Ig gene-like molecule CD31 plays a nonredundant role in the regulation of T-cell immunity and tolerance

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Edited† by N. Avron Mitchison, University College London Medical School, London, United Kingdom, and approved October 1, 2010 (received for review August 9, 2010)

CD31 is an Ig-like molecule expressed by leukocytes and endothelial cells with an established role in the regulation of leukocyte trafficking. Despite genetic deletion of CD31 being associated with exacerbation of T-cell-mediated autoimmune, the contribution of this molecule to T-cell responses is largely unknown. Here we report that tumor and allograft rejection are significantly enhanced in CD31-deficient mice, which are also resistant to tolerance induction. We propose that these effects are dependent on an as yet unrecognized role for CD31-mediated homophilic interactions between T cells and antigen-presenting cells (APCs) during priming. We show that loss of CD31 interactions leads to enhanced primary clonal expansion, increased killing capacity, and diminished regulatory functions by T cells. Immunomodulation by CD31 signals correlates with a partial inhibition of proximal T-cell receptor (TCR) signaling, specifically Zap-70 phosphorylation. However, CD31-deficient mice do not develop autoimmunity due to increased T-cell death following activation, and we show that CD31 triggering induces Erk-mediated prosurvival activity in T cells either in conjunction with TCR signaling or autonomously. We conclude that CD31 functions as a nonredundant comodulator of T-cell responses, which specializes in sizing the ensuing immune response by setting the threshold for T-cell activation and tolerance, while preventing memory T-cell death.

CD31, or platelet endothelial cell adhesion molecule-1 (PECAM-1), is a member of the Ig gene superfamily expressed at high density at the lateral borders of endothelial cells (ECs) and at a lower density on the surface of hematopoietic cells including T lymphocytes (1). By establishing homophilic interactions between apposing ECs and between the endothelium and migrating leukocytes, CD31 has been shown to contribute to EC: EC junctions and promote leukocyte transendothelial cell migration (TEM) (1).

A number of in vivo models of T cell-mediated inflammation, including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), have implicated CD31-mediated interactions in the regulation of T-cell responses. Accelerated progression of EAE resulting in an early increased migration of mononuclear leukocyte infiltration into the central nervous system (CNS) and increased severity of CIA in mice lacking CD31 have been reported (2–4). As CD31 contributes to EC:EC junction integrity, the proinflammatory phenotype observed in these models has been attributed to vascular junction loosening in CD31-deficient endothelium (2, 5), rather than a direct effect on T-cell function.

The expression of this molecule by both T cells and dendritic cells (6) suggests that CD31 is likely to be engaged in homophilic interactions during conventional antigen presentation. Indeed, CD31 engagement on T cells has the potential to directly affect TCR signaling (7–9). Despite these key observations, the role of CD31-mediated interactions in the regulation of T-cell immunity remains to be fully addressed.

In this study, we show that CD31 signals are required to contain the clonal burst and effector-mediated killing following primary T-cell activation, an effect that correlates with partial inhibition of TCR-induced Zap-70 phosphorylation. CD31 signals also promote survival of primed T cells and the activation of the Erk antiapoptotic pathway. Finally, we show that functional CD31 signaling is required for the optimal establishment of regulatory mechanisms and T-cell tolerance.

Results

CD31 regulates T-cell activation and tolerance in vivo. Although lack of CD31 has been clearly associated with a proinflammatory phenotype in models of autoimmunity (2–4), the cellular mechanisms for this effect have been previously attributed solely to junction loosening (2, 5, 10). We therefore sought to assess the contribution of CD31-mediated interactions to the development of immune responses exquisitely T cell-dependent (11) by in vivo models of HY(male)-specific immunity.

First, CD31−/− and WT female recipients were inoculated s.c. to the dorsolateral flank with MB49, a murine bladder carcinoma derived from a male C57BL/6 mouse (12). In WT female recipients tumor growth becomes uncontrollable within 2 wk after inoculation despite the development of a HY-specific T cell-mediated immune response (Fig. 1A). In contrast, tumor growth was largely controlled in CD31−/− recipients up to 27 d postinoculation (Fig. 1A). Second, skin graft experiments were carried out in which WT or CD31−/− female mice received WT male skin. HY mismatch leads to a T cell-mediated rejection of male skin grafts occurring around 4 wk postgrafting (13). As shown in Fig. 1B, skin graft rejection by CD31−/− female recipients was significantly accelerated as compared with WT recipients. Syngeneic female skin grafts survived indefinitely in both WT and CD31−/− female recipients. As revascularization of skin grafts by host endothelium occurs by day 15 in skin grafts, this effect could not be completely due to endothelial leakage.

It has been previously shown that intranasal administration of H2-Db-restricted Dby peptides to female recipients leads to acceptance and tolerance of male skin grafts by a number of mechanisms (13). To assess the influence of CD31-mediated interactions on tolerance induction, WT and CD31−/− female mice were pretreated with three intranasal administrations of 100 µg HY16Dby peptide in PBS or PBS alone, 10 d before grafting with syngeneic male skin. Administration of the HY16Dby pep-
tide resulted in indefinite acceptance of the graft in 100% WT recipients (Fig. 1C). In contrast, a significantly lower number of CD31−/− recipients (33%) retained their skin grafts following a similar pretreatment with the HY\(^{Ab}\)Dby peptide, indicating that loss of CD31 interactions confers relative resistance to tolerance induction. In vitro rechallenge of T cells obtained from the various experimental groups 60 d after grafting is shown in Fig. S1.

**CD31-Mediated Interactions Regulate T-Cell Primary Expansion and Contraction.** On the basis of these findings, we then sought to assess the influence of CD31 signaling on T-cell responses, independently of possible effects due to loss of endothelial integrity in CD31−/− mice.

HY-specific CD8\(^{+}\) T-cell expansion following in vivo priming of female mice with male splenocytes was analyzed. As shown in Fig. 2A and B, expansion of Uty-specific tetramer-positive CD8\(^{+}\) T cells following i.p. immunization with male splenocytes (3 × 10\(^5\) cells i.p.) was enhanced by at least 50% in CD31−/− female recipients as compared with their WT counterpart. However, the percentage of tetramer-positive CD31−/− T cells fell more rapidly compared with that of WT T cells.

Interestingly, a secondary antigenic rechallenge 60 d following primary immunization led to quantitatively similar reexpansion of Uty-specific WT and CD31−/− T cells (Fig. 2A and C), consistent with diminished CD31 expression by memory T cells (Fig. S2).

To further assess the role of CD31-mediated interactions during T-cell recognition of antigen-presenting dendritic cells (DCs), we analyzed the in vitro expansion of either WT or CD31−/− T cells stimulated with either syngeneic (C57BL/6, H2\(^b\)) or allogeneic (BALB/c, H2\(^d\)) fully mature DCs (Fig. 3A) or the expansion of allogeneic BALB/c or syngeneic B6 T cells stimulated with either WT or CD31−/− DCs (Fig. 3B). In both combinations, lack of CD31-mediated interactions led to a larger proportion of T cells entering division and a larger proportion of T cells undergoing more than five divisions. Altogether, these data suggest that CD31 homophilic engagement during DC:T-cell cognate interactions directly down-regulates T-cell expansion during priming. In contrast, CD31 deficiency only mildly affected reexpansion of HY-specific memory T cells in vitro (Table S1), consistent with the in vivo observations (Fig. 2C).

**CD21 Signaling Interferes with T-Cell Receptor (TCR)-Induced Proximal Phosphorylation Events.** CD31 signals have been shown to reduce TCR-induced calcium fluxes due to the recruitment of SH2 domain-containing phosphatases (S). We therefore sought to provide a direct link between the regulation of T-cell activation by CD31 and phosphorylation of proximal intracellular mediators specifically activated by TCR engagement. A prominent event in this process is the phosphorylation leading to the activation of the TCR/CD3/Zap-70 signalosome. Phosphorylation of Zap-70 (Tyr-493) in naïve T cells following antibody-
mediated CD3 activation in the presence or absence of CD31 coligation was analyzed by an anti–P-Zap-70 (Tyr-493) antibody and flow cytometry. As it is shown in Fig. 4, Zap-70 phosphorylation at Tyr-493 following CD3 ligation was partially but consistently inhibited by CD31 triggering. These data suggest that CD31 acts as a down-modulator of proximal TCR signaling.

**CD31 Signals Prevent Cell Death Following Priming and Induce the Antiapoptotic Erk-Mediated Pathway.** Despite enhancing T-cell expansion, we noticed that CD31 deficiency led to an accelerated contraction phase following priming (Fig. 2A and B). The ability of CD31-mediated signals to prevent activation-induced T-cell death (AICD) was therefore investigated in naïve T cells activated by CD3 antibody-mediated triggering in the presence or absence of CD31 coligation. Cell survival following activation was assessed by flow cytometric analysis of Annexin V and 7-AAD staining 3 and 8 d following stimulation. As shown in Fig. 5A, CD31 triggering significantly reduced the percentage of T cells undergoing apoptosis following CD3 stimulation, suggesting that CD31 signals promote T-cell survival after activation. This effect was not detected in CD31−/− T cells.

Given the increased AICD observed in CD31-deficient T cells following activation, we sought to establish whether CD31 activates major survival pathways in T cells and identify potential molecular mediators of these events. The extracellular signal-activated kinase Erk is known to be required for the survival of T cells during mitogenic stimulation (14) and Erk activation in endothelial cells has been shown to depend on the formation of the CD31/SHP-2 signaling complex (15). As it is shown in a representative experiment displayed in Fig. 5B, coligation of CD31 and TCR enhanced and prolonged Erk activation by the TCR. In addition, CD31 triggering was able to induce Erk activation independently of TCR-mediated signals, as shown by the appearance of faint but discrete p44 and the increase of p42 Erk bands in T cells stimulated with CD31 alone, suggesting an autonomous role of this molecule in the intracellular transduction of prosurvival signals. In contrast, CD31 engagement did not result in Akt, IKK/NF-κB, or JNK activation and did not affect their activation by TCR signals.

**CD31 Interactions Protect from T Cell-Mediated Killing.** Given the enhanced tumor clearance by CD31−/− mice and CD31 ability to activate the Erk antiapoptotic pathway, we sought to investigate whether CD31 signals can also affect T cell-mediated killing by an in vivo cytotoxicity assay (13). WT female mice recipients received a coinjection of carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled WT (CFSElow) or CD31−/− (CFSEbright) male splenocytes. Labeled target cells’ persistence in the blood was
Finally, although the relative resistance of CD31−/− mice to antigen-dependent cytolytic killing.

Cumulative data from at least three samples are shown (*P < 0.05). (B) Primary T lymphocytes from WT (left) and CD31−/− mice (right) were challenged in vitro with either anti-CD3 alone or in combination with anti-CD3 for the indicated time points. Whole-cell extracts were subjected to immunoblot analysis. Representative results for Erk, JNK, IKK/NIκB, Akt, and Actin activity are shown. Actin is shown at Bottom for protein loading normalization. Representative data from at least three experiments are shown.

assessed by flow cytometry over a 16-d period. The use of CD31−/− targets delivered to WT recipients, allowed discriminating the effect of CD31 homophilic interactions on cytolytic activity, independently of T-cell expansion (enhanced in CD31−/− mice). As shown in Fig. 6 A and B, CD31−/− targets disappeared significantly more rapidly than CD31-competent cells. In contrast, similar number of female (nonantigenic) WT and CD31−/− splenocytes persisted over the same timeframe (Fig. 6 C and D), suggesting that CD31 acts to protect against antigen-dependent cytolytic killing.

CD31-deficient Tregs display defective regulatory function. Finally, although the relative resistance of CD31−/− mice to tolerance induction by intranasal antigen administration might have been due to the inability of regulatory networks to cope with excessive T-cell expansion, we sought to assess the regulatory activity of CD31−/− and WT Treg cells. Phenotypic analysis of CD4+, CD25brightFoxp3+ T cells showed that CD31 is expressed at high levels by this subset and similar numbers of Tregs were detected in WT and CD31−/− mice (Fig. S3). Similarly to what was observed in conventional CD31−/− T cells, CD31−/− Treg proliferation was enhanced as compared with their WT counterpart (Fig. 7, first two columns). The suppressive ability of WT and CD31−/− CD4+CD25brightFoxp3+ T cells on CD3-induced proliferation of either WT or CD31−/− CD4+CD25− T cells was then analyzed by an in vitro assay. CD31−/− Tregs showed impaired regulatory activity as compared with WT counterpart at low Treg:T-cell ratios (Fig. 7). However, CD31−/− conventional T cells were susceptible to inhibition by WT Tregs, suggesting that direct CD31 interactions between Tregs and Teff are not required for regulation.

Discussion

Here we describe a unique comodulatory function of CD31-mediated interactions in the regulation of T-cell expansion, survival, and tolerance. We show that transplant and cancer T cell-mediated rejection is amplified by lack of CD31 expression in vivo, which is accompanied by resistance to tolerance induction. A combination of several cellular and molecular mechanisms contributes to this function.

CD31-mediated homophilic interactions with antigen-presenting cells (APCs) regulate clonal T-cell expansion following priming, in line with recent reports (16). However, CD31 signaling only mildly affects memory T-cell division following antigen rechallenge, probably due to its lower expression by memory T cells, suggesting that the inhibitory effect of CD31 signaling acts at the level of T-cell priming.

Sourcing the molecular mechanisms at the basis of this immunoregulatory role of CD31 is a current topic of much interest. The most distinctive feature of CD31 is the presence of two immunoreceptor tyrosine inhibitory motifs (ITIMs) in its cytoplasmic domain (17). These ITIMs are phosphorylated following TCR activation (18) and subsequently recruit protein-tyrosine phosphatases, such as the Src homology 2 domain containing protein SHP-2 (8, 17, 19), which is thought to help mediate an inhibitory function in the context of proximal TCR signaling (8). Here we show that Zap-70 phosphorylation is partially inhibited following coligation of TCR and CD31, an effect that can per se explain the attenuation—without abrogation—of T-cell responsiveness.

CD31-mediated signals may also promote the induction of tolerance by increasing the threshold of TCR signaling required—an effect that can also be due to their inhibition of proximal signaling events associated with the TCR/CD3/Zap-70 signalosome, as it has been shown in other negative costimulators such as CTLA-4 (20).

In this context, we have detected a significant decrease in the suppressive activity of CD31-deficient naturally occurring Tregs in vitro, which might contribute to the relative resistance to tolerance induction by CD31-deficient mice. The possibility that excessive expansion of CD31−/− conventional T cells might per se overcome regulatory networks is unlikely as these T cells are susceptible to suppression by WT Tregs. Additionally, homophilic CD31 engagement between Tregs and conventional T cells is not necessary for regulation. As TCR-triggering is necessary to Treg function (21), and CD31 can modulate Zap-70 activity, it would be plausible to speculate that the modulation of proximal TCR signaling (Zap-70) via CD31 engagement by conventional antigen-presenting cells (which are used in our assay) is required for optimal suppressive activity.

Although a cytoprotective effect by CD31-mediated signals on T lymphocytes and other cells has been previously reported (22, 23) the molecular mechanisms underlying this effect are at present controversial. The data presented here show that CD31 triggering selectively and independently induces and enhances TCR-recruited Erk activity, which has been associated with antipapoptotic pathways (18); NF-κB activity, in contrast, is not affected by CD31, in line with other reports (24).

In summary, our data point to a unique role for CD31 as a nonredundant comodulator of T-cell responses. While regulating the size of clonal expansion, selective expression of CD31 by T cells, DCs, and the endothelium might protect them against cytotoxicity by effector T cells, thus redirecting killing to CD31-
negative targets, such as parenchymal cells, without affecting antigen presentation by DCs and extravasation (18).

Unlike CTLA-4 deficiency, loss of CD31 does not lead to the spontaneous development of autoimmunity (25). In our study, in fact, we did not detect clinical signs of inflammatory bowel disease (IBD) or kidney damage in tissue samples from CD31−/− mice up to 22 wk old. The key difference between these two coinhibitors, which appear to use relatively similar signaling pathways (Zap-70 inhibition) to modulate TCR signaling, lies, in our view, in the ability of CD31 to promote T-cell survival following activation, a property that is not shared by CTLA-4. This functional difference is reflected by the differential expression of these molecules during T-cell development and it is likely to underlie a non-overlapping but complementary role of these immunomodulatory receptors in the unfolding of the immune response.

Importantly, the selective influence of CD31 comodulation on different phases of immune responses suggests that targeting this molecule therapeutically might allow a better control of side effects routinely associated with conventional immune suppression.

**Materials and Methods**

**Mice.** Wild-type (WT) C57BL/6 and BALB/c mice were purchased from Harlan-Olac. CD31-deficient mice have been previously described (25) and were used following backcrossing on C57BL/6 background for at least eight generations. Mice were used at the age of 6–8 wk. Experiments were performed under the Home Office Authority (Cambridge, UK) (PPL 70/5872).

Reagents. HY Dby peptides and HYD9/Utby MHC class I tetramers were produced in house.

APC-conjugated anti-mouse CD4 was obtained from Caltag Laboratories. All of the other antibodies used in this study were purchased from BD Biosciences unless specified otherwise. CFSE was purchased from Sigma-Aldrich and added at a final concentration of 1 μM, unless otherwise indicated.

**MB49 Tumor.** MB49 is a cell line derived from a murine bladder carcinoma arising in a male C57BL/6 mouse and subsequently passaged in vivo (12). MB49 cells were injected (5 × 10³/mouse) s.c. to the dorsolateral flank of WT and CD31−/− female mice. The size of the tumor (mm²) [length (mm) × width (mm)] was measured at regular intervals.

**Skin Grafting.** Skin grafting was conducted as previously described (13) using tail skin grafted onto the lateral thorax.

**Intranasal Peptide Administration for Tolerance Induction.** PBS containing 100 μg HYAε/Dby peptide was administered intranasally (20 μl/mouse) on three consecutive days to WT or CD31−/− females anesthetized with isoflurane. Control mice received intranasal PBS. The mice received syngeneic WT male grafts 10 d later.

**In Vivo Killing Assay.** WT or CD31−/− spleen cells (2 × 10⁷/mL in PBS) were labeled with 5 μM or 0.5 μM CFSE (Sigma), respectively, and injected i.v. (2 × 10⁷) into each recipient. Peripheral blood was collected from individual mice at serial time points and was analyzed for the presence of CFSE low and CFSE high donor cells. After lysis of RBC and blockade of FcR, cells were stained with HYD9/Utby-tetramer-PE, anti-CD8-PerCP and analyzed by FACS-Calibur (Becton Dickinson) and FlowJo software (Tree Star).

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**Fig. 6.** Loss of CD31-mediated interactions enhances T cell-mediated killing in vivo. Male (A and B) or female (C and D) CFSE-labeled WT (5 μM) and CD31−/− (0.5 μM) splenocytes were injected i.v. into syngeneic WT female mice. Tail blood samples were taken on days 4, 8, 12, and 16, stained with CD4-APC and CD8-PE-Cy5, and analyzed by flow cytometry. Examples of dot plots (gated on CFSE+ cells) obtained at days 4 and 16 postinjection are shown in A and C. (B and D) Averaged data obtained from at least three recipients (WT vs. CD31−/−, *P* < 0.05).

**Fig. 7.** CD31−/− regulatory T cells from display defective regulatory activity. CD4+CD25high and CD4+CD25− T cells were purified from the lymph nodes of either WT or CD31−/− mice. CD25− responder T cells (10⁵) and 10⁵ T cell-depleted splenocytes (WT) were then added to wells of a 96 U-well plate along with 1 μg/mL anti-CD3ε antibody. Regulatory T cells were then added at ratios of 1:1 to 1:4 to the CD25− cells. After 3 d the cells were labeled. Proliferation was assessed by 3HTr incorporation. The graph shown is representative of three experiments of similar design in which results were averaged using T cells obtained from at least three mice. *P* < 0.02.
Treg Isolation and Suppression Assay. CD4+CD25+ cells were purified from either WT or CD41KO-CD45.2+ mice using a mouse regulatory T-cell isolation kit (Miltenyi). CD4+CD25− cells were purified by labeling cells from the lymph nodes with anti-CD8 (clone 53.6.7), anti-CD45R (B220), anti-CD49b (DX5), anti-I-Ab (M5/114.15.2), and anti-CD25 (PC61.5) FITC-conjugated antibodies (all eBioscience), followed by addition of anti-FITC microbeads (Miltenyi) and depletion of labeled cells using a LD column. Splenocytes were then taken from C57BL/6 mice and depleted of T cells by labeling with 1 μg/mL anti-CD3 antibody (clone 145-2C11, prepared in house). Regulatory T cells were then added at ratios of 1:1–1:4 to the CD4+CD25+ cells. Tritiated thymidine (1 μCi/well) incorporation was measured 72 h later.

In Vitro T-Cell Activation. T cells were isolated from C57BL/6 mice and resuspended at 3 × 10^6/mL. Cells were treated with combinations of 1 μg/mL hamster–anti-mouse CD3 IgG (BD Biosciences); 10 μg/mL rat–anti-mouse CD31 IgG (E-Biosciences) and cross-linked with 0.5 μg/mL rabbit–anti-hamster Ig (BD Biosciences) and 3 μg/mL goat–anti-rat Ig (Biolegend Cambridge Bioscience), respectively.

Measurement of Zap-70 Phosphorylation. Following isolation, T-cell suspensions were “rested” in medium at 37 °C for 1 h before stimulation allowing for endogenous baseline phospho-Zap70 to dissipate. Following CD3 and/or CD31 antibody ligation, cells were harvested at the indicated time points, fixed with 2% PFA for 15 min at 37 °C, washed twice with PBS, permeabilized with 90% ice-cold methanol for 10 min at −20 °C then washed twice with PBS. Intracellular staining was carried out after initially blocking permeabilized cells in RT FACS buffer (0.5% BSA/PBS + Na3VO4) for 30 min and incubated with a dilution of 1:100 of P-Zap70 (Tyr493) antibody (Cell Signaling Technology) for 30 min at room temperature. Cells were washed and stained with surface antibodies for TRC-PeCy5 (eBioscience) at 1:100 and with secondary FITC-F(ab’), fragment donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) at 1:100 for 30 min at room temperature. Cells were then washed in FACS buffer and acquired using the Dako Cytomation MoFlo software.

Immunoblotting Analysis. Whole-cell lysates were prepared from antibody-stimulated WT and CD31−/− primary lymphocytes (see above) using Nonidet P-40 lysis buffer (50 mM hesees pH 8.0, 350 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 20 mM glycerol-2-phosphate, 1 mM PMSF, 1 mM DTT, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and a protease inhibitor mixture; Roche). Equivalent amounts of protein as determined by standard Bradford assay (Bio-Rad) were separated by SDS/PAGE and transferred to a reinforced nitrocellulose membrane (Whatman). Proteins were detected using the following antibodies: phospho-p44/p42 Erk (4377), p44/p42 Erk (9102), phospho-p54/46 JNK (9251), p54/46 JNK (9252), phospho-IKKα/β (2681), IKKγ (2370), phospho-Akt (4058), Akt (9272; Cell Signaling Technology), and Actin (1-19; Santa Cruz Biotechnology).

Statistical Analysis. All results described were obtained from at least three experiments of similar design and are expressed as mean ± SEM. In the in vitro experiments, means of two groups were compared with a one-tailed Student’s t test. In the in vivo experiments, the Student’s t test, the log-rank (Mantel-Cox) test, and the Gehan-Breslow-Wilcoxon multiple comparison tests were used. All reported P values are two-sided. P < 0.05 was considered significant.

ACKNOWLEDGMENTS. We are grateful to A. Ager (University of Cardiff) and E. Simpson (Imperial College London) for critical review of the manuscript and to G. Stamp and M. Moshadian (Imperial College London) for their help with assessing histopathology in CD31−/− mice. F.M.M.-B. is supported by the British Heart Foundation Grants PG/05/136/19997 and RG/09/002. S.N. is supported by funds from the Wellcome Trust (081172Z/06/Z). G.F. is supported by National Institutes of Health Grant R01 CA084040 and Cancer Research UK Program Grant C26587/A8339.