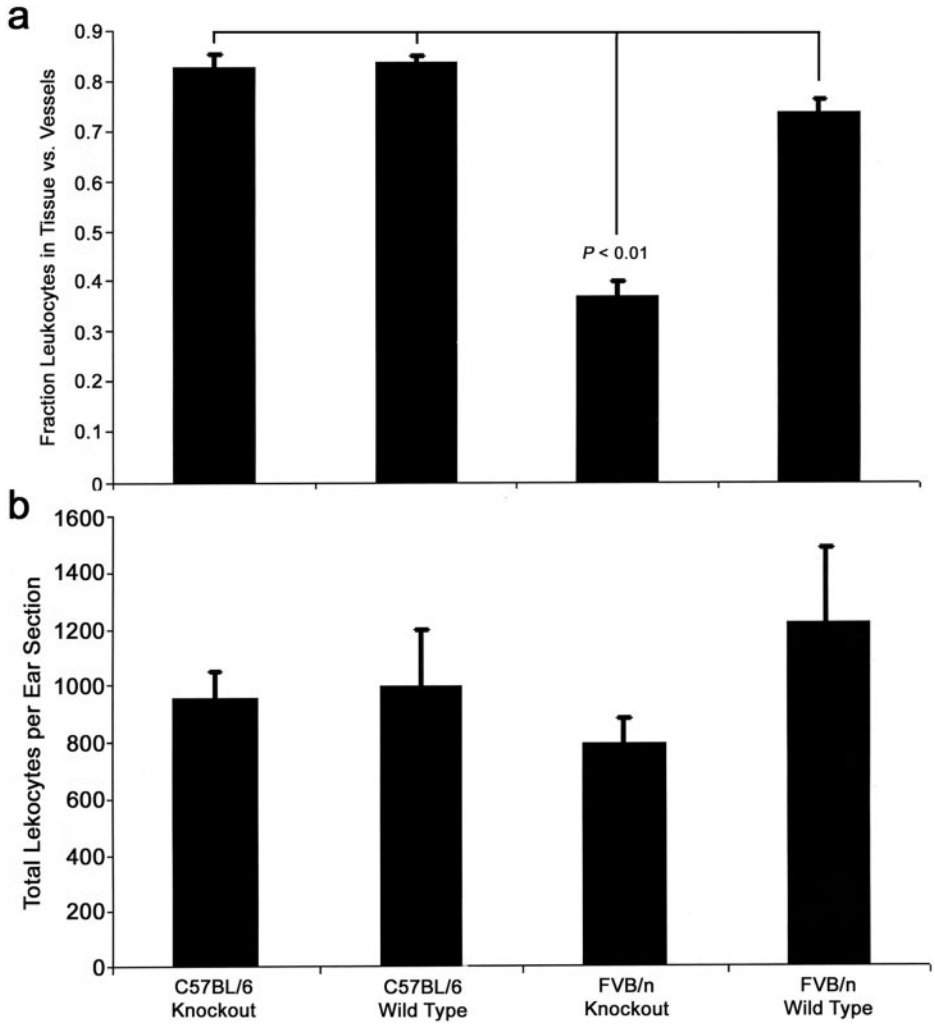

FIGURE 6. Croton oil treatment in C57BL/6 and FVB/n PECAM knockout and wild-type mice. Croton oil was used to test FVB/n and C57BL/6 PECAM knockout mice and wild-type controls during acute dermatitis. On each mouse, croton oil is applied to one ear, and the contralateral ear is used as a control (carrier only). The fraction of total leukocytes in each ear section that were present in soft tissue and not blood vessels was calculated by noting the location and dividing the number in tissue by the total (*a*). FVB/n PECAM knockout mice had reduced numbers of tissue neutrophils compared with wild-type FVB/n or PECAM knockout or wild-type C57BL/6 mice. Total numbers of leukocytes recruited to the ear were similar for all strains tested (*b*); however, in FVB/n PECAM knockout mice, the leukocytes remained mostly in the vasculature. Data shown are means from seven mice per group in one experiment. Error bar is SEM.

the tissues (Fig. 6*a*). The total numbers of leukocytes recruited to the ears was about the same among all mouse strains (Fig. 6*b*). The degree of ear swelling, on average for all four groups, was about +0.10 mm (data not shown). Therefore, this was a selective defect in emigration—not in the ability of these mice to mount the early phases of the inflammatory response.

Discussion

We and others have shown that leukocyte diapedesis can be blocked by Abs against PECAM and sPECAM-Fc in many strains and inflammatory models (8, 10, 13, 19), including transgenic mice expressing soluble mouse PECAM-Fc (11). Some doubt arose about the role of PECAM in regulating diapedesis when the PECAM knockout in C57BL/6 did not show reduced responses in several inflammatory models (14). We show in this study that the reason for these unexpected results is the unique ability of the C57BL/6 strain to compensate for the loss or blockade of PECAM. The same PECAM knockout backcrossed into the FVB/n strain resulted in mice that have poor monocyte and neutrophil extravasation in response to inflammatory stimuli.

The lack of effects on inflammatory function appears to be unique to C57BL/6 mice. Studies in the literature show that inflammation can be blocked by anti-PECAM Abs in other strains such as $CD2F_1$ (8), AKR/J (8), AND (a T cell transgenic derived from SJL) (13), and DBA (19). We have confirmed the result in SJL mice in this report. Neutrophil emigration was also blocked by anti-PECAM Ab in rats (7). Additionally, leukocyte adhesion in synovial vessels was blocked when Abs to PECAM were injected in rats with experimental arthritis (20).

It is well documented that C57BL/6 mice are unique from other inbred mouse strains in a variety of immunological and infectious tests. The strain summary in The Jackson Laboratory Mouse Genome Informatics database (18) lists dozens of classical studies differentiating this strain from others. The opening paragraph about the strain states the following: "C57BL is probably the most widely used of all inbred strains (substrain C57BL/6 alone accounts for over 14% of occasions on which an inbred strain is used), though in many ways it seems to be atypical of inbred strains of laboratory mice. In contrast to 36 other standard inbred strains, it carries a Y chromosome of Asian *Mus musculus* origin (21), and a LINE-1 element derived from *Mus spretus* the frequency of which suggests that up to 6.5% of the genome may be of *M. spretus* origin (22). A probe designated B6-38 to the pseudoautosomal region of the X and Y chromosome has a characteristic *Pst*I pattern of fragment sizes, which is present only in the C57BL family of strains (23)."

The unique compensatory mechanism in CD57BL/6 mice gives us the opportunity to search for new molecules involved in leukocyte extravasation, particularly those that can take over for PECAM function. Other genes that play a role in diapedesis, like CD99 (24), may be able to facilitate the extravasation of leukocytes in these mice. Other adhesion molecules like integrins and junctional adhesion molecules on the leukocytes, and VCAM or

ICAMs on endothelial cells may have alternative roles in C57BL/6 mice. We are currently developing mouse endothelial cultures so we can quantitate the expression of these molecules from different strains. We also want to test each cell type, combining wild-type monocytes with PECAM knockout endothelial cells for example.

Homologs to PECAM may be within the large Ig superfamily; however, there is no immediately obvious PECAM-2 to account for the functional redundancy in C57BL/6 mice. Studies using Apolipoprotein E (ApoE) knockouts in both C57BL/6 and FVB/n strain mice have shown that both strains are susceptible to the development of fatty plaques, but the lesions in FVB/n strain are considerably smaller (25, 26). This is not due to differences in serum cholesterol but appears to be strictly genetic. Quantitative trait locus mapping studies have found several candidate genes for this difference, including the TNF response regulator A20 (27, 28). We are currently working to map differences in our PECAM knockout mice to determine the gene(s) that compensate for the loss of PECAM function, either by genetic deletion or interference, in C57BL/6 mice, by crossing them to FVB/n mice. Initial experiments indicate that the gene(s) from the C57BL/6 mice are dominant.

In summary, these studies show that C57BL/6 mice can uniquely compensate for the loss or inhibition of PECAM by some other means, such as increased gene expression of a known or novel molecule. Importantly, these discoveries also mean that many failed studies using the PECAM knockout in the C57/BL6 strain must be re-evaluated.

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