An immunologist's guide to CD31 function in T-cells

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Summary

Although it is expressed by all leukocytes, including T-, B-lymphocytes and dendritic cells, the immunoglobulin-like receptor CD31 is generally regarded by immunologists as a marker of endothelial cell lineage that lacks an established functional role in adaptive immunity. This perception has recently been challenged by studies that reveal a key role for this molecule in the regulation of T-cell homeostasis, effector function and trafficking. The complexity of the biological functions of CD31 results from the integration of its adhesive and signaling functions in both the immune and vascular systems. Signaling by means of CD31 is induced by homophilic engagement during the interactions of immune cells and is mediated by phosphatase recruitment or activation through immunoreceptor tyrosine inhibitory motifs (ITIMs) that are located in its cytoplasmic tail. Loss of CD31 function is associated with excessive immunoreactivity and susceptibility to cytotoxic killing. Here, we discuss recent findings that have brought to light a non-redundant, complex role for this molecule in the regulation of T-cell-mediated immune responses, with large impact on our understanding of immunity in health and disease.

Key words: CD31, T-lymphocyte, PECAM-1, MHC, tolerance, Cell signaling, Trafficking

Introduction

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a transmembrane homophilic receptor that is expressed by endothelial cells (ECs), platelets, granulocytes, macrophages, dendritic cells (DCs), T- and B-cells and natural killer (NK) cells. As a member of the immunoglobulin (Ig) gene superfamily, CD31 comprises six extracellular Ig folds; it has a molecular mass of 130 kDa and is differentially glycosylated, involving *N*-linked and *O*-linked glycosylation sites (Newton et al., 1999). There are several spliced variants of CD31 – expressed in a cell-type- and species-specific manner in human, rat and mouse – that arise as a result of alternative splicing of either the transmembrane, or one of the cytoplasmic tail exons (Newman and Newman, 2003; Privratsky et al., 2010). In addition to its well-known homotypic or homophilic interaction, a number of putative heterotypic ligands for CD31 have also been identified, including the neutrophil-specific antigen CD177 (NB1) (Sachs et al., 2007) and the ADP-ribosyl cyclase CD38 (Deaglio et al., 1998).

Although all blood lymphocytes express CD31 in the peripheral blood of newborns, a variable portion of blood lymphocytes (memory T-cells) appear to lack this molecule at their surface in adulthood (Kohler and Thiel, 2009; Stockinger et al., 1992). The reason for the apparent loss of CD31 expression on the surface of these cells is discussed below. The homophilic nature of CD31 facilitates its engagement with other CD31-expressing cells, and this includes leukocyte–leukocyte interactions. For instance, T- and B-cells need to interact with each other and with antigen-presenting cells (APCs: macrophages or DCs) to mature or become activated. These interactions have to be tightly regulated during an immune response in order to avoid nonspecific responses, which can lead to organ damage and autoimmunity. The fact that CD31 is not ubiquitously expressed but restricted to immune cells, platelets and ECs suggests that cells of the immune system specifically require its activity. In addition, ECs and platelets have also been described to have important roles in the immune system. ECs are able to present antigens (Pober and Tellides, 2012), and activated platelets are involved in the recruitment of immune cells through the expression of P-selectin (Diacovo et al., 1996). Therefore, the presence of CD31 might also be crucial in establishing interactions of lymphocytes with the endothelium or with platelets to modulate immune responses at the vascular wall and for the recruitment or extravasation of immune cells in inflamed tissues.

Although the CD31 receptor has been classified as a member of the ITIM-bearing immune co-receptors (Newman, 1999; Ravetch and Lanier, 2000), immunologists thus far appear to have neglected the importance of the regulatory functions CD31 has on immune cells. CD31 has been shown to inhibit antigen receptor signaling in T- and B-cells through the action of protein-tyrosine-phosphatases (SHIP, SHP-1 and SHP-2), which are recruited by its ITIMs.

This Commentary will integrate recent studies that have shed new light on the functions of CD31 and that support a fully immunological label for this receptor. Specifically, we will discuss the functional significance of the inhibitory role of CD31 signaling in T-cell activation and tolerance (see Box 1), its contribution to the regulation of T-lymphocyte trafficking and the impact of these functions on human immunomediated diseases.

CD31 is a non-redundant co-modulator of T-cell immunity

The two main reasons for why there has been a lack of interest in a possible role for CD31 in the immune system in the past are that

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Box 1. Key events in the immune response

Immune responses are initiated in lymph nodes, where recirculating naïve T-lymphocytes encounter antigen-presenting DCs that have migrated from the drained organ tissue. Their activation, proliferation and differentiation into memory T-cells requires two signals: engagement of the TCR by MHC–peptide complexes and concomitant delivery of co-stimulatory signals that are mediated by B7 molecules (CD80 and CD86) located on the DC, which engage with CD28 molecules on T-cells. In the absence of co-stimulation, TCR triggering results in T-cell death, a mechanism that is involved in the maintenance of T-cell tolerance against autoantigens. Following activation, T-cells transiently express cytotoxic T-lymphocyte antigen-4 (CTLA-4), a negative co-stimulator that also binds to B7 molecules with higher affinity than CD28. CTLA-4 activation prevents the excessive proliferation of activated T-cells, and it is also thought to be involved in induction of tolerance (Appleman and Boussiotis, 2003). Memory T-cells (effector T-cells) then leave the lymph node and migrate to the site of inflammation, where they exert their effector functions, such as killing of infected cells (cytotoxicity) (Jenkins et al., 2001). Access of T-lymphocytes to these sites is facilitated by their recognition of antigens that are displayed by the endothelium (Ma et al., 2010).

At the end of the immune response, a large fraction of the memory T-cells dies, leaving a cohort of long-lived memory T-lymphocytes, which can quickly react if antigen re-challenge occurs (Jenkins et al., 2001).

As some autoreactive T-cells can mature in the thymus, safeguard mechanisms exist to prevent their activation. These mechanisms can be intrinsic, such as cell death that is induced during activation in the absence of co-stimulation, or extrinsic, such as those mediated by regulatory T-lymphocytes (Wing and Sakaguchi, 2010), which actively suppress the activation and effector function of other T-lymphocytes. As a result of these complex mechanisms, a healthy immune system is prevented from mounting responses against autoantigens. This active immune function is defined 'self-tolerance'. In experimental therapeutic settings, for example to prevent transplant rejection, the delivery of antigen by selective routes, such as oral or nasal administration, can induce tolerance to the graft (Ma et al., 2010).

CD31-deficient mice exhibit a very mild phenotype under steady-state conditions and have a normal number of T-cells (Duncan et al., 1999) and that T-lymphocytes that undergo differentiation (Demeure et al., 1996) and acquire a memory phenotype (Stockinger et al., 1992) do not (apparently) express CD31.

However, the importance of the role played by CD31 in T-cells has recently been outlined by two main lines of investigation. First, under conditions of immunological stress, lack of CD31 affects the extent of T-cell-mediated inflammation in mice (Table 1). For example, progression of experimental autoimmune encephalomyelitis (EAE) is associated with an accelerated and increased migration of mononuclear leukocytes into the central nervous system (CNS) (Graesser et al., 2002), and the severity of collagen-induced arthritis (CIA) is increased in mice lacking CD31 (Graesser et al., 2002; Tada et al., 2003; Wong et al., 2005).

Second, loss of CD31 expression by memory T-cells is only apparent because this molecule is enzymatically shed, rather than transcriptionally downregulated (Fornasa et al., 2010). In fact, in memory T-cells, CD31 signaling can be triggered by cell polarization and subsequent clustering on the same cell membrane (Kishore et al., 2012), and CD31 activity after shedding can be recovered by a peptide (containing CD31 amino acid residues 551 to 574) that is able to homo-oligomerize with the truncated CD31 fragment (Fornasa et al., 2010). In addition, human memory T-cells have been shown to acquire CD31 expression following *trans*-endothelial migration (TEM) as a result of membrane transfer from ECs *in vitro* (Brezinschek et al., 1999).

In naïve T-cells, downregulation of surface expression of CD31 has been associated with homeostatic proliferation, which is responsible for the maintenance of the pool of T-cells when thymic output is reduced (Kohler and Thiel, 2009), but the functional significance of this association has not yet been investigated.

The expression of CD31 by both T-cells and DCs (Ma et al., 2010) suggests that it is likely to engage in homophilic interactions during conventional antigen presentation and T-cell priming. The role of CD31-mediated interaction in T-cell activation has been analyzed recently in CD31-deficient mice, which displayed accelerated and more pronounced allograft and tumor rejection (Ma et al., 2010). These effects correlate with an amplified proliferation of CD31-deficient T-cells specifically following immunization and diminished regulatory T-cell (Treg)-mediated suppression. Notably, these experiments also revealed

Table 1. Models of disease in CD31-deficient mice

Disease model	Effect of CD31 deficiency	Reference
Induction of EAE with MOG peptide in C56BL/6 mice	Enhanced leukocyte extravasation and accelerated onset of EAE	(Graesser et al., 2002)
CIA in DBA/1 mice	Enhanced arthritis	(Tada et al., 2003; Wong et al., 2005)
Exposure to the bacterial endotoxin LPS	Septic shock	(Maas et al., 2005)
Laser-induced and $FeCl_3$ endothelial injury	Accelerated vascular occlusion (thrombosis)	(Falati et al., 2006)
Diet-induced non-alcoholic steatohepatitis	Progressive liver disease	(Goel et al., 2007)
LDLR KO (hypercholesterolemic) mice	Accelerated atherosclerosis	(Goel et al., 2008)
ApoE-deficient (hypercholesterolemic) mice	Inhibited atherosclerosis	(Harry et al., 2008)
Bone marrow hematopoietic cell engraftment	Hypersensitivity to macrophage CSF and receptor activator of NF-κB ligand; osteoclastic bone loss	(Wu et al., 2009)
Lipopolysaccharide (LPS)-induced endotoxemia	Cytokine storm and acute respiratory distress syndrome due to accumulation of cytokine-producing leukocytes at sites of inflammation	(Privratsky et al., 2010).
Tumor and skin allograft	Enhanced tumor and skin allograft rejection	(Ma et al., 2010)
Systemic *Salmonella* infection	Delayed pathogen clearance despite increased T-cell activation due to enhanced T-cell susceptibility to AICD	(Ross et al., 2011)
Generation of endothelial cell barrier *in vitro*	Loss of endothelial barrier integrity	(Privratsky et al., 2011)

that CD31-deficient target cells are more susceptible to T-cell-mediated cytotoxicity. The enhanced expansion of CD31-deficient T-cells, however, was accompanied by an increased activation-induced cell death (AICD) of CD31-deficient T-cells. The lack of CD31 expression did not affect the magnitude of T-cell responses to antigen re-challenge (Ma et al., 2010).

A recent study investigated the role of CD31-mediated interactions in T-cell protection against AICD in a model of *Salmonella* infection in CD31-deficient mice and described a delayed pathogen clearance, despite increased T-cell activation (Ross et al., 2011). T-cell activation subsequent to infection appears to be continuous and leads to an enhanced inappropriate susceptibility of CD31-deficient T-cells to AICD. However, this increased apoptosis at the earliest stages of activation delays but does not abrogate pathogen clearance caused by the increased proliferative capacity of CD31-deficient T-cells, which eventually leads to a slow accumulation of activated T-cells (Ross et al., 2011). In addition to reducing AICD indirectly (i.e. by inhibiting T-cell proliferation), CD31-mediated signals have also been shown to induce extracellular-signal-regulated kinase (Erk) activity, which is associated with anti-apoptotic pathways, independently of T-cell receptor (TCR) triggering (Ma et al., 2010).

Therefore, CD31 is necessary to promote the controlled activation of T-cells and their survival. Although the role of CD31 in these events appears to selectively regulate the early stages of T-cell activation, the extent and kinetics of memory responses are not affected. The possibility that CD31 signaling might influence T-cell differentiation into effector or central memory T-cells has not been reported.

The apparently conflicting results for CD31 function from the studies of autoimmunity (organ-specific enhanced severity) and infection (systemic, delayed clearance) reflect the complex role that CD31 interactions have in regulating immunity in a cell-specific context and dependent on the T-cell differentiation stage. In addition, given that the increased expansion of CD31-deficient T-cells is compensated by enhanced death, the severe exacerbation of autoimmunity, transplant and tumor rejection in CD31-deficient mice cannot be fully explained by CD31-mediated effects on primary T-cell activation. As will be discussed below, in contrast to being lost, the regulatory role of CD31 shifts towards the migration of specific T-cells once they have been activated.

In addition to its engagement by antigen-presenting DCs, CD31 triggering occurs during the interaction of T-cells with CD31-expressing ECs and platelets. Antigen presentation by IFNγ-activated ECs promotes T-cell migration through these ECs and their subsequent activation in antigen-rich tissue (Marelli-Berg et al., 2007; Pober and Tellides, 2012). Platelets are also gaining attention owing to their regulatory role both in nonspecific inflammatory processes and in T-cell-dependent adaptive immune responses (Semple et al., 2011). It is possible that platelet-expressed CD31 might engage the CD31 at the surface of T-cells, thus preventing their excessive activation.

The above studies point to an essential function of CD31 signaling in increasing the threshold for T-cell activation. Deciphering the molecular mechanisms underlying this immunoregulatory role of CD31 is a subject of considerable current interest. The most distinctive feature of CD31 is the presence of two ITIMs in its cytoplasmic domain (Newman and Newman, 2003; Privratsky et al., 2010). These ITIMs are phosphorylated following TCR activity (Newman et al., 2001) and subsequently recruit protein-tyrosine phosphatases (PTPs), such as the Src homology 2 (SH2)-domain-containing protein SHP-2 (Newman et al., 2001), leading to inhibition of TCR signaling (Newton-Nash and Newman, 1999) (Fig. 1). The relative contribution of each ITIM to CD31 signaling is at present unknown.

The engagement of CD31 at the surface of T-cells with a recombinant CD38 (which is an alternative CD31 ligand) is able to inhibit the activation of the Jun-N-terminal kinase (JNK), nuclear factor (NF)-κB and interferon-regulatory-factor 3 (IRF-3) pathways, which correlates with a dampened cytokine production (Rui et al., 2007). More recently, phosphorylation of the tyrosine-protein kinase ZAP-70 (Zap70), a key upstream mediator of TCR signaling, has been shown to be partially inhibited following interaction of the TCR with CD31 (Ma et al., 2010), an effect that can per se explain the attenuation of T-cell proliferation.

Similar observations have been made with respect to B-cells, in which CD31 has been shown to inhibit the B-cell receptor (BCR) signaling (Henshall et al., 2001). As a consequence, a hyper-reactive phenotype has been described in CD31-deficient B-cells, which was accompanied by the production of autoantibodies in elderly CD31-deficient mice (Wilkinson et al., 2002).

In summary, CD31 prevents lymphocyte hyper-reactivity by raising the activation threshold of their antigen–receptor signaling and might thus contribute to the establishment of peripheral tolerance, the key mechanism that prevents the development of autoimmunity (Box 1).

Is CD31 involved in T-cell tolerance?

Overexpression of a soluble CD31 protein can lead to T-cell hypo-responsiveness *in vitro* (Prager et al., 2001) and reduced frequency of activated T-cells *in vivo* (Groyer et al., 2007), suggesting that CD31 signals contribute to T-cell tolerance. CD31 might promote the induction of tolerance through the inhibition of TCR-induced proximal signaling events, such as Zap70 phosphorylation, as has been shown for other negative co-stimulators such as CTLA-4 (Guntermann and Alexander, 2002). However, unlike CTLA-4 deficiency, loss of CD31 does not lead to the spontaneous development of overwhelming T-cell proliferation and severe autoimmunity in mice early after birth (Tivol et al., 1995; Waterhouse et al., 1995). The key difference between CTLA-4 and CD31, which appear to use similar signaling pathways to modulate TCR signaling (PTP recruitment and Zap-70 inhibition), 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 also reflected by the different expression of these molecules during and after T-cell activation. Specifically, CD31 expression is reduced by shedding in activated T-cells, whereas CTLA-4 expression is transiently induced after T-cell activation, suggesting a non-overlapping but complementary role for these immune-modulating receptors in the unfolding immune response.

Although CD31-deficient mice do not develop the severe autoimmune disorder and lympho-proliferation that are observed in CTLA-4-deficient mice, they do display resistance to induction of T-cell tolerance against HY-mismatched skin grafts following intranasal administration of an antigenic peptide, suggesting that CD31 mediates tolerance-permissive signals in T-cells (Ma et al., 2010).

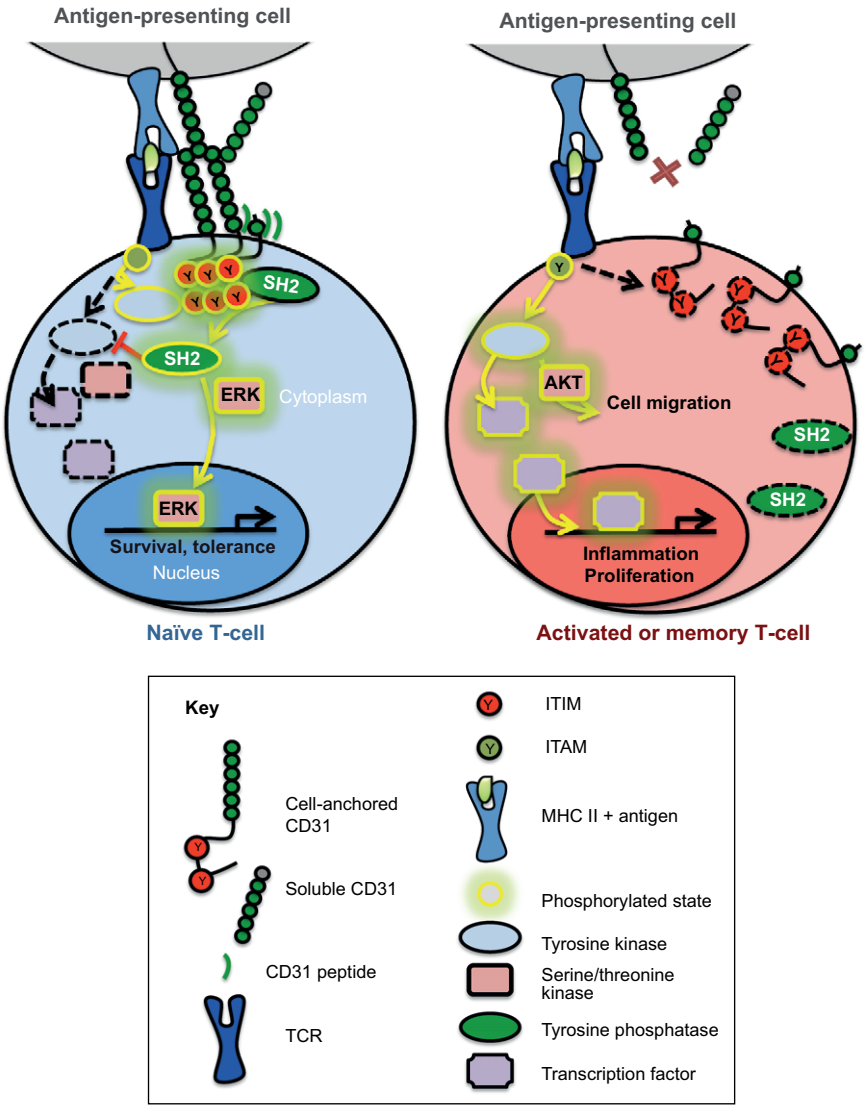

Fig. 1. CD31 signaling pathways in T-lymphocytes. (Left) Stimulation of the T-cell receptor (TCR), by the antigen-loaded MHC molecules of antigen-presenting cells, triggers the recruitment of tyrosine kinases, which drive the signaling cascade leading to cell activation. The T-cell-anchored CD31 co-clusters with the stimulated TCR molecules, provided that it is *trans*-homophilically engaged with either the cell-anchored CD31 of the interacting antigen-presenting cell or with a soluble form of CD31. The tyrosine kinases associated with the activated immunoreceptor tyrosine-based activation motif (ITAM) can hence phosphorylate also the nearby immunoreceptor tyrosine inhibitory motifs (ITIMs) in the cytoplasmic tail of CD31. This raises the activation threshold of naïve T-cells as the phosphorylated ITIMs of CD31 recruit and activate SH2-domain-containing protein-tyrosine phosphatases (such as SHP-1 and SHP-2), which eventually dephosphorylate the tyrosine kinases involved in cell activation. In parallel, the CD31-ITIM–SH2 phosphatase signaling pathway elicits the activity of certain serine/threonine kinases, such as ERK1/2, which are necessary for cell survival in a quiescent state. (Right) In the absence of the *trans*-homophilic sequence of CD31 (lost by extracellular cleavage by activated or memory T-cells or missing because of total deletion of the gene encoding CD31), the activation threshold of T-cells is lowered because CD31 molecules cannot co-cluster with the stimulated ITAM receptor, and the cascade that leads to the nuclear translocation of transcription factors and promotes cell proliferation and production of inflammatory mediators is unrestrained. Moreover, lack of CD31 signaling favors Akt phosphorylation downstream of the stimulation of other ITAM receptors (e.g. chemokine CXCL10-receptor binding) in activated T-cells, which leads to cell migration. Interestingly, this situation can be reverted by agents, such as homotypic CD31 peptides, that, as shown on the left, are able to restore or sustain the CD31-ITIM–SH2 phosphatase signaling and raise the (re)activation threshold of memory T-cells.

The resistance of CD31-deficient mice to T-cell tolerance induction might be due to either an intrinsic hyper-reactivity of CD31-deficient T-cells or the inability of APCs, particularly immature DCs that physiologically express high levels of CD31 (Ma et al., 2012), to deliver inhibitory signals to T-cells. Alternatively, as the intranasal tolerance model relies on the involvement of regulatory T-cells (Tregs), it is possible that their function is impaired in the absence of CD31. Although a direct link between Treg function and CD31 expression has not yet been reported, the use of a CD31-agonist peptide has been shown to drive the enrichment of the Treg compartment at the expense of the effector T-cell (Teff) compartment *in vivo* (Fornasa et al., 2012). Activation of the CD31 signaling pathway by the agonist might therefore tilt the Treg–Teff balance by reducing Teff differentiation. Indeed, the tyrosine phosphatase (SHP-2) that is recruited by the CD31 ITIM motifs can contemporaneously interfere with signaling pathways involved in the differentiation of Teff cells and promote those involved in Treg generation, such as the Gab–Erk–MAPK pathway (Nishihara et al., 2007).

A number of studies have associated CD31 expression with T-cell subsets with immunosuppressive function (Prager et al., 2001; Prager et al., 1996; Torimoto et al., 1992), and a notable decrease in the suppressive activity of naturally occurring Tregs following the loss of CD31 expression has been reported *in vitro* (Ma et al., 2010) and *in vivo* (Haas et al., 2007), but the molecular mechanism underlying this effect is at present unclear. The homophilic engagement of CD31 between Tregs and conventional T-cells has been shown not to be necessary for regulation (Ma et al., 2010). As TCR-triggering is necessary for Treg function and CD31 can modulate Zap70 activity, one can speculate that the modulation of Treg-proximal TCR signaling (Zap70) through engagement of CD31 by APCs is required for their optimal suppressive activity.

CD31 and T-cell trafficking

In contrast to the large number of studies that have established a role for CD31 in facilitating neutrophil and monocyte TEM (Newman and Newman, 2003), the contribution of CD31-mediated interactions to lymphocyte trafficking has been largely

overlooked. Early studies showed that the engagement of CD31 on T-cells can lead to so-called 'inside-out signaling' and induce the activation of β_2 and β_1 integrins (Piali et al., 1995; Tanaka et al., 1992) and suggested a potential mechanism by which CD31 mediates the interaction of lymphocytes with components of the venular wall during TEM (Miller et al., 2001). However, further investigations reported inconsistent findings. For instance, an *in vitro* study analyzing the phenotype of human T-lymphocytes that migrated through cytokine-treated human endothelial cell monolayers failed to detect an enrichment of CD31-expressing T-cells among the migrated cells, which mostly consist of memory lymphocytes, and led the authors to conclude that CD31 is not involved in T-cell migration (Bird et al., 1993).

By contrast, more recent *in vitro* studies that used the blocking of CD31 with antibodies could directly implicate CD31 in T-lymphocyte TEM, in particular in the migration of human effector memory T-cells that had been induced by antigen-presenting ECs (Manes et al., 2010; Manes and Pober, 2011).

Another recent study investigated the potential role for CD31-mediated interactions in the regulation of T-cell trafficking *in vivo* by separately assessing the effect of CD31 deficiency in T-cells and in the endothelium and provided conclusive evidence of a direct contribution of CD31-mediated interactions in the regulation of T-cell trafficking (Ma et al., 2012). In this study, CD31 was shown to facilitate the access of naïve T-cells to secondary lymphoid tissue. This effect might have been masked in CD31-deficient mice that display normal colonization of lymphoid tissue through compensatory mechanisms that involve a complex network of molecules that participate in lymphocyte TEM, including CD99, junctional adhesion molecules (JAMs) and intercellular adhesion molecules (ICAMs) (Nourshargh and Marelli-Berg, 2005).

Moreover, the loss of homophilic engagement of CD31 between T-cells and the endothelium also impaired the constitutive recirculation of effector T-cells and inflammation-induced T-cell extravasation to antigen-rich sites, suggesting that CD31-mediated T-cell–endothelium interactions facilitate the recirculation of memory T-cells, as suggested by a previous *in vitro* study (Manes et al., 2010).

However, a selective lack of CD31 expression in the endothelium resulted in enhanced antigen-specific memory T-cell extravasation under inflammatory conditions, in line with previous observations in experimental models of autoimmune disease (Graesser et al., 2002; Tada et al., 2003; Wong et al., 2005). Antigen recognition at the endothelium, which facilitates the extravasation of antigen-specific T-cells, involves the engagement of antigen-loaded major histocompatibility complex (MHC) molecules that are displayed by the endothelium by the TCR of the migrating lymphocytes. MHC triggering has been shown to induce the rapid translocation of RhoA, a major modulator of the cytoskeleton, to the cell membrane, where it mediates formation of F-actin stress fibers and reorganization of the cytoskeleton (Boulday et al., 2004), leading to increased contractility of ECs and a transient increase in endothelial permeability (Ma et al., 2012).

CD31 might be required to re-establish rapidly endothelial continuity, which is compromised transiently by the engagement of MHCs between endothelial and migrating antigen-specific T-cells (Fig. 2). In fact, by inducing dephosphorylation of β-catenin and enhancing anchorage of the vascular endothelial (VE)-cadherin complex, CD31 might contribute to the stabilization of endothelial adherens junctions (Biswas et al., 2006).

Importantly, this role represents a further molecular mechanism for the enhanced extravasation of T-cells that has

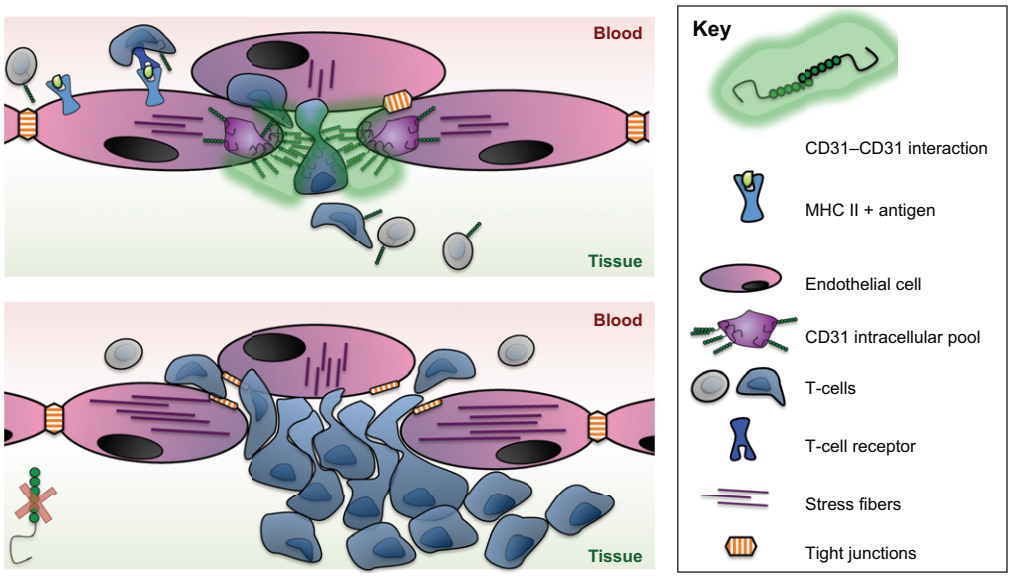

Fig. 2. CD31 signaling re-establishes endothelial integrity following migration of antigen-specific T-cells. (Upper) Endothelial cells (ECs) can upregulate MHC molecules and present antigen to antigen-specific migrating T-cells. The ligation of endothelial MHC to the TCR of the extravasating lymphocytes induces reorganization of the actin cytoskeleton, formation of stress fibers and endothelial contraction. However, *trans*-homophilic CD31 interactions between apposing ECs and migrating, interacting lymphocytes might preserve the cohesion of endothelial cells and prevent the disruption of tight junctions. During lymphocyte migration, more CD31 molecules are recruited from the endothelial intracellular pools to support this event. (Lower) Loss of CD31 in the endothelium results in the loosening of the tight junctions, and the lack of CD31 either in the endothelium or on lymphocytes favors an uncontrolled and nonspecific lymphocyte extravasation.

been observed following induction of EAE in CD31-deficient mice (Graesser et al., 2002). Studies of chimeric mice that express CD31 selectively either on ECs or on leukocytes revealed that endothelial, but not leukocyte, CD31 can partially protect against excessive inflammation in the inflammatory disease models of EAE (Graesser et al., 2002). Of note, T-cell infiltration in EAE is promoted by antigen presentation by the brain endothelium (Sobel et al., 1984).

Molecular mechanisms of differential CD31 activity in naïve and memory T-cells

The findings discussed above suggest that CD31-mediated interactions regulate the activation and migration of naïve T-lymphocytes. However, although CD31 also regulates the trafficking of memory T-cells, it does not appear to affect their activation (Ma et al., 2010). A possible explanation for this discrepancy is the higher expression of CD31 by ECs compared with DCs, which might result in a more effective homophilic engagement of memory T-cells – that also express low amounts of CD31 – with ECs than with DCs.

Besides such effects of the different levels of CD31 expression in different cell types, there is also evidence that suggests that CD31 signaling can occur in memory T-cells independent of any *trans*-cellular engagement. Activated, but not naïve, CD31-deficient T-lymphocytes display enhanced responses to soluble chemokines (i.e. independent of *trans*-cellular interactions) both *in vitro* and *in vivo* (Kishore et al., 2012). The role of CD31 in inhibiting the response of memory T-cell to chemokines was shown to depend on the polarization of memory T-cells, which allows CD31 molecules to cluster and segregate to the leading edge. This clustering of CD31 promotes its interactions with nearby CD31 molecules on the same cell membrane, resulting in the initiation of CD31 signaling.

Such dynamic homophilic *cis* CD31 interactions have been described previously in human embryonic kidney and erythroleukemia cells that have each been transfected with vector encoding CD31 (Zhao and Newman, 2001), but the molecular domains that mediate these interactions have not been identified.

In naïve T-cells, CD31, despite being expressed at higher levels, is evenly distributed on the cell surface, and it is exclusively activated by *trans*-cellular interactions. The physiological relevance of the selective effects of CD31 signaling on the proliferation of naïve T-cells and the responsiveness of activated T-cells to chemokines must lie, in our view, in the differences in their trafficking and proliferative potential. We propose that CD31 regulatory signals are adapted in response to the inflammatory potential of specific events leading up to the immune response, as we explain below.

Naïve T-cells continuously recirculate through secondary lymphoid tissue in response to chemokines that are constitutively expressed in secondary lymphoid organs, such as CCL19 and CCL21 (Kishore et al., 2012). This type of trafficking does not generate inflammation – hence chemotaxis of naïve T-cells does not require regulation by CD31.

However, CD31 signaling is activated in naïve T-cells when they engage with antigen-presenting DCs, with the effect of preventing the excessive expansion of the T-cells following activation (Ma et al., 2010). Upon antigenic stimulation, T-cells are reprogrammed into memory T-cells to respond to inflammation-induced chemokines such as CXCL10, which allow them to gain access to non-lymphoid tissue, where they perform their effector function, which can lead to nonspecific tissue damage if it is not controlled (Marelli-Berg et al., 2008). The proliferative potential of memory T-cells is considerably reduced compared with that of naïve T-cells and is therefore unlikely to require stringent regulation by CD31. Instead, CD31 contributes to the regulation of memory T-cell function by modulating their chemotactic responses.

Relative impact of the adhesive and signaling functions of CD31

The question of whether CD31 function is only defined by its signaling properties or also by its role as an adhesion receptor is an as-yet-unresolved issue among vascular biologists.

The effects of blocking CD31 with antibodies are consistent with an adhesive function of this molecule as antibodies to CD31 have been shown to reduce leukocyte infiltration and disease severity dramatically in *in vivo* models of T-cell-mediated inflammation, including collagen-induced arthritis (CIA) (Ishikaw et al., 2002); (Decking et al., 2001) and experimental autoimmune encephalitis (Qing et al., 2001); (Bogen et al., 1994; Liao et al., 1997; Reinke et al., 2007; Vaporciyan et al., 1993).

Paradoxically, in similar disease models, genetic deletion of the gene encoding CD31 results in disease acceleration (Graesser et al., 2002; Tada et al., 2003; Wong et al., 2005), as discussed above. This issue is further complicated by the observation that the C57BL/6 mouse strain, which has been used in most of these studies, can compensate better than other strains for the loss of PECAM function with regard to monocyte and neutrophil migration (Schenkel et al., 2004).

There are a number of possible explanations for this discrepancy that suggest a selective removal of the adhesive, but not the signaling, function of CD31 when antibodies are used. First, blockade with antibodies or recombinant molecules is mostly used following disease induction, thus possibly bypassing the effect that CD31 inhibition might have on the amplification of T-cell clonal expansion, thereby only affecting T-cell transmigration. Second, it is possible that endothelial CD31 that is engaged in *trans*-endothelial homophilic interactions (i.e. actively signaling) might not be easily accessible to antibodies and recombinant molecules, especially considering that a large cytoplasmic pool of CD31 molecules is stored in a network of vesicles within the endothelium (Mamdouh et al., 2003).

Alternatively, using the same amount of an antibody to CD31 might elicit different effects on T-cells or the endothelium (i.e. crosslinking and activation or blockade, depending on the relative availability of CD31 on the endothelial and lymphocyte surface). Finally, given that CD31-'blocking' reagents are defined by their ability to interfere with leukocyte migration rather than measuring the activation state of intracellular mediators downstream of CD31, it is possible that CD31-targeting antibodies and agonists that are bound to T-cells might have actually triggered CD31 signaling and induced its inhibitory functions, leading to amelioration of the disease.

In support of this interpretation, transgenic mice that chronically express high levels of circulating recombinant CD31 molecules (sPECAM-Fc) develop an earlier onset of EAE (Reinke et al., 2007). It is tempting to speculate that an overly high concentration of circulating CD31 ligand might have prevented CD31 signaling in T-cells by interfering with CD31 clustering, an effect known to occur in the presence of

over-saturating concentrations of T-cell-stimulating antibodies (Roosnek et al., 1987).

In our view, the contrasting conclusions drawn from studies that use either genetic ablation or antibody blockade of CD31 might reflect differential effects on its adhesive or signaling functions that are elicited in ECs and T-cells.

Of mice and men: clinical relevance of CD31 functions in the immune system

The immunoregulatory role of CD31 and its interactions has recently begun to be appreciated also in human diseases. For instance, a link between CD31 and T-cell proliferation and apoptosis has also been found in studies of human T-cells, which showed that $CD31^{low}$ T-cells proliferate more readily (Prager et al., 2001).

In addition, low expression of CD31 has been found on a subpopulation of human T-cells that had recently emigrated from the thymus (Gomez et al., 2003; Kilpatrick et al., 2008; Kimmig et al., 2002), which accumulate with age. This subpopulation of T-cells might also have an enhanced proliferative capacity, and it has been proposed that these cells enhance susceptibility to autoimmune disease (Kohler and Thiel, 2009). By contrast, the acquisition of CD31 by tumor cells provides them with resistance to apoptotic death and to immune effectors, leading to a poorer prognosis of patients (Bergom et al., 2005; Darom et al., 2006; Khattab et al., 2009; Sapino et al., 2001).

Several reports have highlighted an association between genetic variants of CD31 and increased graft-versus-host disease (GVHD) and the severity of atherosclerosis. Eleven different single-nucleotide polymorphisms (SNPs) exist within the gene encoding human CD31, but thus far only three have been associated with disease (Novinska et al., 2006). Although the effects of these SNPs on CD31-mediated adhesion or signaling functions have not yet been determined, it is interesting to note that the SNP at position 670 is located in the cytoplasmic domain within the ITIM motifs, which are important for signal transduction and regulation of expression of CD31 on the cell surface (Listi et al., 2004; Listì et al., 2007). It is tempting to speculate that the SNP at position 670 affects the intracellular pathways that are activated by CD31, which could be related to the occurrence of disease, but the molecular details need to be determined experimentally.

The development of GVHD upon bone marrow transplantation (BMT) in human leukocyte antigen (HLA)-matched transplants is often attributed to a mismatch of minor histocompatibility antigens (mHags), which are poorly defined in humans (Behar et al., 1996). A number of studies have shown an association between the occurrence of allelic SNPs of the gene encoding CD31 and the incidence of acute GVHD in patients who had received bone marrow or hematopoietic stem cell transplants (Behar et al., 1996; El-Chennawi et al., 2006; Grumet et al., 2001). This association has been related to the generation of polymorphic CD31-peptides (mHags) that might induce immune responses; this has clinical implications as matching for alleles encoding CD31 might reduce the incidence of GVHD in patients undergoing BMT. Alternatively, these mutations could also influence the binding affinity of CD31, its structure and signaling capacity (Goodman et al., 2008) and might affect CD31-mediated regulation of T-cell function. The expression of loss-of-function CD31 mutants could lead to an enhanced immunoreactivity and could potentially result in severe GVHD (when such a variant is expressed by the donor) or graft versus leukemia (GVL; when the variant is expressed by the recipient). This suggests that the presence of CD31 mutations can potentially be used as a predictive marker of BMT outcome.

CD31 has also been implicated in the development of atherosclerosis and of its clinical complications. Loss of CD31 at the surface of circulating T-cells is positively correlated to the occurrence of atherothrombosis in mice and of abdominal aortic aneurysm in patients (Caligiuri et al., 2005; Caligiuri et al., 2006), and CD31 ablation by genetic targeting leads to an enhanced lesion formation throughout the arterial tree, apart from the aortic arch, in experimental atherosclerosis (Goel et al., 2008). A few studies have described a link between the development of atherosclerosis in humans and the presence of CD31 SNPs. Interestingly, the CD31 SNPs that have been associated with atherosclerosis are the same as those found to be implicated in GVHD (Elrayess et al., 2004; Listi et al., 2004; Listì et al., 2007). However, the functional significance of these SNPs has yet to be explored.

Interestingly, administration of a CD31-derived peptide that encompasses the murine CD31 residues 551 to 574, which is able to suppress T-cell and macrophage activation, not only delays the onset of and noticeably increases survival from atherosclerotic complications such as abdominal aortic aneurysm (Fornasa et al., 2012) but also from lethal GVHD (Chen et al., 1997), suggesting that CD31 can also predict the outcome of atherosclerosis progression. Moreover, the immunomodulatory activity of CD31 could also be exploited pharmacologically in therapeutic settings.

Immune functions of CD31: future directions

Collectively, the current data point to a unique role of CD31 as a non-redundant co-modulator of T-cell responses, as summarized in Fig. 3. However, the potential influences of CD31-mediated interactions on the development and function of the immune system have yet to be fully investigated.

For example, given its high expression by thymocytes and its ability to fine-tune TCR signaling, CD31 might play an important role as a regulator of positive and negative selection of thymocytes and contribute to shaping the T-cell repertoire, a possible role that remains as yet unexplored.

In the ensuing immune response, CD31 regulates the size of clonal expansion by inhibiting excessive proliferation and preventing apoptosis. The selective expression of CD31 by T-cells, DCs and the endothelium might protect them against cytotoxicity by effector T-cells, thus directing their activity to CD31-negative targets, such as parenchymal cells, without affecting antigen presentation by DCs and extravasation.

Intriguingly, the role of CD31 in the formation of the most important interaction of the adaptive immune cells – the immunological synapse – has never been evaluated. This platform of communication between immune cells is crucial for the activation of the immune response, and it will be interesting to study CD31 expression and its possible roles here.

The involvement of CD31 in cytoprotection and cytolytic killing also suggests that it might be implicated in NK cell function, which is another as-yet-unexplored role.

Despite these pending issues, the ability of CD31 to regulate different aspects of T-cells, including their activation, survival and trafficking, clearly establishes its role as a key player in the dynamic tuning of both ensuing and established immunity as well as a potential target for therapeutic intervention. This complex

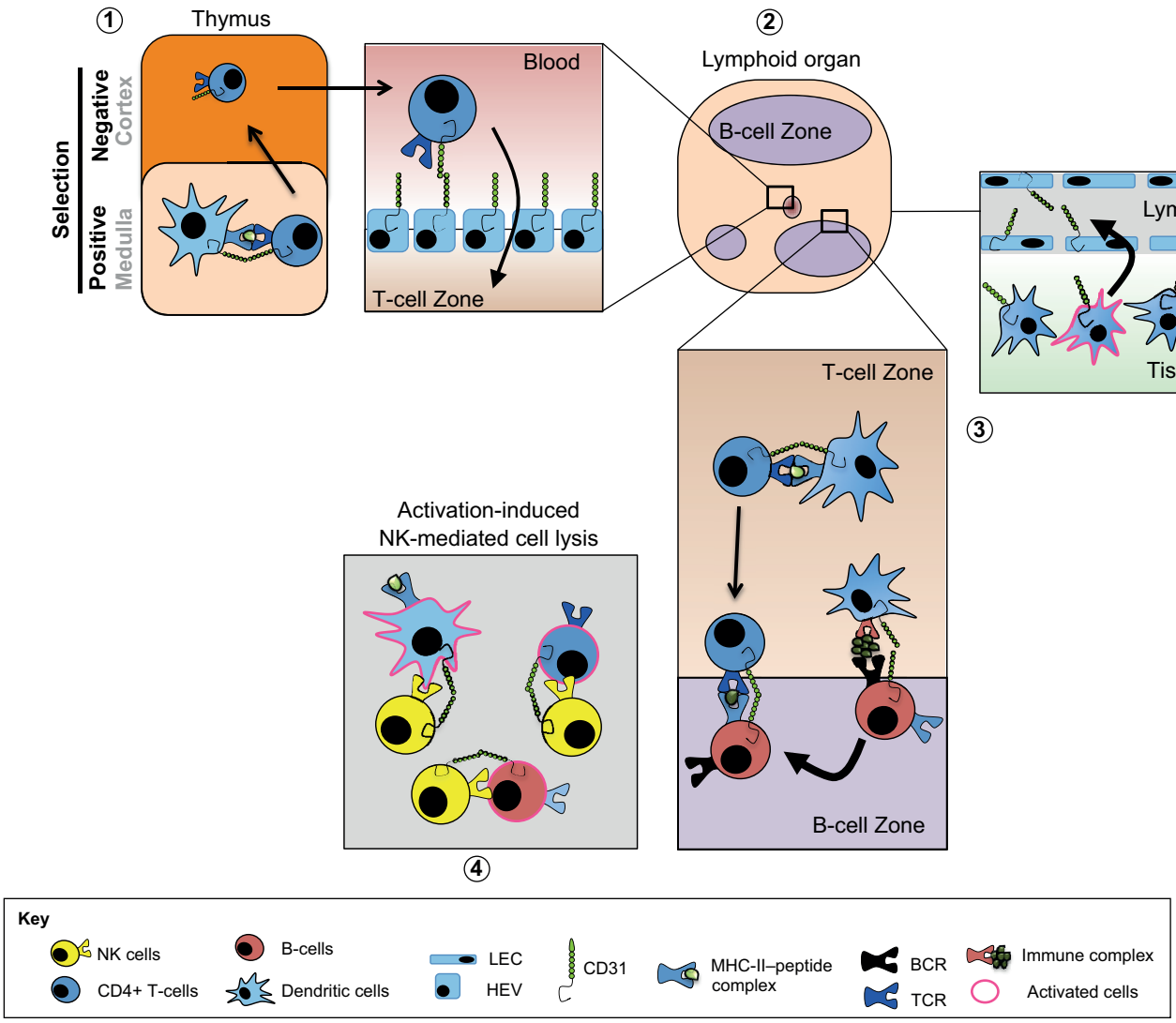

Fig. 3. Putative functions of CD31 in the regulation of the immune system. (1) In the thymus, CD31 interactions might affect the positive and negative selection of thymocytes by regulating TCR signaling. (2) In mature naïve T-cells, CD31 contributes to the regulation of homeostatic expansion and facilitates their access to lymphoid organs. (3) T-cell expansion and survival following activation are also regulated by CD31-mediated interactions with DCs; these interactions, in turn, protect DCs from cytolytic killing. CD31–CD31 interactions might also be important to control the expansion of antigen-specific B-cells that are stimulated by T-cells and/or dendritic cells bearing antibody–antigen immune complexes. (4) Finally, activation-induced natural killer (NK)-mediated cell lysis of T- and B-lymphocytes and of dendritic cells might also be regulated by CD31–CD31 interactions between these immune cells. BCR, B-cell receptor; HEV, high endothelial venule; LEC, lymphatic endothelial cell; MHC, major histocompatibility complex; TCR, T-cell receptor.

regulatory role is coordinated and exerted differentially depending on the cells in which CD31 is expressed, its cellular compartmentalization and the biological process taking place.

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