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CD31 Exhibits Multiple Roles in Regulating T Lymphocyte Trafficking In Vivo

Liang Ma,* Kenneth C. P. Cheung,† Madhav Kishore,† Sussan Nourshargh,† Claudio Mauro,† and Federica M. Marelli-Berg‡

The role of CD31, an Ig-like molecule expressed by leukocytes and endothelial cells (ECs), in the regulation of T lymphocyte trafficking remains contentious. Using CD31-deficient mice, we show that CD31 regulates both constitutive and inflammation-induced T cell migration in vivo. Specifically, T cell:EC interactions mediated by CD31 molecules are required for efficient localization of naive T lymphocytes to secondary lymphoid tissue and constitutive recirculation of primed T cells to nonlymphoid tissues. In inflammatory conditions, T cell:EC CD31-mediated interactions facilitate T cell recruitment to Ag-rich sites. However, endothelial CD31 also provides a gate-keeping mechanism to limit the rate of Ag-driven T cell extravasation. This event contributes to the formation of Ag-specific effector T cell infiltrates and is induced by recognition of Ag on the endothelium. In this context, CD31 engagement is required for restoring endothelial continuity, which is temporarily lost upon MHC molecule ligation by migrating cognate T cells. We propose that integrated adhesive and signaling functions of CD31 molecules exert a complex regulation of T cell trafficking, a process that is differentially adapted depending on cell-specific expression, the presence of inflammatory conditions and the molecular mechanism facilitating T cell extravasation. The Journal of Immunology, 2012, 189: 4104–4111.

P latelet/endothelial cell adhesion molecule 1, also known as CD31, is a member of the Ig gene superfamily expressed at high density at the lateral borders of endothelial cells and at a lower density on the surface of hematopoietic cells including T lymphocytes (1). By establishing homophilic interactions between endothelium- and leukocyte-expressed CD31, this molecule has been shown to facilitate leukocyte transendothelial migration (TEM) in vitro and in vivo (1).

In contrast to the relatively large number of studies addressing the role of CD31 in neutrophil and monocyte transmigration, the role of CD31 in lymphocyte trafficking has been largely overlooked and remains contentious. Early studies showed that ligation of CD31 on T cells can generate inside-out signaling that can induce β2 and β1 integrin activation (2, 3). These findings suggested a potential mechanism via which CD31 may mediate interaction of lymphocytes with components of the venular wall during transmigration (4). Additional investigations reported inconsistent findings. An in vitro study analyzing the phenotype of human T lymphocytes migrated through cytokine-treated human endothelial cell monolayers failed to detect CD31+ T cell enrichment in the migrated population, which mostly consisted of memory lymphocytes, concluding that this molecule is not involved in T cell migration (5).

However, more recent in vitro studies using Ab blockade of CD31 have directly implicated the CD31-CD99 axis in T lymphocyte TEM, particularly in human effector memory T cell migration induced by Ag-presenting endothelial cell monolayers (6, 7). A role for CD31 in the regulation of T cell–mediated inflammation has also been indirectly suggested by in vivo studies in CD31-deficient mice. Genetic deletion of CD31 led to enhanced T cell–mediated inflammatory responses and disease severity in experimental autoimmune encephalomyelitis and collagen-induced arthritis models (8, 9). The proinflammatory phenotype observed in these models was attributed to vascular junction loosening in CD31-deficient endothelium (10).

A potential role of CD31 dysfunction in the pathogenesis of T cell–mediated human diseases has only recently been recognized. CD31 polymorphisms affecting its intracellular signaling domains have been associated with increased graft-versus-host disease severity (11–15) and loss of CD31 expression by the endothelium has been observed in vascular walls chronically exposed to turbulent shear stress, which are characteristic sites of atherogenic development (16, 17). The molecular basis of these associations has not been clarified.

In this study, we have investigated a potential role for CD31-mediated interactions in the regulation of T cell trafficking in vivo by separately assessing the effect of CD31 deficiency by either T cells or the endothelium. Our observations are consistent with a complex regulatory role for CD31 on T lymphocyte migration, which is differentially exerted depending on its expression by T cells or the endothelium and the molecular mechanism mediating T cell extravasation.

Materials and Methods

Mice

CD31−/− and wild type (WT) mice were generated as previously described (18) and used at the age of 8–10 wk. Experiments were performed under the Home Office authority (PPL 70/5872).
Reagents

All the Abs used in this study were purchased from BD Biosciences unless specified otherwise. The cell linker PKH26 and CFSE were purchased from Sigma-Aldrich (Gillingham, U.K.). For T cell labeling, PKH26 or CFSE were added at final concentrations of 5 and 1 μM, respectively.

**HY-specific memory T cells**

Memory CD4+ and CD8+ T cells specific for the male-specific minor transplantation Ag HY peptide Dby epitope restricted by H-2A* and for the Uty epitope restricted by H-2D*, respectively, were obtained from WT and CD31-/- mice by two fortnightly i.p. immunizations of female mice with male splenocytes, as described previously (19, 20).

**Recruitment of circulating T cells into tissues**

Labeled T cells (10^6/mouse) were injected i.v. T cells were incubated at 37°C for 30 min and then washed three times with PBS before injection. After 24 h, mice were sacrificed, tissues were sampled and either processed for flow cytometric analysis (lymph nodes and spleen) or embedded in optimal cutting temperature compound (Agar Scientific, Stansted, U.K.), snap-frozen and stored until analysis. Tissue infiltration by T cells was assessed by wide-field fluorescence microscopy. In the peritoneal recruitment models, infiltration models, and labeled T cells were injected i.d. into mice that had received an i.p. injection of IFN-γ (PeproTech, London, U.K.; 600 U/ml) 24 h earlier. Enrichment of labeled T cells in the peritoneal lavage was assessed 24 h later by cyttofluorimetric analysis respectively.

Wide-field fluorescence microscopy and flow cytometry

Frozen tissue sections were laid onto Polysine Microscope slides (VWR International, Lutterworth, U.K.), left to dry overnight, and then mounted in Vectorshield mounting medium for fluorescence with DAPI (Vector Laboratories, Peterborough, U.K.), to visualize the nuclei. Slides were visualized with a Coolview 12-ccd CCD camera (Photonic Science, Newbury, U.K.) mounted over a Zeiss Axiovert S100 microscope equipped with Metamorph software (Zeiss, Welswyn Garden City, U.K.). ×10 and ×20 NA 0.6 objectives and standard epi-illuminating fluorescein and rhodamine fluorescence filter cube were used, and 12-bit image data sets were generated. Tissue infiltration was quantified by randomly selecting 10-10× magnified fields and assessing the number of fluorescent cells in each field, as described previously (20). Quantification of T cell infiltrates observed by wide-field fluorescence microscopy was performed using a specifically designed software to run in the LabView (V7.1, National Instruments) environment (20), which allows identification of single fluorescent cells within the tissue. The number of infiltrating cells obtained (from 10 samples from each animal) was then averaged and assessed statistically. Infiltration is expressed as the mean of fluorescent cells per ×10 field in a given experimental condition ±SD. The presence of labeled cells in the peritoneal lavage was analyzed by flow cytometry using a FACScalibur (Becton Dickinson, Mountain View, CA) and FlowJo (Tree Star, Ashland, OR).

**Endothelial cell culture**

Murine endothelial cells (ECs) purified from female WT and CD31-/- mouse lung tissue were isolated as described previously (21). After recovering, ECs were serially subcultured at 37°C with 5% CO2 in DMEM (Life Technologies, Paisley, U.K.) supplemented with 2 mM glutamine (Life Technologies), 100 μM penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 μM m-HEPES (Life Technologies), 1% nonessential amino acids (Life Technologies), and 50 μM 2-mercaptoethanol (Sigma-Aldrich), with freshly added 20% heat-inactivated FCS (Labtech International, Ringmer, U.K.), and 75 μg/ml EC growth supplement (Sigma-Aldrich) in 2% gelatin-coated (Sigma-Aldrich) tissue culture flasks (BD Biosciences, Oxford, U.K.). At confluence, ECs were detached from the culture flasks using trypsin/EDTA (Life Technologies) and passaged. In all the experiments, ECs were used between passages 3 and 4.

**Measurement of transendothelial resistance**

WT and CD31-/- ECs were treated with 300 U/ml IFN-γ for 48 h to induce MHC molecule expression. EC (5 × 10^5/well) were then seeded onto a 3-μm-pore polycarbonate transwell inserts (diameter 6.5 mm; Costar, High Wycombe, U.K.) in EC medium at 37°C with 5% CO2 for 24 h to form a monolayer. MHC molecule upregulation remained stable for up to 72 h following the removal of IFN-γ. Treatment with IFN-γ did not modify the level of CD31 expression by WT ECs (not shown). Prior to transendothelial resistance (TER) measurements, the well contents were replaced with fresh EC media to remove nonadherent ECs in the upper chamber. TER was measured with an Epithelial Voltohmeter with a chopstick-type electrode (World Precision Instruments, Sarasota, FL) stabilized at 148 ± 2 Ω. In Abscroslinking experiments, the Abs were added at the indicated concentrations immediately after the first TER measurement (time 0).

**Actin cytoskeleton staining**

ECs (10^5) were seeded onto each well of 24-well plates containing glass coverslips (VWR International) coated with 100 μg of 2% gelatin (Sigma-Aldrich). They were incubated overnight at 37°C with 5% CO2 in EC media to form a monolayer. EC monolayers were then fixed with 4% buffered paraformaldehyde (Sigma-Aldrich) for 20 min at 4°C, washed three times with PBS and stained with 1 ng/ml tetramethyl rhodamine B isothiocyanate-conjugated phalloidin (Sigma-Aldrich) for 30 min at 37°C. Coverslips were extensively washed, air dried, and mounted in Vectashield (Vector Laboratories) mounting medium for fluorescence with DAPI (Vector Laboratories) on glass slides. The slides were analyzed with wide-field fluorescence microscopy.

**Quantification of stress fibers**

Quantification of stress fibers was performed as described previously (22). ImageJ software was used to generate line profiles. A graphic depiction was then generated where the x-axis represented the distance across the cell, the y-axis represented the level of fluorescence, and each immunofluorescence intensity spike represented an individual stress fiber crossed by the line. To distinguish the true stress fibers from the background, we also drew several lines outside the cells and determined the intensities on the lines. The fluorescence level of 100 was set as the cutoff because the fluorescence intensity (FI) outside the cells was never greater than this value. We randomly selected six cells and three regions in each cell for quantification. The FI was classified into two levels: low intensity (FI < 2000) and high intensity (FI ≥ 2000). The number of stress fibers at high intensity was quantified.

**Statistical analysis**

The results are expressed as means ± SD. Each experiment was repeated at least three times. Significant differences between multiple groups were identified by one-way ANOVA, followed by Newman-Keuls multiple comparison test. Whenever two groups were compared, Student unpaired t tests were performed. A p value <0.05 was regarded as significant.

**Results**

**CD31-deficient naive T cells display defective homing to secondary lymphoid tissue**

In physiologic conditions, naïve T lymphocytes continuously recirculate through secondary lymphoid tissue (23). Although CD31 is expressed on both high endothelial venules in the lymph nodes (24) and sinusoids in the spleen (25), the contribution of this molecule to naïve T cell migration to secondary lymphoid tissue has not yet been investigated. We therefore sought to assess the effect of CD31 deficiency on the ability of adoptively transferred naïve T cells to migrate to lymph nodes and spleen. Naïve T cells were isolated from lymph nodes obtained from CD31-/- mice and WT littermates based on CD44 expression. Phenotypic characterization of WT and CD31-/- T cells did not reveal any significant differences in the array of molecules analyzed, including CCR7 and CD62L as well as other adhesion and chemokine receptors, thus excluding potential indirect effects owing to different expression of secondary lymphoid organ-selective homing receptors (Supplemental Fig. 1A). T cells were then labeled with PKH26 and injected i.v. (10^6/mouse) into syngeneic WT recipients. Localization of labeled T cells to lymph nodes (LN) and spleen was assessed by flow cytometry 24 h later. As shown in Fig. 1, CD31-/- CD4+ (Fig. 1A, 1B) and CD8+ (Fig. 1C, 1D) naïve T cells localization to both LNs and spleen was partially but significantly impaired, suggesting that CD31-mediated interactions contribute to naïve T cell homing to secondary lymphoid tissue.

To assess the functional effects of reduced migration of CD31-/- T cells in the secondary lymphoid organs, naïve CD31-/- T cells derived from female mice were labeled with CFSE and injected into WT male recipients (i.e., to induce activation by recognition
of the male Ag HY (19, 20). T cell localization and division were assessed 72 h later by flow cytometry. As it is shown in Fig. 1E, although significantly fewer CD31^{2/2} T cells accessed the lymph nodes, they underwent a higher number of divisions likely because of a lack of CD31-mediated interactions with resident Ag-presenting dendritic cells, as we have previously described (26), leading to similar numbers of activated T cells compared with their WT counterpart.

CD31 facilitates constitutive trafficking by memory T cells

It has been suggested that lack of CD31 interactions as a consequence of genetic deletion might lead to loosening of the endothelial barrier and unregulated migration of primed T cells to sites of autoimmune inflammation (10). However, the absence of spontaneous T cell inflammation and signs of endothelial leakage in CD31^{2/2} mice (18) suggests that other mechanisms might be in place which underlay the enhanced T cell infiltration in these models. We therefore sought to study the contribution of CD31-mediated interactions on effector memory T cell trafficking.

Effector memory HY (male Ag)-specific T cells (Supplemental Fig. 1B) were generated by immunization of female WT and CD31^{2/2} mice with syngeneic WT male splenocytes, as previously described (26).

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** CD31 promotes naive T cell migration to secondary lymphoid tissue. Naive T cells (CD44^{high}) isolated by cell sorting from lymph nodes of C57BL/6 mice were labeled with PKH26 and injected intravenously (10^7) into WT and CD31^{2/2} mice. The localization of CD4^{+} (A, B) and CD8^{+} (C, D) PKH26-labeled T cells in LN and spleen harvested 24 h later was assessed by flow cytometry. (B) and (D) represent cumulative data from at least three recipients (*p < 0.001). (E) Naive CD31^{2/2} T cells derived from female mice (10^7) were labeled with CFSE and injected into WT male recipients. T cell localization and division were assessed 72 h later by flow cytometry. The graph on the right side summarizes cumulative data from at least six recipients (*p < 0.001).

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** CD31 is required for constitutive memory T cell trafficking. (A) HY-specific H2-A^b-restricted WT (PKH26-labeled) and CD31^{2/2} T cells (CFSE-labeled) were coinjected i.v. into syngeneic female mice. (B) PKH26-labeled HY-specific H2-A^b-restricted WT T cells were injected i.v. into either WT or CD31^{2/2} syngeneic female recipients. T cell localization in the indicated tissues was assessed 24 h later by wide-field fluorescence microscopy and software-based automatic cell counting (20). To minimize the effect of arbitrary choice of field, tissue infiltration was quantified by randomly selecting ten ×10-magnified fields from tissue samples from at least three animals and assessing the number of fluorescent cells in each field. Each panel shows the average number of cells detected in 10 sections from at least four recipients. The mean T cell infiltration ± SD observed is shown (*p < 0.05).
panels transferred i.v. into CD31 population from cumulative data from at least four animals (mean from cumulative data from at least four animals (mean

played significantly impaired ability to access nonlymphoid, pared with their WT counterpart (Fig. 2A) CD31

memory T cells (10^7/mouse), and T cell localization to several

female recipients treated i.p. with 600 U IFN-

Selective loss of endothelial CD31 leads to enhanced Ag-driven T cell migration. PKH26-labeled WT HY-specific T cells (10^7/mouse) were

FIGURE 4. T cell–expressed CD31 enhances memory T cell access to Ag-rich tissue

Previous work has suggested that human T cell migration in response to Ag presentation by the endothelium is supported by CD31 and CD99 engagement (6, 7). To investigate the contribution of CD31-mediated interactions to memory T cell recruitment to antigenic sites in inflammatory conditions, we first compared the recruitment of HY-specific CD4+ WT and CD31−/− T cells from the circulation into the peritoneal cavity of male (antigenic) and female (nonantigenic) WT mice. To induce local upregulation of MHC molecules and Ag presentation, mice received an i.p. injection of IFN-γ (600 U) 48 h prior to adoptive transfer (19, 20). In this model, localization of HY-specific T cells is dependent on TCR triggering by the endothelium, as HY-specific T cells selectively localize in the peritoneal cavity of IFN-γ-treated male, but not female mice (19). PKH26-labeled WT or CD31−/− HY-specific T cells were injected i.v. (10^7/mouse) into male or female WT mice and their recruitment to the peritoneal cavity was assessed 24 h later. Significant amounts of HY-specific WT CD4+ (Fig. 3A) and CD8+ (Fig. 3B) T cells were recruited in the peritoneal lavage of WT male recipient. Migration of HY-specific CD31−/− T cells to the peritoneal cavity of IFN-γ-treated WT male mice was significantly decreased, although the enhancing effect of Ag recognition was not completely abrogated. As expected, few WT and CD31−/− HY-specific T cells migrated to the peritoneal cavity of IFN-γ-treated female mice. These data suggest that CD31 expression by T cells enhances, but is not required

Constitutive trafficking to nonlymphoid tissues by primed CD31−/− T cells in the absence of inflammatory or antigenic stimuli was first analyzed. WT female mice (nonantigenic) were coinjected with PKH26-labeled WT or CFSE-labeled CD31−/− HY-specific memory T cells (10^6/mouse), and T cell localization to several tissues was assessed 16 h later by wide-field microscopy. Compared with their WT counterpart (Fig. 2A) CD31−/− T cells displayed significantly impaired ability to access nonlymphoid, nonantigenic, noninflamed tissue, with the exception of the gut. Because lack of CD31 expression by EC has been suggested to lead to passive leakage of memory T cells into nonlymphoid tissue (10), we then addressed the effect of selective lack of CD31 expression by EC on constitutive T cell trafficking. HY-specific PKH-26-labeled WT T cells were injected i.v. (10^7/mouse) into WT or CD31−/− female recipients. Unexpectedly, migration of WT T cells into nonlymphoid tissue of CD31−/− recipients was also severely impaired (Fig. 2B).

These data suggest that CD31-mediated interactions between T cells and the endothelium are required for constitutive trafficking of effector memory T cells into nonlymphoid tissue.

FIGURE 3. CD31 regulates specific T cell localization into antigenic inflammatory sites. (A and B) WT male and female mice received 600 U IFN-γ or PBS i.p. Two days later, mice received an i.v. injection of PKH26-labeled HY-specific WT or CD31−/− T cells (10^7/mouse). The presence of CD4+ (A) and CD8+ (B) labeled T cells in the peritoneal cavity was assessed 16 h later by flow cytometry. Because of the presence of an autofluorescent population of non-T cells often detected in FL-2 (also in control mice that received IFN-γ but no T cells; data not shown) (49) cells were double-stained with APC-conjugated anti-CD4 and anti-CD8 (or anti-CD3 in some experiments) Abs after harvesting. The percentage of PKH26 (FL-2)–labeled T cells gated in the CD4+ or CD8 T cell population from cumulative data from at least three animals (mean ± SD) are shown in the left panels, and representative dot plots are depicted on the right panels. *p < 0.03, **p < 0.01, significant versus female mice.

HY-specific WT T cells into CD31−/− recipients

FIGURE 4. Selective loss of endothelial CD31 leads to enhanced Ag-driven T cell migration. PKH26-labeled WT HY-specific T cells (10^7/mouse) were transferred i.v. into CD31−/− male or female recipients treated i.p. with 600 U IFN-γ 48 h earlier. The presence of CD4+ (A) and CD8+ (B) labeled T cells in the peritoneal cavity was assessed 16 h later by flow cytometry. The percentage of PKH26 (FL-2)–labeled T cells gated in the CD4+ or CD8 T cell population from cumulative data from at least four animals (mean ± SD) are shown in the right panels, and representative dot plots are depicted in the left panels. *p < 0.05, **p < 0.01, significant versus female mice. (C) Migration of HY-specific WT or CD31−/− T cells into the peritoneal cavity of CD31−/− female recipients treated i.p. with 600 U IFN-γ 48 h earlier. The percentage of PKH26 (FL-2)–labeled T cells gated in the CD4+ or CD8 T cell population from cumulative data from at least four animals (mean ± SD) are shown. *p < 0.05, significant versus CD31−/− recipients.
for migration of specific effector CD4+ and CD8+ T cells to nonlymphoid Ag-rich tissue.

Endothelial CD31 regulates the size of T cell extravasation to inflammatory sites in vivo

To selectively assess the relative contribution of endothelial-expressed CD31 to Ag-driven T cell recruitment, the ability of HY-specific WT T cells to migrate from the circulation into the peritoneal cavity of IFN-γ-treated male and female WT and CD31−/− mice was compared. PKH26-labeled HY-specific WT T cells were injected i.v. (10⁷/mouse) into male or female CD31−/− mice that had received an i.p. injection of IFN-γ (600 U) 48 h earlier. The localization of HY-specific T cells to the peritoneal cavity of male CD31−/− recipients was significantly enhanced compared with that observed in WT male mice (Fig. 4A, 4B). In contrast, few HY-specific T cells localized in the peritoneal cavity of either WT or CD31−/− female recipients, suggesting that Ag-dependent recruitment is still operational in the absence of endothelial CD31.

Further analysis revealed that the amount of HY-specific T cells retrieved in the peritoneal cavity of CD31−/− female recipients (i.e., Ag-independent), although small, was decreased compared with those retrieved in WT recipients, suggesting that enhanced extravasation as a consequence of loss of endothelial CD31 selectively occurs during Ag-induced migration (Fig. 4C).

Similar observations were made when CD31-deficient T cells were injected into CD31−/− recipients (Supplemental Fig. 2), suggesting that the effect of CD31 loss by the endothelium is dominant over the lack of CD31 expression by T cells.

Mechanisms of endothelial CD31-mediated regulation of Ag-specific T cell recruitment

Our finding that loss of CD31 expression by the endothelium leads to enhanced Ag-specific memory T cell extravasation is difficult to reconcile with the general inhibitory effect of lack of T cell:EC interactions occurring on both constitutive and inflammation-induced trafficking by a loss of endothelial integrity.

In addition, previous studies of leukocyte migration through CD31−/− endothelium in inflammatory conditions did not reveal enhanced extravasation as a result of lack of endothelial CD31 expression (18–27). We reasoned that the increased T cell migration through CD31-deficient endothelium following cognate Ag recognition must therefore be related to specific molecular interactions occurring between Ag-presenting endothelium and migrating Ag-specific T cells, i.e., triggering of endothelial MHC-peptide complexes. To address this issue, we investigated whether changes in endothelial permeability (measured as electrical resistance) were induced by MHC class I molecule triggering in the presence or absence of CD31 coengagement.

First, electrical resistance through IFN-γ-treated WT and CD31−/− EC monolayers following Ab-ligation of MHC class I molecules was measured and compared over time. As it is shown in Fig. 5A, MHC molecule triggering led to a quick and similar reduction in resistance (i.e., increased permeability) by both WT

**FIGURE 5.** CD31 interactions promote the recovery of endothelial integrity following increased permeability induced by MHC molecule triggering. (A) WT and CD31−/− ECs (6 × 10⁴/well) previously treated with 300 U/ml IFN-γ for 48 h were grown to form a monolayer on a 3-μm-pore polycarbonate transwell inserts (diameter 6.5 mm; Costar) in EC media at 37°C with 5% CO2. Prior to TER measurements, the well contents were replaced with fresh EC media to remove non-adherent ECs in the upper chamber. In some wells, EC were stimulated with 1 μg/ml mouse–anti-mouse H-2Ld/H-2Db (MHC class I, BD Biosciences) or isotype control and 0.5 μg/ml rat–anti-mouse IgG as secondary cross-linking Ab. (B) Cell death was evaluated microscopically by Trypan blue staining. To normalize for differences from cultures from three different experiments of similar design, data are expressed as the ratio of death between controls and those treated with ICAM ligation (rat–anti-mouse CD54 5 μg/ml plus goat–anti-rat Ig 2.5 μg/ml; BioLegend). TER was measured with a trans-epithelial voltometer stabilized at 148 ± 12 Ω at the indicated time points. *p < 0.05, **p < 0.01.
and CD31−/− endothelium. However, while resistance of the WT endothelium returned to baseline levels within 6 h, resistance of the CD31-deficient endothelium remained significantly higher for up to 24 h after MHC stimulation. MHC triggering did not induce EC death (Fig. 5B); therefore, the increased endothelial permeability was unlikely to be due to cell loss.

To rule out that a difference in cultured WT and CD31 EC was responsible for this effect, experiments were performed in which MHC molecule triggering was performed in the presence or absence of CD31 coligation in IFN-γ–treated WT EC monolayers.

As it is shown in Fig. 5C, coligation of CD31 molecules led to a faster recovery of endothelial resistance, suggesting that CD31 signals contribute to reestablishing endothelial integrity induced by MHC molecule engagement. Importantly, resistance of endothelial monolayers was not affected by ICAM-1 Ab-mediated stimulation either in the presence or absence of CD31 coligation (Fig. 5D), suggesting that this effect is specific to MHC molecule signaling (i.e., to migrating T cells).

As endothelial contractility leading to decreased endothelial resistance is associated with cytoskeletal rearrangements, particularly with F-actin polymerization and stress fiber formation (28), we further analyzed EC cytoskeletal rearrangements following MHC triggering with or without CD31 ligation. As it is shown in Fig. 6A, MHC triggering induced formation of large bundles of F-actin stress fibers. Stress fibers are contractile actin filaments that are typically associated at both their ends to focal adhesions. CD31 coligation led to increased interendothelial cell adhesion areas in which the stress fibers converged in large bundles of F-actin. This is reflected by the increased number of stress fibers measured in the EC that underwent coligation of MHC and CD31 molecules (Fig. 6B). Overall, these data suggest that CD31-mediated signals serve to reestablish endothelial integrity following cognate recognition by migrating T cells.

**Discussion**

The present study provides direct evidence of a nonredundant role for CD31-mediated interactions in the regulation of T cell trafficking. CD31 interactions between migrating T cells and the endothelium and between apposing endothelial cells both participate to facilitating and coordinating T cell extravasation, and appear to involve both CD31 adhesive and signaling functions.

First, our data highlight a previously unknown direct role of CD31 in promoting naive T cell access to secondary lymphoid tissue. This effect might have been masked in mice constitutively lacking CD31 expression, which display normal colonization of lymphoid tissue, by compensatory mechanisms (18–29).
by the redundant and complex network of molecules involved in lymphocyte TEM (30). In addition, enhanced Ag-induced T cell division consequent to the loss of CD31-mediated interactions with dendritic cells (26) might compensate for the reduced entry in the lymph nodes. In CD31-competent mice, blockade of CD31 interactions, which does not allow for compensatory mechanisms to take place, might reduce naive T cell access to lymphoid tissue and subsequent priming, thus explaining the attenuation of T cell–mediated autoimmunity (31–35).

In line with these studies, we show that effector T cell recirculation in steady state and T cell extravasation to Ag-rich sites are also impaired by the loss of CD31 homophilic interactions between T cells and the endothelium, suggesting that these interactions facilitate memory T cell recirculation and targeted localization, as previously suggested by in vitro studies (7).

However, selective lack of CD31 expression by the endothelium results in enhanced Ag-specific memory T cell extravasation in inflammatory conditions, as observed in a number of studies performed in CD31 mice (8, 10, 36) in which loss of CD31 also affected the endothelium. Increased T cell extravasation only becomes apparent during migration of Ag-specific T cells through Ag-presenting endothelium.

MHC triggering is known to deliver a number of signals to the endothelium. Engagement of MHC class I on the surface of EC was shown to lead to ERK activation through an mTORC2-dependent pathway (37). Similarly, exposure of EC to anti-MHC class I Abs promoted proliferation through the mTOR pathway (38). MHC class II engagement in brain endothelial cells was shown to be directly coupled to IL-6 production via a CAMP/PKA-dependent intracellular pathway (39, 40).

In this study, we show that MHC ligation also induces stress fiber formation, leading to endothelial contractility and a transient increase in endothelial permeability. In line with our findings, MHC class I engagement on EC was previously shown to induce a rapid translocation of RhoA to the cell membrane associated with F-actin stress fiber formation and cytoktes (41).

Our observations are consistent with the possibility that in this context, CD31 is required to rapidly reestablish endothelial continuity, which is transiently compromised by endothelial MHC molecule engagement by migrating Ag-specific T cells.

CD31 signals are known to facilitate VE-cadherin complex anchorage by preventing β catenin phosphorylation and degradation, thus supporting EC junction stability (42). Other junctional molecules have been proposed to interfere with increased endothelial permeability induced by inflammatory mediators. For example, the junction molecule JAM-C has been shown to regulate endothelial contractility and VE-cadherin–mediated interendothelial adhesion (43) in response to histamine and VEGF stimulation.

We also report that stress fiber assembly is induced by MHC, but not ICAM-1 molecule triggering, suggesting that this mechanism may specifically support transmigration of Ag-specific T cells into the tissue following cognate recognition of the endothelium.

This effect, which has been described in both the murine and human systems (19, 44–47) has been shown to sustain the development of specific T cell infiltrates and pathology in a number of experimental models of disease (47, 48), including experimental autoimmune encephalomyelitis (in which severity is increased by CD31 deficiency) (10). Cognate recognition of the endothelium selectively enhances T cell TEM (19, 47), a process in which CD31 interactions are integrated. We propose that in this instance, interendothelial CD31 interactions serve to facilitate the reestablishment of endothelial continuity following lymphocyte TEM.

Overall, our data are consistent with a complex and nonredundant role of CD31 in the regulation of the anatomy of immune responses, mediating either promigratory or antimigratory effects dictated by specific cellular expression and the mechanism of T cell extravasation. Together with its function as a regulator of T cell activation and survival (26), the ability of CD31 to regulate T cell trafficking identifies this molecule as a key player in the dynamic tuning of both ensuing and established immunity as well as a potential target for therapeutic intervention.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Phenotype of WT and CD31-/- T cells. Expression of the molecules indicated above each set of panels by WT and CD31-/- naïve (panel A) and HY-specific effector T cells (panel B) was assessed by flow cytometry. Staining with an isotype-matched control antibody is indicated by the light grey profiles.
Supplemental Figure 2. Lack of endothelial CD31 enhances antigen-induced T cell migration irrespectively of CD31 expression by migrating T cells. PKH26-labeled CD31-/- HY-specific T cells (10^7/mouse) were transferred i.v. into CD31 -/- male or female recipients treated i.p. with 600U IFN-γ 48 hours earlier. The presence of CD3+ T cells in the peritoneal cavity was assessed 16 hours later by flow cytometry. Representative dot plots are shown in panel a. The percentage of PKH26 (FL-2)-labeled T cells gated in the CD3+ T cell population from cumulative data from at least three animals (mean ± SD) are shown in panel b. *: significative versus female mice (p<0.03).