Genetic Evidence for Functional Redundancy of Platelet/Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD31-Deficient Mice Reveal PECAM-1-Dependent and PECAM-1-Independent Functions

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Platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31), a member of the Ig superfamily, is expressed strongly at endothelial cell-cell junctions, on platelets, and on most leukocytes. CD31 has been postulated to play a role in vasculogenesis and angiogenesis, and has been implicated as a key mediator of the transendothelial migration of leukocytes. To further define the physiologic role of CD31, we used targeted gene disruption of the CD31 gene in embryonic stem cells to generate CD31-deficient mice. CD31-deficient mice (CD31KO) are viable and born at the expected Mendelian frequency, remain healthy, and exhibit no obvious vascular developmental defects. In response to inflammatory challenge, polymorphonuclear leukocytes of CD31KO mice are arrested between the vascular endothelium and the basement membrane of inflammatory site mesenteric microvessels, confirming a role for CD31 in the migration of neutrophils through the subendothelial extracellular matrix. Normal numbers of leukocytes are recovered from inflammatory sites in CD31KO mice, however, suggesting that the defect in leukocyte migration across basal lamina observed in the absence of CD31 may be compensated for by the use of other adhesion molecules, or possibly an increased rate of migration. Homing of T lymphocytes in vivo is normal, and CD31KO mice are able to mount a cutaneous hypersensitivity response normally. In addition, CD31-mediated homophilic adhesion does not appear to play a role in platelet aggregation in vitro. This study provides genetic evidence that CD31 is involved in transbasement membrane migration, but does not play an obligatory role in either vascular development or leukocyte migration. The Journal of Immunology, 1999, 162: 3022–3030.
implying involvement of CD31 in the development of blood islands and vessels.

In addition to direct cell-cell adhesion, it would appear that engagement of CD31 facilitates outside-in signaling. A number of studies have demonstrated a CD31-dependent increase in the integrin-mediated adhesion of a variety of leukocytes, including monocytes, PMN, NK cells, lymphokine-activated killer cells, CD34+/CD38− hemopoietic progenitor cells, and T lymphocytes (22–28). A potential role for CD31 in platelet function has also been proposed. Distinct signaling complexes involving CD31 were formed during human platelet aggregation (29), and CD31 has been proposed as a costimulatory agonist receptor capable of modulating integrin function in human platelets during adhesion and aggregation (30). In vivo, treatment with anti-CD31 Abs delayed platelet adhesion/aggregation in the mouse (31, 32).

In this study, we show that CD31KO mice are viable and undergo normal vascular development, suggesting that CD31 is not critical for vasculogenesis. Although electron-microscopic examination of mesenteric postcapillary venules revealed an accumulation of PMN at the perivascular BM in CD31KO mice, similar numbers of leukocytes migrate into the peritoneal cavity in both wild-type (WT) and CD31KO mice after challenge with IL-1β or thiglycollate (TG). We observed no defect in migration of PMN in vitro that could account for these disparate findings. No defect in T lymphocyte homing to lymphoid organs, or in the mounting of a cutaneous hypersensitivity response, is evident in CD31KO mice. No dependence on CD31-mediated adhesion in ADP-stimulated platelet-platelet in vitro aggregation was found. In conclusion, the creation of a CD31-deficient mouse confirms that CD31 is involved in TEM of neutrophils at the level of passage across the BM. However, PECAM is not essential for the processes of leukocyte migration and vascular development, implying the existence of compensatory mechanisms in the mouse.

Materials and Methods

Generation of CD31-deficient mice

A 129J mouse genomic library was screened with a CD31 cDNA probe, and a phage clone containing exons 6–8 was isolated. A targeting vector was designed to disrupt exon 7 by inserting a PGKNeo resistance expression cassette in reverse orientation of CD31 transcription. The targeting vector (20 μg) was linearized with Kpnl and electroporated into 5 × 106 E14K embryonic stem (ES) cells (derived from 129/Ola mice) maintained on a layer of mitomycin C-treated embryonic fibroblasts in DMEM, supplemented with leukemia-inhibitory factor, 15% FCS, l-glutamine, and 2-MiM glutamine. ES cells were grown in 0.1% serum and cultured in 100 μg/ml G418 for 8 days. Homologous recombinants were identified by PCR using the following primers: 5′-AGG TAA GGA CCT ACA GGT GTG TTC-3′ plus 3′-CTT CCT CTT GCT TTA CGG TAT C-5′, yielding a mutant band of kb. PCR conditions as follows: 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C for 30 cycles. Colonies positive for PCR were genotyped by Southern blotting analysis using a PCR-amplified flanking probe and a neo-specific probe (data not shown). Chimeric mice were produced by microinjection of targeted ES cells into 3.5-day C57BL/6 blastocysts that were subsequently transferred to CD1 pseudopregnant foster mothers. Chimeric males were mated with C57BL/6 mice, and subsequently stained with mAbs in 100 μl of PBS (2 mM EDTA/5% BSA and 0.1% azide) for 30 min at 4°C. Cells were analyzed for single-, double- and triple-color immunofluorescence using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). mAbs used were MEC 13.3 and 390 (anti-CD31), M1/70 (anti-CD11b, Mac-1), RB6-8C5 (anti-Ly-6G, Gr-1), and 145-2C11 (anti-CD3) (all mAbs from PharMingen, San Diego, CA).

PBL, RBC, and platelet counts

Circulating cell numbers were determined on a Sysmex F-800 semiautomated hemolytic analyzer and by direct counting using a hemocytometer. Additional determinations of blood composition were performed by PACS analysis of blood.

Preparation of whole embryo sections

Immunohistochemistry for CD31 (anti-CD31, clone MECD13.3, 1:100; PharMingen) was performed on sectioned material, as previously described (33).

IL-1β- and TG-induced peritonitis model

Acute peritonitis was induced by i.p. injection of 10 ng of murine rIL-1β (Genzyme, Cambridge, MA) in 0.5 ml PBS or by 1.5 ml of 4% TG broth (Difco, Detroit, MI). At various time points, mice were sacrificed by CO2 inhalation, and peritoneal exudate cells were harvested by lavage with 7 ml of PBS containing 2 mM EDTA and 50 U/ml heparin. Cells recovered were washed three times in Ca2+/Mg2+-free PBS and counted using a hemocytometer. Cytosposms were prepared to perform differential counts, and infiltrating cells were subsequently identified as either PMN, monocytes, macrophages, or lymphocytes.

Mouse air pouch model

Air pouches were formed by the dorsal s.c. injection of 6 ml sterile air on day 0 and 5 ml on day 3. On day 6, 10 ng murine IL-1β (Genzyme), approximately 105 Formalin-inactivated Staphylococcus aureus (American Type Culture Collection, Manassas, VA; 25923), or 100 ng murine macrophage inflammatory protein-1α (Genzyme) was injected locally into the air pouch in 1.5 ml vehicle (0.5% carboxymethyl cellulose, CMC, medium viscosity, Sigma, St Louis, MO). At various time points, air pouches were washed with 2 ml PBS containing 2 mM EDTA and 50 U/ml heparin. Collected cells were washed three times in Ca2+/Mg2+-free PBS and counted, and cytospins were performed.

Electron-microscopic examination of mesenteric postcapillary venules

Portions of mesentery containing blood vessels were dissected out of the original sample (4 h postinjection of 10 ng IL-1β or 1.5 ml TG), immersed overnight in fixation buffer (2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2), postfixed in 1% aqueous osmium tetroxide, rinsed in water, dehydrated in ethanol, and embedded in epoxy resin. One-micrometer sections were initially cut, stained with toluidine blue, and screened by light microscopy for the presence of postcapillary venules. Samples containing postcapillary venules were ultra-thin-sectioned, and the resultant sections were collected onto both mesh grids and formvar-coated keyhole grids. Sections were contrast enhanced with uranyl acetate and lead citrate before examination under a CM120 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). Five mesenteric portions were analyzed from three WT, and five from three CD31KO mice, allowing the scanning of 4.1 mm of vessel in the WT and 4.12 mm in the CD31KO. The postcapillary venules studied in both WT and CD31KO exhibited the same number of EC, with approximately the same thickness of basal lamina and number of pericytes. Leukocytes within postcapillary venules were included for analysis only if they showed both nucleus and cytoplasm in the plane of the section and if they were considered to be potentially transmigrating, i.e., they were either proximal to the endothelium (within one cell diameter or in contact with endothelium), or trapped between the endothelium and the BM. Migrating leukocytes were identified either as PMN or monocytes based on their morphology. Counts of PMN and monocytes were compared using the Student’s t test or, when the distribution was skewed, the Wilcoxon’s rank sum test. The distribution of leukocytes in the proximal and trapped position was compared between the CD31KO and WT mice using the χ2 test with continuity correction factor.

Contact hypersensitivity model

Mice were sensitized on days 1 and 2 with oxazalone (Sigma; freshly dissolved in a 4:1 acetone/paraffin oil mixture) on the shaved abdomen (50
μl) and right front paw (5 μl). On day 5, mice were challenged with 1% oxazalone in the same solvent mixture. For challenge, 10 μl of oxazalone was applied topically to both dorsal and ventral surfaces of the right ear, while the left ear was treated with vehicle alone. After 24 h, both ears were measured with a force calibrated digital thickness gauge, and data were expressed as percentage of increase in ear thickness between right and left ears.

**Analysis of lymphocyte trafficking**

Lymphocytes (5 × 10^5/ml) were taken from mesenteric and peripheral lymph nodes and labeled with 100 μCi of ^51^Cr in PBS. Labeled cells were centrifuged over a FCS gradient to remove dead cells and debris, washed three times, and resuspended at 3 × 10^6/ml in PBS. A total of 100 μl labeled cells was injected i.v. via the tail vein, while an uninjected fraction of labeled cells was kept for determination of total counts injected. At 2- or 24-h postinjection, mice were sacrificed and lymphoid organs were removed for determination of ^51^Cr activity using a gamma counter.

**In vitro analysis of bEND3 cell transmigration**

Transwell membranes (5 μm, 24 mm; Costar, Cambridge, MA) were coated with 1% gelatin in PBS. After 30 min, the gelatin was removed and 2 × 10^6 bEND-3 cells (kindly provided by Dr. Werner Risau, Max Planck Institute for Physiology and Clinical Research, Bad Nauheim, Germany) were added to each membrane and cultured for 3 days, after which the membranes were washed twice in adhesion media (RPMI containing 10% FCS). A total of 500 μl adhesion media containing 100 ng/ml human rIL-8 (Genzyme) was added to the lower wells, and 2 × 10^5 PMN was added to the top well, respectively.

**Results**

**Generation of CD31 KO mice**

CD31 null mice were generated using homologous recombination in E14K embryonic stem (ES) cells. The targeting vector consisted of a neomycin resistance gene inserted into exon 7 of the CD31 gene (Fig. 1). Of nine ES cell clones selected on the basis of G418 resistance for 0% and PPP as a reference for 100% platelet aggregation.

**FIGURE 2.** Lack of cell surface CD31 expression in CD31KO mice. A. The histogram shows CD31 expression on PBL from CD31WT (bold line), CD31^{+/−} (light line), and CD31KO (dashed line) mice, as assessed by FACS analysis of PBL using anti-CD31 mAb MEC13.3. Ten thousand viable lymphocytes were gated in each case. Transverse sections through the ventricle of the heart of WT (B) and CD31KO (C) of E11.5 (dpc 11.5) embryos confirm that CD31 expression is absent in CD31 null EC. The endocardium, the innermost layer of the heart, consists of an endothelial lining that is continuous with the endothelium of the vessels entering and leaving the heart. Endocardial cells stain positive for CD31 in the WT, but not CD31KO (arrowheads in A). Blood vessels (arrows in A) in the pericardium (p) also stain positive for CD31 in the WT, but not the CD31KO mutant. Scale bar = 250 μm.

and converted to percentage of platelet aggregation, using PRP as a reference for 0% and PPP as a reference for 100% platelet aggregation.
while CD31KO mice display only background fluorescence. Interestingly, analysis of CD31 heterozygous mice (CD31<sup>+/−</sup>) revealed an approximately 50% decrease in cell surface staining for CD31 compared with WT mice, indicating that loss of one functional allele reduces CD31 protein synthesis and subsequent cell surface expression. CD31-specific staining was also undetectable on leukocytes obtained from spleen, lymph nodes, and thymus of CD31KO mice. An identical pattern of cell surface staining was obtained using mAb 390.

Analysis of whole embryos revealed a complete absence of CD31 staining of the endothelial cells lining the endocardium of CD31 null E11.5 (DPC 11.5) embryos (Fig. 2), compared with that of WT embryos (Fig. 2B), confirming that mutation of the CD31 gene results in the absence of cell surface expression on both vascular endothelium and circulating leukocytes.

**PBL, RBC, and platelet counts in CD31KO mice**

No significant difference in the number of PBL was apparent between WT and CD31KO mice, either before, or after, injection of TG i.p. (Table I). TG challenge resulted in an overall reduction in PBL numbers of approximately 50% in both WT and CD31KO mice. Circulating monocyte, macrophage, PMN, and lymphocyte numbers in CD31KO mice were normal, as was the cellularity of thymus, spleen, and lymph nodes (Table II).

**In vivo leukocyte migration in CD31KO mice**

To investigate the role of CD31 in TEM, we examined leukocyte influx into acute inflammatory sites using three in vivo models: IL-1β- or TG-induced peritonitis, and the mouse air pouch model. Injection of IL-1β or TG into the peritoneal cavity of the mouse results in a rapid influx of inflammatory cells into the area that, at 4 h, consists mainly of PMN. Contrary to previously reported results in which PMN transendothelial migration into sites of acute inflammation in wild-type mice is clearly blocked by anti-CD31 Ab (34–37), influx of PMN into the peritoneal cavities of CD31KO mice injected with 10 ng IL-1β or 1.5 ml TG was identical to that observed in wild-type mice, as assessed by the number of leukocytes in lavage fluid at 4 h postinjection (Table III). The proportion of PMN in these samples was approximately equivalent in WT and CD31KO mice (approximately 90%) after IL-1 (data not shown) or TG (Table III).

To assess the role of CD31 in PMN and mononuclear phagocyte migration at a later time point after inflammatory challenge, mice were injected i.p. with 1.5 ml of 4% TG. Cells collected by lavage 24 h after challenge with TG were counted, and differential counts were performed to determine cell type. As shown in Table III, total cell numbers obtained 24 h after TG were identical in WT and CD31KO mice. Differential counts on recovered cells revealed no significant differences in the cell populations recovered: PMN and mononuclear cells recovered from both WT and CD31KO were present at approximately equivalent percentages of total cells, although as at 4 h, CD31KO mice exhibited a small increase in the percentage of PMN recovered.

The role of CD31 in migration across an alternative site of vascular, the mouse air pouch, was also examined. Proinflammatory stimuli or chemoattractants can be introduced locally into the air pouch, and subsequent cell migration can be assessed by lavage of the resulting infiltrate (38). Injection of IL-1β (39) or nonviable (Formalin-inactivated) Gram-positive bacteria, S. aureus (40), resulted in an influx of largely PMN into the air pouch cavity at 4 h, and of PMN, macrophages, and monocytes after 24 h. CD31KO mice again displayed no defect in leukocyte migration in response to either IL-1β or S. aureus (Table III). The relative influx of the various cell types, as assessed by differential counts of cells recovered from the air pouch, was also essentially the same in WT and CD31KO mice (Table III). In addition, no significant difference was apparent in monocyte, macrophage, PMN, or lymphocyte

<table>
<thead>
<tr>
<th>Table I. Circulating blood cell numbers and composition before and after TG challenge in CD31KO mice&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td><strong>Total WBC</strong> (&lt;sup&gt;x&lt;/sup&gt;10&lt;sup&gt;9&lt;/sup&gt;/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>PBL PRE-TG (n = 17)</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>CD31KO</td>
</tr>
<tr>
<td>PBL POST-TG (n = 12)</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>CD31KO</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total WBC numbers and percentages of circulating leukocytes before (PRE-TG) and 4 h after (POST-TG) i.p. injection with 1.5 ml 4% TG. Cell numbers were determined by direct counting using a hemocytometer. Percentages are calculated from forward/side scatter profiles obtained by FACS analysis of blood from tail vein.

<sup>b</sup> Percent neutrophils was also calculated from percent Gr-1<sup>+</sup> cells in Mac-1/Gr-1 double-stained samples.

<sup>a</sup> p < 0.05, ** p < 0.005 for CD31KO vs WT, (Student’s t test). All other comparisons not statistically significant (p > 0.05).

<table>
<thead>
<tr>
<th>Table II. Circulating RBC and platelet count and cellularity of lymphoid organs in CD31KO mice&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td><strong>RBC (&lt;sup&gt;x&lt;/sup&gt;10&lt;sup&gt;9&lt;/sup&gt;/mm&lt;sup&gt;3&lt;/sup&gt;)</strong></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>CD31KO</td>
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</tbody>
</table>

<sup>a</sup> Circulating blood cell counts were performed using a Sysmex F-800 semi-automated hematological analyzer. Total leukocyte numbers in unfractionated spleen and thymus are quoted. Lymph node (LN) cellularity is based on single popliteal LN from mice. All data are mean ± SEM for five mice in each group.
migration between WT and CD31KO mice in response to macrophage inflammatory protein-1α (41), demonstrating that C-C chemokine-mediated TEM was normal in the absence of CD31 (data not shown).

Interestingly, despite the recovery of equivalent numbers of leukocytes from the peritoneal cavities of WT and CD31KO mice injected i.p. with 10 ng IL-1β, electron-microscopic examination of the mesenteric postcapillary venules of these mice revealed trapping of PMN at the perivascular BM in CD31KO mice (Fig. 3, A and B). As shown in Table IV, in CD31KO mice there was a significant decrease in the number of PMN that were situated proximally to the endothelium or contact with the endothelium, and a corresponding significant increase in the number of PMN found between the EC and BM (trapped cells). This phenomenon appears specific to PMN in the case of IL-1β stimulation, the number of monocytes proximal to endothelium or

Table III. In vivo leukocyte migration in CD31KO mice

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Time (h)</th>
<th>Site</th>
<th>n</th>
<th>Cell Recovered (×10⁶)</th>
<th>% PMN</th>
<th>% Mo</th>
<th>% Mø</th>
<th>% Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4</td>
<td>i.p.</td>
<td>WT</td>
<td>5</td>
<td>1.09 ± 0.30</td>
<td>30.30 ± 5.90</td>
<td>19.00 ± 2.90</td>
<td>39.90 ± 4.20</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>i.p.</td>
<td>CD31KO</td>
<td>5</td>
<td>0.75 ± 0.14</td>
<td>23.80 ± 6.30</td>
<td>17.50 ± 1.50</td>
<td>50.50 ± 4.70</td>
</tr>
<tr>
<td>TG</td>
<td>4</td>
<td>i.p.</td>
<td>WT</td>
<td>5</td>
<td>13.23 ± 1.87</td>
<td>85.00 ± 1.79</td>
<td>5.80 ± 1.59</td>
<td>7.80 ± 0.58</td>
</tr>
<tr>
<td>TG</td>
<td>4</td>
<td>i.p.</td>
<td>CD31KO</td>
<td>5</td>
<td>13.14 ± 2.05</td>
<td>94.75 ± 1.31</td>
<td>1.25 ± 0.25</td>
<td>2.50 ± 0.87</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4</td>
<td>i.p.</td>
<td>WT</td>
<td>5</td>
<td>8.69 ± 0.60</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4</td>
<td>i.p.</td>
<td>CD31KO</td>
<td>5</td>
<td>9.44 ± 1.80</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TG</td>
<td>24</td>
<td>i.p.</td>
<td>WT</td>
<td>13</td>
<td>21.23 ± 1.40</td>
<td>51.35 ± 4.48</td>
<td>8.66 ± 0.88</td>
<td>37.89 ± 4.73</td>
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<tr>
<td>TG</td>
<td>24</td>
<td>i.p.</td>
<td>CD31KO</td>
<td>14</td>
<td>21.36 ± 1.64</td>
<td>61.80 ± 2.76</td>
<td>6.08 ± 1.06</td>
<td>28.72 ± 1.84</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4</td>
<td>AP</td>
<td>WT</td>
<td>5</td>
<td>6.31 ± 1.85</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4</td>
<td>AP</td>
<td>CD31KO</td>
<td>5</td>
<td>6.78 ± 0.84</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S. aureus</td>
<td>4</td>
<td>AP</td>
<td>WT</td>
<td>5</td>
<td>3.38 ± 0.71</td>
<td>93.00 ± 1.73</td>
<td>0.00 ± 0.00</td>
<td>5.75 ± 1.90</td>
</tr>
<tr>
<td>S. aureus</td>
<td>4</td>
<td>AP</td>
<td>CD31KO</td>
<td>5</td>
<td>4.78 ± 0.69</td>
<td>94.80 ± 1.80</td>
<td>1.6 ± 0.87</td>
<td>3.40 ± 1.70</td>
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<tr>
<td>S. aureus</td>
<td>24</td>
<td>AP</td>
<td>WT</td>
<td>15</td>
<td>4.14 ± 0.51</td>
<td>73.90 ± 2.70</td>
<td>6.52 ± 0.79</td>
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<tr>
<td>S. aureus</td>
<td>24</td>
<td>AP</td>
<td>CD31KO</td>
<td>15</td>
<td>3.64 ± 0.45</td>
<td>68.72 ± 5.40</td>
<td>8.37 ± 2.29</td>
<td>14.11 ± 2.24</td>
</tr>
</tbody>
</table>

*Mean ± SEM of total cell numbers recovered from either the peritoneal cavity (i.p.) or the air pouch (AP) of WT or CD31KO mice at times indicated after injection of stimuli are quoted. Mean ± SEM percentages of neutrophils (PMN), monocytes (Mo), macrophages (Mø), or lymphocytes were determined from cytospin preparations of lavage samples. When n > 10, means are derived from three separate experiments; when n = 5, means are from one experiment. ND, not determined.

FIGURE 3. PMN appear trapped at the BM of mesenteric postcapillary venules of CD31KO mice, but not WT mice. A, A representative electron micrograph of leukocytes migrating into the peritoneal cavity of CD31KO mice 4 h after i.p. injection of IL-1β, showing four PMN trapped between the EC and BM of the vessel wall. Arrows indicate vascular endothelium; size bar = 5 μm. B, Higher magnification micrograph of another PMN in the process of TEM, situated between the EC and BM of the vessel. Arrows again indicate vascular endothelium; size bar = 2 μm. C and E, Light micrographs of WT and CD31KO postcapillary venules, respectively, obtained from mice 4 h post-TG injection; scale bar = 25 μm. D and F, Electron micrographs from the same samples, WT and CD31KO, respectively, showing leukocyte accumulation between EC and BM in the mutant CD31KO postcapillary venules. Scale bar = 5 μm.
trapped at the BM being equivalent in WT and CD31KO mice. The accumulation of leukocytes at the BM of CD31KO mice was also clearly apparent after i.p. injection of TG (Fig. 3, E and F), with large numbers of leukocytes visible between EC and the BM in all five CD31KO mice observed. None of the five WT mice tested displayed this phenomenon. Thus, CD31 is required for the normal migration of PMN, but apparently not monocytes, across the BM at an early time point after an inflammatory stimulus.

**In vitro leukocyte migration in CD31KO mice**

Despite slightly lower numbers of circulating PMN after TG injection in CD31KO mice (Table I), and the observed accumulation of PMN at the BM, similar numbers of PMN were recovered from peritoneal lavage 4 h post-TG in both WT and CD31KO (Table III). A possible explanation for this phenomenon could be that CD31KO PMN have a higher rate of migration than their WT counterparts. Augmented rate of migration across EC could also conceivably manifest itself as an accumulation of PMN at the BM.

In an attempt to elucidate the mechanism underlying the accumulation of PMN at the BM in CD31KO mice, PMN migration in vitro across bEND3 cells in response to IL-8 was investigated. As shown in Fig. 4, CD31-deficient PMN migrate across bEND3 cells to the same extent, and with the same kinetics, as WT PMN, suggesting that an increased rate of TEM is not an explanation for our findings.

**T lymphocyte trafficking in CD31KO mice**

Given the expression of CD31 on approximately 50% of human lymphocytes (7) and on almost all circulating lymphocytes in the peripheral blood of mice (Fig. 2A), it was of interest to assess the ability of T lymphocytes to undergo transendothelial migration in the absence of CD31. Two in vivo models were used: 1) oxazalone-mediated contact sensitivity, which in the mouse involves both mononuclear and PMN infiltration, and 2) the homing of labeled donor lymphocytes to lymphoid organs of recipient mice.

Leukocyte influx after oxazalone sensitization and subsequent challenge was assessed in WT, CD31KO, and LFA-1-deficient (42) mice. After topical oxazalone treatment, ear thickness in WT mice was increased postchallenge by approximately 100% over that of the control, vehicle-treated ear (Fig. 5A). As a positive control, ear swelling was not apparent in LFA-1-deficient mice, demonstrating that CD11a-mediated cell-cell adhesion and/or activation is essential for the mounting of a normal cutaneous hypersensitivity response. In contrast, on challenge with oxazalone, CD31KO mice exhibited an identical response to that of WT mice, indicating no role for CD31 either in the priming or effector phase of the cutaneous hypersensitivity response.

To address the role of CD31 in tissue-specific lymphocyte homing, mesenteric and peripheral lymph node cells from WT mice were labeled with Na$_2$CrO$_4$ and injected i.v. into both WT and CD31KO mice. Quantification of radiolabel present in various lymphoid organs of the recipient mice showed no difference in tissue-specific homing of WT cells between WT and CD31KO recipient mice. This finding was observed at both 2 h (Fig. 5B) and 24 h (Fig. 5C) postinjection of labeled donor WT cells, and clearly shows that CD31 present on the EC of the lymphoid organ vasculature of the recipient is not required for the homing of lymphocytes to lymphoid organs. Homing of CD31KO and WT lymph node cells in WT mice was also examined, and at 2 h postinjection, no difference in counts was found in WT recipients that had received WT or CD31KO donor cells (data not shown), implying that leukocyte CD31 has no role in the homing of resting T lymphocytes.

**Platelet function in CD31KO mice**

In addition to leukocytes and EC, CD31 is also expressed on the surface of platelets. To examine the potential role of CD31 in platelet-platelet interaction, we assessed platelet aggregation in vitro in response to varying doses of ADP, a potent proaggregatory agent. The aggregation response to ADP was identical in both wild-type and CD31KO mice, with half-maximal responses occurring at essentially the same concentration of ADP (Fig. 6). These results suggest that platelet CD31 is not critically involved in the
Discussion

The complex interaction of EC, both with other EC and with components of the extracellular matrix, is a common feature of vasculogenesis and angiogenesis (43, 44). The expression of CD31 on all continuous EC linings in vivo (18), and the original observation that an anti-CD31 Ab blocked initiation of EC-EC contact (8), suggested an important role for CD31 in these processes. Although the formation of EC tubes in vitro and in a murine model of angiogenesis was disrupted in the presence of anti-CD31 Ab (20, 45), another study reported that capillary tube formation in vitro and wound healing in a SCID mouse model were disrupted only when an anti-CD31 Ab and an anti-vascular endothelial (VE) cadherin Ab were present simultaneously (46). Differential tyrosine phosphorylation of CD31 also correlated with angioblast differentiation during the formation of blood islands and vessels in the murine conceptus (21), and with the induction of an EC migratory phenotype (47), and transfection with full-length PECAM resulted in the promotion of cell-cell adhesion and the loss of migratory ability in fibroblasts (48). However, the normal development and fertility of the CD31KO mice reported in this study clearly argue against an essential requirement for CD31 in the processes of vasculogenesis and angiogenesis in the mouse, and imply that the molecule is functionally redundant in this respect.

It has been proposed that CD31 may play a role in hemopoiesis, either through the formation of a stromal cell/extracellular matrix network, in the commitment of multipotential progenitor cells to myeloid and lymphoid lineages, or through the exit of hemopoietic cells via the bone marrow sinus endothelia into the circulation (7). There have been, however, no published reports to substantiate these hypotheses, and the essentially normal number and composition of hemopoietic cells found in the CD31KO mouse suggest that CD31 is not crucial for the normal production of peripheral blood cells.

Large numbers of monocytes and PMN migrate from the peripheral blood into tissues in response to inflammatory stimuli. The final stage in the emigration of leukocytes into tissues involves the movement of leukocytes across EC and their subsequent passage through the BM into the subendothelial tissues (1). This step has been shown to be mediated, at least in part, by CD31 (16), and many studies, both in vitro and in vivo, have demonstrated the ability of anti-CD31 Ab to block leukocyte migration (3, 34–37, 49). Given the abundant number of reports detailing the importance of CD31 in the TEM of monocytes and PMN, the testing of this observation in the CD31KO mouse was a primary objective. Surprisingly, no significant differences in leukocyte TEM were detected between CD31KO and WT mice. Total numbers of cells recovered and the cellular composition of the lavage were similar. Monocytes and lymphocytes underwent TEM in essentially equal numbers in both CD31KO and WT mice independent of the stimulus used, organ systems studied, or time point of observation. It should be noted that, in none of the cited studies demonstrating a role for CD31 in leukocyte emigration was TEM blocked completely, suggesting that CD31-independent pathways normally

![Figure 5](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

**FIGURE 5.** T lymphocyte migration is normal in CD31KO mice. A, Ear thickness in WT, CD31KO, and LFA-1KO mice after oxazalone sensitization and subsequent local challenge. Data shown as percentage of increase in thickness of the treated ear over that of the control, untreated ear. Data are shown as mean ± SD for five mice per group in a single experiment, representative of two others. B and C, Lymphocyte trafficking in WT (solid bars) and CD31KO (open bars) mice. 51Cr-labeled WT lymph node cells were injected i.v. into WT and CD31KO recipients, and lymphoid organs were removed at 2 h (B) or 24 h (C) for quantification of label. Results are shown as mean ± SD of three mice per group, representative of two independent experiments. PLN, peripheral lymph nodes; MLN, mesenteric lymph nodes; PP, Peyer’s patches.

![Figure 6](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

**FIGURE 6.** ADP induced in vitro platelet aggregation in CD31-deficient platelets is normal. PRP prepared from the pooled blood of five WT and five CD31KO mice was used as a source of platelets, resuspended at 1 × 10^5 platelets/ml in autologous PPP as diluent. Addition of ADP resulted in equal half-maximal responses in both WT and CD31-deficient platelets. Open symbols represent WT platelets; closed symbols represent CD31KO platelets. Data are shown as mean ± SD of four experiments in which five mice of each genotype were pooled for each individual experiment.
exist, although they are quantitatively less important. These CD31-independent pathways are apparently sufficient to support acute inflammation in mice that have developed in the absence of CD31. Interestingly, although similar numbers of cells were recovered from the peritoneal cavities of both CD31KO and WT mice after i.p. injection of IL-1β or TG, electron-microscopic examination of the postcapillary venules in mesentery taken from treated animals revealed a disparity. Only in the CD31KO samples were leukocytes routinely visible between the EC lining, the lumen of the vessels, and the perivascular BM. Selecting only leukocytes whose nuclei were present in the field of view (which could therefore be classified as either PMN or monocytes), this accumulation of leukocytes was found to be specific to PMN 4 h after IL-1β injection.

Liao et al. (49) showed that mAbs to domain 6 of human CD31, which have no effect on TEM, block migration of monocytes across the subendothelial basal lamina. A parallel to this finding was demonstrated in vivo: administration of an anti-CD31 Ab inhibited extravasation of leukocytes in rat mesenteric microvessels 4 h after IL-1β treatment (50). The mechanism underlying this phenomenon remains unclear, although proteoglycans may interact in some indirect way with domain 6 of leukocyte CD31 (49), with subsequent increases in adhesive interactions and/or proteolytic degradation of the BM components (50). Outside-in signaling involving CD31 has been demonstrated in a number of systems; amplification of integrin-mediated adhesion on CD31 ligation has been shown in human PMN, monocytes, T cell subsets, murine lymphokine-activated killer cells, and human NK cells (22–28). The trapping of PMN at the BM in the CD31KO mouse suggests that CD31 is indeed involved in trans-BM passage. That an equal number of cells were recovered after peritoneal lavage from both WT and CD31KO mice suggested to us that CD31 may affect the rate of passage (while not affecting the overall outcome) of the migration process, via a decrease in the rate of passage across the BM or, possibly, an increase in the rate of passage across the EC. Our study of in vitro migration of CD31-deficient PMN over a period of 4 h in response to a chemotactic gradient of IL-8 would appear to argue against an increased rate of passage across EC as being the mechanism behind the observed accumulation of PMN at the BM. It must be pointed out, however, that the in vitro system used does not fully mimic the in vivo situation, in that bEND3 cells express CD31, unlike the CD31KO EC. The accumulation of PMN at the BM could be explained by a reduction in the ability of PMN to pass through this subendothelial barrier in the absence of CD31, for reasons yet to be elucidated, but possibly related to an absence of CD31-mediated activatory signals. The equal cell counts recovered in this study suggest that if there is a retardation of passage across BM in CD31KO mice, then it must be transient and subtle, and could be compensated for by an increased ability of CD31-deficient PMN to migrate through the interstitium. This hypothesis would reconcile the disparate findings of equal numbers of cells recovered from lavage fluid at 4 h postinflammatory stimulus and of PMN accumulation at the BM in CD31KO mice. In summary, our in vitro findings in the CD31KO mouse indicate that the role of CD31 in migration across the basal lamina is most likely independent of any role for CD31 in migration across the intercellular junctions of endothelium.

The apparent importance of CD31 in the modulation of lymphocyte function (25, 26, 28), and the expression of CD31 on almost all murine PBL (Fig. 2A) suggested that CD31 may be involved in lymphocyte migration. However, in an oxazalone-mediated model of contact sensitivity, CD31KO mice did not display the reduction in ear swelling expected if the absence of CD31 were to affect either the priming or effector phase of this response. Similarly, specific homing of radiolabeled WT or CD31KO lymph node cells to lymphoid organs in a WT host was equivalent. Neither was the homing of labeled CD31KO cells injected into WT recipient mice impaired. CD31 does not therefore appear to play a role in T lymphocyte migration.

CD31 is also highly expressed on the surface of platelets, although its role here remains unclear. Differential CD31 phosphorylation has been reported in nonaggregated and aggregated platelets (51, 52), and the protein tyrosine phosphatase SHP-2 has been shown to interact with CD31 during platelet aggregation (29). In addition, it has recently been reported that CD31 can act as a costimulatory molecule for integrin-mediated adhesion and aggregation in platelets (30). In vivo, CD31 has been implicated in platelet aggregation in a model of EC injury in mouse cerebral arterioles in which platelet aggregation is dependent on EC injury without exposure of basal lamina (31, 32). In these studies, treatment with two independent anti-CD31 mAbs doubled the time required for platelet aggregates to form after EC damage. The finding in this study that ADP-induced in vitro aggregation of CD31KO platelets is normal is in agreement with an ex vivo study on the effect of anti-CD31 Ab on platelet aggregation in response to ADP and arachidonate (32). Taken together, these data suggest that CD31 expression on platelets is not essential for platelet-platelet aggregation, but that EC injury may expose or activate CD31 on EC, resulting in augmentation of aggregate formation. The relative roles of EC and platelet CD31 in hemostasis therefore remain unknown, and are currently under investigation.

There has been a plethora of reports published recently detailing studies demonstrating the importance of CD31 in vascular development and angiogenesis, leukocyte migration, and functional regulation of integrins via outside-in signaling. Based on these reports, a null mutation of the gene encoding CD31 would be expected to result in a variety of phenotypes involving both vascular development and/or transendothelial migration of leukocytes. This study of such a CD31-null mouse provides genetic evidence that CD31 does not have an essential exclusive role in any of these biological processes, and that CD31-deficient mice are able to utilize CD31-independent pathways to sustain leukocyte migration in inflammatory situations. Whether these CD31-independent pathways are mediated through the compensatory induction of known, but normally redundant, adhesion molecules in the KO mouse cannot be fully resolved until these molecules are identified and their functional role examined in both CD31KO and WT mice. Identification of the cell adhesion molecules used by CD31KO mice for TEM may yield valuable information about CD31-independent pathways of TEM in humans and WT animals. The modification of these molecules may provide important adjuncts to antiinflammatory therapies aimed at inhibiting CD31.

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References

Leukocyte transendothelial migration in CD31-deficient mice


