Molecular mechanisms of endothelial cytoprotection by CD31-mediated signals

By Chat Pan Kenneth Cheung

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The William Harvey Research Institute,
Barts and The London School of Medicine and Dentistry
at Queen Mary University of London

Supervised by:
Prof Federica Marelli-Berg
Dr Claudio Mauro

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Abstract

Maintenance of vascular integrity during inflammation is a major challenge for the cooperation between the immune and the vascular systems. Continuous exposure to inflammatory stimuli is a risk for dysfunction of the vascular endothelial cells. The objectives of this study were to assess 1) the cytoprotective role of CD31 to TNFα and cytotoxic T lymphocytes in both in vitro and in vivo allotransplantation settings and 2) the role of CD31 in the regulation of T cell: EC interactions during leukocyte extravasation in response to inflammatory stimuli. My results first showed that the Ig-family member CD31, which is expressed by endothelial but not epithelial cells, is necessary to prevent primary EC death induced by TNFα and cytotoxic T lymphocytes in vitro. Combined qRT-PCR array and biochemical analysis showed that, upon engagement of TNF-R with TNFα on ECs, CD31 becomes activated and, in turn, modulates the pro-apoptotic transcriptional programme induced by TNFα via activation of Erk and Akt pathways. Specifically, Akt activation by CD31 signals prevents the localization of the forkhead transcription factor FoxO3 to the nucleus, thus inhibiting transcription of the pro-apoptotic genes CD95/Fas and Caspase 7 and de-repressing expression of the anti-apoptotic gene cFlar. Both CD31 intracellular ITIM motifs are required for its pro-survival function. Importantly, CD31 gene transfer is sufficient to recapitulate the cytoprotective mechanisms in CD31-negative pancreatic beta cells, which become resistant to immune-mediated rejection when grafted in fully allogeneic recipients. Secondly, using CD31-deficient mice, I 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. I 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.
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Lastly, my sense of gratitude also to those who directly or indirectly have lent their hand in this venture.

It's one of the hardest challenges in my life, but it is worthy!
DECLARATION

I, Kenneth Cheung, confirm that the research included within this thesis is my own work.

I carried out all of the research and analyses, except for transplantation of pancreatic cells in mice and the in vivo experiments as detailed in Chapter 2.16-2.17 & 4.1.3 – 4.1.4, which were performed by Dr Liang Ma.

In addition, I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party’s copyright or other Intellectual Property Right, or contain any confidential material.

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List publications

Publications arising from this thesis

CD31 signals confer immune privilege to the vascular endothelium


CD31 Exhibits Multiple Roles in Regulating T Lymphocyte Trafficking In Vivo

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List of abbreviations

AJ: Adherens junction
CD: Cluster of differentiation
CFSE : Carboxyfluorescein Succinimidyl Ester
CTL : Cytotoxic T lymphocyte
DAPI : 4',6-diamidino-2-phenylindole
DMSO : Dimethyl sulphoxide
DMBA : 7,12-Dimethylbenz(a)anthracene
EC : Endothelial cell
EDTA : Ethylenediaminetetraacetic acid
FACS : Fluorescence-activated cell sorting
FCS : Foetal Calf Serum
FLAR: Flice-inhibitory protein
GFP : Green fluorescent protein
IFNγ : Interferon gamma
IL-x : Interleukin-x
i.p. : Intraperitoneal
i.v. : Intravenous
KO: Knock out
mAb : Monoclonal antibody
MHC: Major Histocompatibility Complex
PECAM-1: Platelet endothelial cell adhesion molecule-1 (CD31)
PBS : Phosphate Buffered Saline
PBMC : Peripheral blood mononuclear cells
PD-1: Programmed Death 1

PDL-1/2: Programmes Death Ligand 1/2

pMHC : peptide-MHC complex

s.c. : subcutaneous

Tc : T cell

TJ: Tight Junction

WT : Wild Type
Chapter 1 - Introduction
1.1 The vascular system

The vascular system is often seen to comprise the circulatory system, which distributes blood. Arteries that carry oxygenated blood away from the heart to the body; veins that carry blood from the body back to the heart. Capillaries which are the tiny blood vessels between arteries and veins that distribute oxygen-rich blood to tissues and organs.

Figure 1. The circulatory system. The circulatory system is made of the vessels that carry blood and lymph through the body. Arteries and veins carry blood throughout the body, delivering oxygen and nutrients to the tissues and taking away waste products. Picture adapted from McKesson Health Solutions, LLC.
Due to its function, the vascular system is integrated with all other body systems.

Examples include:

- **Respiratory system.** As blood flows through the capillaries in the lungs, carbon dioxide is given up and oxygen is picked up. The carbon dioxide is expelled from the body through the lungs, and the oxygen is taken to the body tissues from the blood.

- **Digestive system.** As food is digested, blood flows through the intestinal capillaries and picks up nutrients, such as glucose (sugar), vitamins, and minerals. These nutrients are delivered to the body tissues by the blood.

- **Kidneys and urinary system.** Waste materials from the body tissues are filtered out from the blood as it flows through the kidneys. The waste material then leaves the body in the form of urine.

The vascular system also functions as a ‘route’ for cell translocation throughout bodily compartments: thus, migratory cells such as stem cells and leukocytes can cross the vascular wall in both directions without altering the vessel integrity. This important function is mediated and regulated by interactions between migratory cells and the endothelium, and physiologically occurs at the level of postcapillary venules.
1.1. 1 The lymphatic system

The lymphatic system is part of the circulatory system and a vital part of the immune system, comprising a network of lymphatic vessels that carry a clear fluid called lymph directionally towards the heart.

The lymphatic system has multiple interrelated functions:

- It is responsible for the removal of interstitial fluid from tissues
- It absorbs and transports fatty acids and fats as chyle from the digestive system
- It transports white blood cells to and from the lymph nodes into the bones
- The lymph transports antigen-presenting cells (APCs), such as dendritic cells, to the lymph nodes where an immune response is stimulated.

The other main function is that of defense in the immune system. Lymph is very similar to blood plasma: it contains lymphocytes and other white blood cells. It also contains waste products and debris of cells together with bacteria and protein. Associated organs composed of lymphoid tissue are the sites of lymphocyte production. Lymphocytes are concentrated in the lymph nodes. The spleen and the thymus are also lymphoid organs of the immune system. The tonsils are lymphoid organs that are also associated with the digestive system. Lymphoid tissues contain lymphocytes, and also contain other types of
cells for support. The system also includes all the structures dedicated to the circulation and production of lymphocytes (the primary cellular component of lymph), which also includes the bone marrow, and the lymphoid tissue associated with the digestive system (Figure 2).

![Diagram of the lymphatic system](image)

**Figure 2 The lymphatic system.** The lymphatic system primarily consists of lymphatic vessels, which are similar to the circulatory system's veins and capillaries. The vessels are connected to lymph nodes, where the lymph is filtered. The tonsils, adenoids, spleen and thymus are all part of the lymphatic system. Picture adapted from Wikimedia Commons.

The blood does not come into direct contact with the parenchymal cells and tissues in the body (except in case of an injury causing rupture of one or more blood vessels), but constituents of the blood first exit the microvascular exchange blood vessels to become interstitial fluid, which comes into contact with the parenchymal cells of the body. Lymph is the fluid that is formed when interstitial fluid enters the initial lymphatic vessels of the
lymphatic system. The lymph is then moved along the lymphatic vessel network by either intrinsic contractions of the lymphatic passages or by extrinsic compression of the lymphatic vessels via external tissue forces (e.g., the contractions of skeletal muscles), or by lymph hearts in some animals. The organization of lymph nodes and drainage follows the organization of the body into external and internal regions; therefore, the lymphatic drainage of the head, limbs, and body cavity walls follows an external route, and the lymphatic drainage of the thorax, abdomen, and pelvic cavities follows an internal route. Eventually, the lymph vessels empty into the lymphatic ducts, which drain into one of the two subclavian veins, near their junction with the internal jugular veins.

1.1.2 Lymphoid organs

The thymus and the bone marrow constitute the primary lymphoid organs involved in the production and early clonal selection of lymphocyte tissues. Bone marrow is responsible for both the creation of T cells and the production and maturation of B cells. From the bone marrow, B cells immediately join the circulatory system and travel to secondary lymphoid organs in search of pathogens. T cells, on the other hand, travel from the bone marrow to the thymus, where they develop further. Mature T cells join B cells in search of pathogens. The other 95% of T cells begin a process of apoptosis (programmed cell death).

The central or primary lymphoid organs generate lymphocytes from immature progenitor cells.
Secondary or peripheral lymphoid organs, which include lymph nodes and the spleen, maintain mature naive lymphocytes and initiate an adaptive immune response. The peripheral lymphoid organs are the sites of lymphocyte activation by antigens. Activation leads to clonal expansion and affinity maturation. Mature lymphocytes recirculate between the blood and the peripheral lymphoid organs until they encounter their specific antigen.

Secondary lymphoid tissue provides the environment for the foreign or altered native molecules (antigens) to interact with the lymphocytes. It is exemplified by the lymph nodes, and the lymphoid follicles in tonsils, Peyer's patches, spleen, adenoids, skin, etc. that are associated with the mucosa-associated lymphoid tissue (MALT).

In the gastrointestinal wall the appendix has mucosa resembling that of the colon, but here it is heavily infiltrated with lymphocytes.

The tertiary lymphoid tissue typically contains far fewer lymphocytes, and assumes an immune role only when challenged with antigens that result in inflammation. It achieves this by importing the lymphocytes from blood and lymph.
1.1. 3 Thymus

The thymus is a primary lymphoid organ and is the site of maturation for T cells, the lymphocytes of the adaptive immune system. The loss or lack of the thymus results in severe immunodeficiency and subsequent high susceptibility to infection (Miller, 2002).

1.1. 4 Spleen

The spleen synthesizes antibodies in its white pulp and removes antibody-coated bacteria and antibody-coated blood cells by way of blood and lymph node circulation. A study published in 2009 using mice found that the spleen contains, in its reserve, half of the body's monocytes within the red pulp. These monocytes, upon moving to injured tissue (such as the heart), turn into dendritic cells and macrophages while promoting tissue healing. The spleen is a center of activity of the mononuclear phagocyte system and can be considered analogous to a large lymph node, as its absence causes a predisposition to certain infections.

Like the thymus, the spleen has only efferent lymphatic vessels. Both the short gastric arteries and the splenic artery supply it with blood. The germinal centers are supplied by arterioles called *penicilliary radicles*.
Up to the fifth month of prenatal development the spleen creates red blood cells. After birth the bone marrow is solely responsible for hematopoiesis. As a major lymphoid organ and a central player in the reticuloendothelial system, the spleen retains the ability to produce lymphocytes. The spleen stores red blood cells and lymphocytes. It can store enough blood cells to help in an emergency. Up to 25% of lymphocytes can be stored at any one time.

1.1. 5 Lymph nodes

A lymph node is an organized collection of lymphoid tissue, through which the lymph passes on its way back to the blood. Lymph nodes are located at intervals along the lymphatic system. Several afferent lymph vessels bring in lymph, which percolates through the substance of the lymph node, and is then drained out by an efferent lymph vessel. There are between five and six hundred lymph nodes in the human body, many of which are grouped in clusters in different regions as in the underarm and abdominal areas. Lymph node clusters are commonly found at the base of limbs (groin, armpits) and in the neck, where lymph is collected from regions of the body likely to sustain pathogen contamination from injuries.

The substance of a lymph node consists of lymphoid follicles in an outer portion called the cortex. The inner portion of the node is called the medulla, which is surrounded by the cortex on all sides except for a portion known as the hilum. The hilum presents as a depression on the surface of the lymph node, causing the otherwise spherical lymph node to be bean-shaped or ovoid. The efferent lymph vessel directly emerges from the lymph node.
node at the hilum. The arteries and veins supplying the lymph node with blood enter and exit through the hilum.

The region of the lymph node called the paracortex immediately surrounds the medulla. Unlike the cortex, which has mostly immature T cells, or thymocytes, the paracortex has a mixture of immature and mature T cells. Lymphocytes enter the lymph nodes through specialised high endothelial venules found in the paracortex.

A lymph follicle is a dense collection of lymphocytes, the number, size and configuration of which change in accordance with the functional state of the lymph node. For example, the follicles expand significantly when encountering a foreign antigen. The selection of B cells, or B lymphocytes, occurs in the germinal center of the lymph nodes.

Lymph nodes are particularly numerous in the mediastinum in the chest, neck, pelvis, axilla, inguinal region, and in association with the blood vessels of the intestines.

**Figure 3 Lymph node anatomy.** Follow the flow of lymph from the afferent lymphatic vessel, through the interior of the node, to the efferent lymphatic vessel. Picture adapted from Wikimedia Commons.
1.1. 6 Other lymphoid tissue

Lymphoid tissue associated with the lymphatic system is concerned with immune functions in defending the body against infections and the spread of tumors. It consists of connective tissue formed of reticular fibers, with various types of leukocytes, (white blood cells), mostly lymphocytes enmeshed in it, through which the lymph passes. Regions of the lymphoid tissue that are densely packed with lymphocytes are known as lymphoid follicles. Lymphoid tissue can either be structurally well organized as lymph nodes or may consist of loosely organized lymphoid follicles known as the mucosa-associated lymphoid tissue (Figure 4).

**Figure 4 Regional lymph nodes.** Each lymph node chain is responsible for draining a particular body region or structure. For example, the lymph node chains in your armpits drain your arms and upper chest, while the chains in your groin are responsible for filtering lymph from your feet, legs, genitals, and lower abdominal wall. Picture adapted from Wikimedia Commons.
1.1.7 Lymphatic vessel

The lymphatic vessels, also called lymph vessels, conduct lymph between different parts of the body. They include the tubular vessels of the lymph capillaries, and the larger collecting vessels—the right lymphatic duct and the thoracic duct (the left lymphatic duct). The lymph capillaries are mainly responsible for the absorption of interstitial fluid from the tissues, while lymph vessels propel the absorbed fluid forward into the larger collecting ducts, where it ultimately returns to the bloodstream via one of the subclavian veins. These vessels are also called the lymphatic channels or simply lymphatics.

The lymphatics are responsible for maintaining the balance of the body fluids. Its network of capillaries and collecting lymphatic vessels work to efficiently drain and transport extravasated fluid, along with proteins and antigens, back to the circulatory system. Numerous intraluminal valves in the vessels ensure a unidirectional flow of lymph without reflux (Vittet, 2014). Two valve systems are used to achieve this one directional flow—a primary and a secondary valve system. The capillaries are blind-ended, and the valves at the ends of capillaries use specialized junctions together with anchoring filaments to allow a unidirectional flow to the primary vessels. The collecting lymphatics, however, act to propel the lymph by the combined actions of the intraluminal valves and lymphatic muscle cells (Heppell et al., 2015) (Figure 5).
1.2 Endothelium

The endothelium is a layer of tightly connected cells lining the inner surface of blood vessels. The endothelium functions as a major barrier at the interface between blood and tissues by limiting entry of plasma, cells, and molecules from the circulation into the organ parenchyma. In the microcirculation, the function of microvascular endothelium is to control blood perfusion by regulating vasomotor response to vasoactive hormones or metabolites to ensure adequate nutrient supply that meets the demand of tissues.

The semipermeable property of the endothelium - comprising the wall of capillaries and postcapillary venules - enables plasma fluid, nutrients, and cells to move into surrounding tissues and metabolic byproducts to be taken into the circulation. These microvessels serve as the major site for blood–tissue exchange and thus are crucial in fluid and metabolic homeostasis. Appropriate regulation of microvascular fluid hydrodynamics and endothelial barrier function is vital to support normal tissue viability and organ function.
1.2.1 Paracellular and transcellular pathways

Classically, blood fluids, solutes, and circulating cells cross the endothelium via two routes: paracellular and transcellular (Figure 6).

Figure 6. Transcellular and paracellular permeability pathways across the microvascular endothelium. Barrier function of the microvasculature is provided by closely apposed ECs of the microvessel walls. Paracellular pathways allow the selective passage of solutes and fluids across the endothelium. Solute can also traverse the cell interior via receptor-mediated vesicle endocytosis originating at caveolae, or via vacuole-vesicular organelles (VVOs) that can fuse with trafficking vesicles and form open transcellular pores via the transcellular pathway (Yuan and Rigor, 2010). Picture adapted from Regulation of Endothelial Barrier Function by Sarah Yuan, Robert Rigor.
1.2. 2 Paracellular permeability, cell-cell junctions

The paracellular pathway is responsible for the majority of leakage of blood fluids and proteins across the microvascular endothelium under pathophysiological conditions. The structural and functional integrity of these junctions are a major determinant of paracellular permeability. There are discontinuities or fenestrations between ECs among certain types of tissues or organs, such as the kidney and the liver, where there are sufficiently large spaces to permit the passage of large molecules or proteins (Michel and Curry, 1999). In other organs, most EC-EC interfaces are tightly kept together by intercellular junctions. Two types of intercellular junctions have been characterized as the cell–cell adhesive barrier structures in the microvascular endothelium: the adherens junction (AJ) and the tight junctions (TJ) (Mehta and Malik, 2006) (Figure 7).

Figure 7. Endothelial cell junctions. ECs of the microvessel wall are joined together by intercellular junction proteins: adherens junctions (AJs), tight junctions (TJs) and/or gap junctions. Barrier function in most vascular beds is provided by AJs. Some specialized microvascular beds rely upon TJs for additional barrier function. Gap junctions, formed of connexins, primarily facilitate signaling between ECs, and do not directly contribute to barrier
ECs are anchored to the basement membrane via focal adhesions. Focal adhesions and intercellular junctions are interconnected via cytoskeleton. Barrier function is dependent upon the stability and integrity of these three elements. Picture adapted from Regulation of Endothelial Barrier Function by Sarah Yuan, Robert Rigor.

1.2. 3 Adherens junctions

AJ are the most ubiquitous type of endothelial cell–cell junction and form the major determinant of endothelial barrier to macromolecules in many organs and tissues (Mehta and Malik, 2006).

Vascular Endothelium (VE)-cadherin (CD144) is a key component of AJs. It is a calcium-dependent cell–cell adhesion glycoprotein composed of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail. Early studies using blocking antibodies to VE-cadherin increased monolayer permeability in cultured cells (Corada et al., 2001) and vascular defects (embryonic lethal) have been reported in two mouse models of VE-cadherin deficiency (Carmeliet et al., 1999). VE-cadherin functions as a classic cadherin by imparting to cells the ability to adhere in a homophilic manner with neighbouring cells (trans-interaction). VE-cadherin has a transmembrane domain followed by a cytoplasmic tail that directly associates with β-catenin and γ-catenin (plakoglobin). They are highly homologous members of the Armadillo protein family might act as positive (plakoglobin) and negative (β-catenin) regulators to the strength of the junction. Noted, this molecules could act as a linker between cadherin/catenins complex and actin based cytoskeleton thus directly increasing or decreasing junction cohesion and strength (Lampugnani et al., 1995). p120-catenin can also binds to the cytoplasmic domain of VE-cadherin and is thought to regulate cadherin function, stability,
and availability at the cell surface. The catenins can also transduce biochemical signals for cell–cell communications (Harris and Nelson, 2010; Wang et al., 2006) (Figure 8).

Other proteins such as junctional adhesion molecules (JAMs), ESAM-1, CD99 and CD31 have also been suggested to participate to EC: EC junctions via homophilic trans-cellular interactions. JAM proteins can bind to zona occludens 1 (ZO-1), a linker protein that connects tight junction proteins to α-catenin, as well as to signaling molecules and proteins that stabilize the actin cytoskeleton.

Figure 8. Adherens junctions. AJs are ubiquitous throughout the vasculature. The intercellular adhesion protein vascular endothelial (VE)-cadherin is principally responsible for barrier function. VE–cadherin homophilic intercellular binding is stabilized by calcium ions and by intracellular connections to the actin cytoskeleton: α-, β-, γ and p120-catenin connect VE–cadherin to actin microfilaments. Other junction proteins contribute to AJ structure, including Junction adhesion molecules (JAM-A, -B and -C) and platelet-EC adhesion molecule CD31. JAMs connect to the actin cytoskeleton via zona occludens (ZO)-1 and α-catenin, which may stabilize AJs. CD31 facilitates cell–cell binding with circulating blood cells.
1.2. 4 Tight junctions

Compared to AJs, TJs are less common in the peripheral microvasculature. TJs are mainly expressed in the microvascular endothelium of some specialized tissues, for example, the blood–brain or blood–retinal barriers (Hawkins and Davis, 2005). TJs impart additional barrier function, preventing the passage of much smaller molecules (<1 kDa), even restricting the flow of small inorganic ions (e.g., Na⁺).

These junctions depend on interactions of a number of proteins: occludin, claudins (3/5), and JAM-A. Occludin and claudins are integral membrane proteins, each with four transmembrane domains and two extracellular loop domains. The extracellular loop domains of occludin or claudins form homotypic binding with the extracellular domains of on neighboring ECs. JAM-A, a member of the immunoglobulin superfamily of proteins, is also present in tight junctions, though the role of JAM-A in tight junctions is not understood. Occludin, claudins, and JAM-A are connected to the actin cytoskeleton via zona occludens proteins (ZO-1, ZO-2) and α-catenin (Hawkins and Davis, 2005) (Figure 9).
Figure 9 Tight junctions. Tight junctions (TJs) are found in most vascular beds; however, TJs contribute to microvascular barrier function only in a few specialized tissues, including the brain, retina and testicles. The intercellular junction proteins mainly responsible for TJ barrier function are occludin and claudin-5. TJ protein intercellular binding is stabilized by connections to the actin cytoskeleton via ZO-1, ZO-2 and α-catenin. JAM-A also contributes to TJ structure. Picture adapted from Regulation of Endothelial Barrier Function by Sarah Yuan, Robert Rigor.

1.2. 5 Transcellular permeability, vesicular transcytosis

Transcytosis represents an important pathway of endothelial transcellular permeability to macromolecules (Chidlow and Sessa, 2010; Komarova and Malik, 2010). The mechanism involves vesicle-mediated endocytosis at the endothelial luminal membrane, followed by transeptosis across the cell, and exocytosis at the basolateral membrane. Alternatively this process can be done by clusters of interconnected vesiculo-vacuolar organelles (VVOs) that form channel-like structures 80–200 nm in diameter, spanning the cell interior (Dvorak et al., 1996).
Vesicle-mediated transcytosis occurs when albumin binds to gp60 receptors on the EC surface (Hu and Minshall, 2009; Minshall et al., 2000). Endocytosis and exocytosis are mediated by caveolae, lipid raft microdomains that form “cave-like” invaginations in the plasma membrane (Predescu and Palade, 1993) (Figure 6).

1.2. 6 The endothelial cytoskeleton

The endothelial cytoskeleton is composed of microtubules, intermediate filaments and actin filaments in most cell types (Prasain and Stevens, 2009). These structures are important for EC morphology, adhesion, and barrier function. The actin cytoskeleton is most centrally important for regulation of endothelial permeability.

1.2. 7 Microtubules

Microtubules are tubular structures composed of polymers of heterodimeric subunits of alpha and beta tubulin. The 13 parallel polymeric filaments are arranged in a ring structure. In ECs, microtubules are cross-linked to actin filaments and can affect endothelial permeability through effects on actin filaments. The stability of microtubules is determined by dynamic polymerization and depolymerization that regulate the permeability.
Dynamic instability refers to the coexistence of assembly and disassembly at the ends of a microtubule. The microtubule can dynamically switch between growing and shrinking phases in this region. Tubulin dimers can bind two molecules of GTP, one of which can be hydrolyzed subsequent to assembly. During polymerization, the tubulin dimers are in the GTP-bound state (Weisenberg, 1972). The GTP bound to α-tubulin is stable and it plays a structural function in this bound state. However, the GTP bound to β-tubulin may be hydrolyzed to GDP shortly after assembly. The assembly properties of GDP-tubulin are different from those of GTP-tubulin, as GDP-tubulin is more prone to depolymerization (Weisenberg et al., 1976). A GDP-bound tubulin subunit at the tip of a microtubule will tend to fall off, although a GDP-bound tubulin in the middle of a microtubule cannot spontaneously pop out of the polymer. Since tubulin adds onto the end of the microtubule in the GTP-bound state, a cap of GTP-bound tubulin is proposed to exist at the tip of the microtubule, protecting it from disassembly. When hydrolysis catches up to the tip of the microtubule, it begins a rapid depolymerization and shrinkage. This switch from growth to shrinking is called a catastrophe. GTP-bound tubulin can begin adding to the tip of the microtubule again, providing a new cap and protecting the microtubule from shrinking. This is referred to as "rescue" (Mitchison and Kirschner, 1984).

Depolymerization of microtubules can also activate guanine nucleotide exchange factors, and signaling through Rho family GTPases, leading to actin stress fiber formation (Birukova et al., 2004). Signaling through Rho kinases has further consequences for endothelial barrier function (See 1.2.12-1.2.13).
1.2. 8 Intermediate filaments

Intermediate filaments are formed of heterogeneous polymeric protein arrangements (Wang and Stamenovic, 2002). The principle intermediate filament protein monomer is vimentin in most cells. They control the EC structure, and are expressed most abundantly in cells exposed to shear stress, e.g., aortic ECs. Intermediate filaments are connected to cell–cell junction proteins and to focal attachments to the basement membrane.

1.2. 9 Actin filaments

Actin filaments are linear polymers of filamentous (F)-actin (Prasain and Stevens, 2009). The stability of actin filaments is dependent upon the concentration of globular (G)-actin within the cell. Upon treatment of ECs with hyperpermeability-inducing agents, such as thrombin or histamine, actin filaments organize into linear, parallel bundles across the cell interior (stress fibers). Stress fiber formation is often accompanied by a contractile cell morphology and formation of gaps between adjacent ECs. In contrast, when ECs are treated with barrier-protective agents such as sphingosine-1-phosphate, actin filaments re-organize and localize at the cell periphery, appearing to strengthen cell–cell contacts.

1.2. 10 Signalling mechanisms in the regulation of endothelial permeability

In microvessels, permeability responses are initiated by endothelial surface receptors, followed by activation of a variety of intracellular signaling molecules, including second
messengers, kinases, phosphatases, and GTPases. Some of these mediators important for the signaling of endothelial barrier function will be mentioned in the following sections.

1.2. 11 Src kinases.

Src family kinases are known to mediate microvascular endothelial hyperpermeability through phosphorylation of tyrosine residues on numerous proteins involved in endothelial cell–cell and cell–matrix adhesions (Patterson et al., 1992). For example, Src kinase is required for the endothelial hyperpermeability in response to VEGF (Eliceiri et al., 1999), tumor necrosis factor TNFα (Nwariaku et al., 2002) and reactive oxygen species (ROS) (Kevil et al., 2001). At cell–cell junctions, activated neutrophils and pro-inflammatory cytokines, such as TNFα, cause Src family kinase-dependent phosphorylation of VE–cadherin, an event believed to destabilize the adherens junction complex. Thus protein phosphorylation regulated by protein kinases (as opposed to protein phosphatase-catalyzed dephosphorylation) is a major determinant of endothelial barrier function (Hu et al., 2008).

1.2. 12 Small GTPases

GTPases are a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP) (Scheffzek and Ahmadian, 2005). The GTP binding and hydrolysis takes place in the highly conserved G domain common to all GTPases.

All regulatory GTPases have a common mechanism that enables them to switch a signal transduction chain on and off. Toggling the switch is performed by the unidirectional
change of the GTPase from the active, GTP-bound form to the inactive, GDP-bound form by hydrolysis of the GTP through intrinsic GTPase-activity, effectively switching the GTPase off. This reaction is initiated by GTPase-activating proteins (GAPs), coming from another signal transduction pathway. It can be reversed (switching the GTPase on again) by Guanine nucleotide exchange factors (GEFs), which cause the GDP to dissociate from the GTPase, leading to its association with a new GTP. This closes the cycle to the active state of the GTPase; the irreversible hydrolysis of the GTP to GDP forces the cycle to run only in one direction. Only the active state of the GTPase can transduce a signal to a reaction chain (Figure 10).

**Figure 10 Small GTPase-mediated control of endothelial barrier function.** Small GTPases play central roles in endothelial barrier dysfunction (e.g., RhoA) and barrier protection (e.g., Rac-1 or Cdc-42). Small GTPases are bound to GDP in the inactive state, stabilized by GDP-dissociation inhibitors (GDIs). Upon release from GDI binding, GDP can be exchanged for GTP by GTP-exchange factors (GEFs). Small GTPases become active once in the GTP-bound state. Inactivation occurs when GTP is exchanged for GDP by GTPase-activating proteins (GAPs). Picture adapt from. REGULATION OF SMALL GTPases BY GEFs, GAPs, AND GDIs by Jacqueline Cherfils and Mahel Zeghouf.
1.2. 13 Rho Cascade - stress fiber formation

Activation of RhoA increases microvascular endothelial permeability (Spindler et al., 2010), whereas basal RhoA activity maintains normal physiological barrier function in microvascular endothelium, i.e., basal permeability is increased upon exposure to RhoA inhibitors. RhoA is also centrally involved in endothelial hyperpermeability through effects on multiple cellular events including actin stress fiber formation and increased actomyosin contractility. Hyperpermeability in response to thrombin is mediated through activation of Rho kinase (ROCK), which in turn phosphorylates and inactivates MLCP.

In contrast to RhoA, two other small GTPases, Rac-1 and Cdc42, decrease endothelial permeability and improve barrier function (Kumar et al., 2009). These small GTPases are activated downstream of barrier protective signaling in response to sphingosine-1-phosphate (S1P), or during resolution (late stages) of hyperpermeability caused by inflammatory agents. Therefore, activation of Cdc42 and Rac-1 is often considered a feedback/repair mechanism following endothelial barrier dysfunction.

1.2. 14 Dysregulation of endothelial barrier function

The endothelial barrier is a dynamic system that undergoes constant remodeling with steady-state arrival on a moment-to-moment basis. Dysregulation of endothelial barrier function happens in different disease states and injury considerations, such as inflammation, trauma, ischemia/reperfusion injury, diabetes mellitus, multiple sclerosis, thrombosis, metastatic tumor development, infectious disease, and sepsis, as well as during...
exposure to certain drugs or toxicants. Endothelial barrier dysfunction is characterized by leakage of fluid, proteins, or small molecules. Excessive flux of these molecules across the endothelium, termed hyperpermeability, which leads to vascular diseases can be seen. It clinically manifests as accumulation of plasma-like, protein-rich fluid in the extravascular space leading to tissue swelling, termed edema.

Physical forces (such as shear stress) or biological factors (such as inflammatory mediators) also cause changes in endothelial permeability by altering the synthesis and expression of cell–cell junction proteins or cell–matrix adhesion molecules in the long term (e.g., during chronic inflammation or angiogenesis), or by inducing conformational reorganization of the junction or focal adhesion complexes when stimulated by acute inflammatory agents, such as histamine, or during leukocyte transendothelial migration. Activated leukocytes release reactive oxygen species and enzymes that further perpetuate and prolong barrier dysfunction (Yuan and Rigor, 2010).

1.2. 15 Inflammation

Inflammation is a multi-faceted reaction to tissue injury and/or infection. Uncontrolled and persistent inflammation underlies many of the most common diseases in western societies. The cardinal signs of inflammation were first characterized by Celsus in the first century A.D. as rubor (redness), tumor (swelling), calor (heat), and dolor (pain). These cardinal signs are largely the result of two main components of inflammatory responses: 1) increased vascular permeability; 2) the emigration from the blood vessel, accumulation, and activation of leukocytes in the inflamed tissue (Lawrence et al., 2002). The modulation of vascular permeability and the recruitment of leukocytes rely on junctional adhesion
molecules which mediate intercellular communication amongst adjacent EC and between EC: leukocytes. Cellular adhesion molecule-mediated interactions allow leukocytes to home to the site of inflammation (leukocyte extravasation), influence the release of inflammatory mediators that activate both cell types, and are important for the maintenance of vascular barrier function (endothelial tight junction). Consequently, cellular adhesion molecules mediated-interactions are vitally important to the initial activation, maintenance, and subsequent resolution of inflammation.

Noted, platelets, prostaglandins and Cyclooxygenase (COX) enzymes are also essentials early components of the inflammatory response. Prostaglandins are local hormones (paracrine) produced in the body, that have diverse effects in the body, including transmission of pain formation to brain, modulation of the hypothalamic thermostat, and inflammation. Normally Cyclooxygenase (COX) produces prostaglandins most of which are proinflammatory and thromboxanes which are responsible for the aggregation of platelets that form blood clots. Heart attacks are primarily caused by blood clots, and their reduction with the introduction of small amounts of aspirin has been seen to be an effective medical intervention (American Heart Association: Aspirin in Heart Attack and Stroke Prevention).

1.2. 16 Leukocyte extravasation

Leukocyte extravasation from the blood flow and into sites of tissue inflammation is a tightly controlled process that involves the multi-step action of traffic signals and adhesion molecules that mediate rolling, adhesion and transendothelial migration (Figure 11).
Leukocyte extravasation occurs mainly in post-capillary venules, where hemodynamic shear forces are minimized. Leukocyte-EC interactions during extravasation include capturing, rolling, activation, binding, strengthening of the binding and spreading, intravascular creeping, paracellular migration or transcellular migration. Leukocyte recruitment is halted whenever any of these steps is suppressed.

The basic mechanisms which support leukocyte extravasation are similar in all leukocyte subtypes.
Figure 11 The leukocyte-extravasation cascade. Leukocyte extravasation from the blood into inflamed tissues follows a multistep cascade that involves the sequential action of molecular signals and adhesion molecules. Selectins (such as P-selectin and E-selectin) initiate leukocyte tethering and rolling along inflamed endothelium. Rolling slows down circulating leukocytes, bringing them into close proximity with EC and allowing binding of chemokines (such as CC-chemokine ligand 5 and CXC-chemokine ligand 8, also known as IL-8) that are displayed on inflamed endothelium to their specific G-protein-coupled chemokine receptors on leukocytes. Activation of chemokine receptors triggers intracellular signalling pathways that activate leukocyte integrins.

1.2. 17 Multiple adhesion cascades

Upon recognition of and activation by pathogens, resident macrophages in the affected tissue release cytokines such as IL-1, TNFα and chemokines. IL-1, TNFα and C5 (Monk et al., 2007) cause the ECs of blood vessels near the site of infection to express cellular adhesion molecules, including selectins.
1.2. 18 Rolling

Selectin molecules on the inner wall of the vessel bind to carbohydrate ligands on the circulating leukocytes like velcro, with relatively low affinity. This causes the leukocytes to slow down and begin rolling along the inner surface of the vessel wall. During this rolling motion, transitory bonds are formed and broken between selectins and their ligands.

1.2. 19 Activation and tight adhesion

At the same time, chemokines immobilized on the EC surface by glycosaminoglycans (GAGS) cause surface integrin molecules to switch from the default low-affinity state to a high-affinity state. This is assisted through juxtacrine activation of integrins by chemokines and soluble factors released by ECs. In the activated state, integrins bind tightly to complementary receptors expressed on ECs, with high affinity. This causes the immobilization of the leukocytes, despite the shear forces of the ongoing blood flow.

1.2. 20 Transmigration

The cytoskeletons of the leukocytes are reorganised in such a way that the leukocytes are spread out over the ECs. In this form, leukocytes extend pseudopodia and pass through gaps between ECs. Once through the endothelial cell barrier, leukocytes exhibit sub-endothelial cell motility and extension of ventral membrane protrusions to seek permissive sites in the pericyte sheath and venular basement membrane for their continued migration through venular walls. In the interstitial tissue, leukocytes migrate in an amoeboid manner.
that is largely autonomous from the molecular composition of the extracellular environment.

**Pericytes.** In addition to endothelial cells, pericytes are a cellular component of capillaries and post-capillary and collecting venules, and closely associate with the underlying endothelium through their many long protrusions (Hirschi and D'Amore, 1996). They exhibit morphological and phenotypical differences depending on vessel type, vascular bed, developmental stage, species and the pathological conditions under which they are found. In contrast to endothelial cells, which form a confluent polarized monolayer that is anchored away from the vessel lumen to its basement membrane, in most tissues the pericyte network is loosely distributed around endothelial cells and is embedded in the venular basement membrane. The extent of pericyte coverage around microvessels varies substantially between different organs, suggesting different levels of barrier function in different tissues. Leukocyte migration through the pericyte sheath can occur by both paracellular and transcellular routes (see 1.2.1), but details of the associated mechanisms and the potential role of pericytes in the regulation of leukocyte transmigration remain unclear.

**Venular basement membrane.** The venular basement membrane is generated by both endothelial cells and pericytes. Basement membranes provide an adhesive and structural support for the generating cell, and in venules provide a substantial barrier to migrating cells (Rowe and Weiss, 2008). Leukocyte migration through this structure occurs in several ways, including through biochemically permissive sites. For example, regions expressing laminin 511 are proposed to be anti-migratory, whereas laminin 411 reportedly promotes migration (Hallmann et al., 2005).
1.2. 21 Selectins

Selectins are adhesion molecules specialised in establishing cell contact for the recruitment of leukocytes from the flowing blood stream. They are expressed shortly after cytokine activation of ECs by tissue macrophages. Activated ECs initially express P-selectin molecules, but within two hours after activation E-selectin expression is favoured. Endothelial selectins bind carbohydrates on leukocyte transmembrane glycoproteins, including sialyl-Lewis X. This binding is known to play a vital role in cell-to-cell recognition processes. The sialyl Lewis X determinant, E-selectin ligand carbohydrate structure, is constitutively expressed on granulocytes and monocytes and mediates inflammatory extravasation of these cells. Resting T and B lymphocytes lack its expression and are induced to strongly express sialyl Lewis X upon activation. The sialyl Lewis X determinant is expressed preferentially on activated Th1 cells but not on Th2 cells.

**P-selectins (CD62P):** P-selectin is expressed on activated ECs and platelets. P-selectin is a prototype adhesion molecule–chemoattractant pair that appears on the EC surface subsequent to stimulation by secretion from Weibel-Palade bodies.

Synthesis of P-selectin can be induced by thrombin, leukotriene B4, complement fragment C5a, histamine, TNFα or LPS. These cytokines induce the externalization of Weibel-Palade bodies in ECs, presenting pre-formed P-selectins on the EC surface. P-selectins bind PSGL-1 as a ligand (McEver et al., 1989).
**E-selectins (CD62E):** E-selectin is expressed on activated ECs. Synthesis of E-selectin follows shortly after P-selectin synthesis, induced by cytokines such as IL-1 and TNFα. E-selectins bind PSGL-1 and ESL-1.

**L-selectins (CD62L):** L-selectins are constitutively expressed on naive t cells, and are known to bind GlyCAM-1, MadCAM-1 and CD34 as ligands.

Suppressed expression of some selectins results in a slower immune response. If L-selectin is not produced, the immune response may be ten times slower, as P-selectins (which can also be produced by leukocytes) bind to each other. P-selectins can bind each other with high affinity, but occur less frequently because the receptor-site density is lower than with the smaller E-selectin molecules. This increases the initial leukocyte rolling speed, prolonging the slow rolling phase.

### 1.2. 22 Integrin-mediated leukocyte rolling

Integrins participate in rolling and mediate firm leukocyte adhesion. Cell lines expressing alpha4beta7-integrin roll on immobilized recombinant mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1), and lymphocytes can roll on immobilized vascular cell-adhesion molecule 1 (VCAM1) by engaging their cell-surface ligand very late antigen 4 (VLA4; also known as alpha4beta1-integrin) (Gordon et al., 2002). VLA4-dependent rolling is mostly seen for monocytes and monocyte-like cell lines (Grabsch et al., 2001) and T cells. In vivo, VLA4 supports lymphocyte rolling in venules of the central nervous system (CNS) in conjunction with P-selectin (Grant et al., 2002) or can directly mediate rapid adhesion independent of P-selectin engagement (Gunn et al., 1998).
βeta2-integrins also support rolling. When resting mouse neutrophils suspended in their native whole blood roll on a substrate of recombinant E-selectin and intercellular adhesion molecule 1 (ICAM1), engagement by E-selectin induces an intermediate affinity conformation of lymphocyte function-associated antigen 1 (LFA1; also known as alphaLbeta2-integrin) (Handschel et al., 1999), which allows it to transiently bind to its ligand ICAM1 on the substrate. In at least one study, rolling of human lymphocytes was shown to be enhanced and slowed when ICAM1 was co-expressed with L-selectin ligands in a human vascular endothelial cell line (Heckmann et al., 1998). Similarly, LFA1 expressed by K562 erythroleukaemia cells supported rolling on ICAM1, suggesting that these cells maintain LFA1 in an intermediate affinity state. Recent structural evidence suggests that LFA1 can change its conformation and thereby increase its ligand-binding affinity under shear stress. Taken together, these studies support an important role for the integrin LFA1 in its intermediate affinity conformation as a rolling and signalling molecule.

When mice are treated with tumour-necrosis factor (TNF), endothelial cells express E-selectin and increased amounts of ICAM1, and the rate of neutrophil rolling in the venules becomes slow (below 5 μm per second). Slow rolling in vivo was shown to require not only E-selectin, but also engagement of beta2-integrins, specifically LFA1 or macrophage receptor 1 (MAC1; also known as CD11b–CD18 and alphaMbeta2-integrin). A role for MAC1 in mediating slow rolling in this model is consistent with an early report demonstrating activation of the adhesive capacity of MAC1 following E-selectin engagement (Weston et al., 2015).
1.2. 23 Chemokines

Chemokines are chemotactic cytokines that control the migratory patterns and positioning of immune cells. Chemokine function is critical for all immune cell movement ranging from the migration required for immune cell development and homeostasis, to that required for the generation of primary and amnestic cellular and humoral immune responses, to the pathologic recruitment of immune cells in disease. Chemokines constitute the largest family of cytokines, consisting of approximately 50 endogenous chemokine ligands in humans and mice (Supplementary 1).

Noted, there are two major chemokine sub-families based upon the position of cysteine residues, i.e., CXC and CC. All members of the CXC chemokine sub-family have an intervening amino acid between the first two cysteines; members of the CC chemokine sub-family have two adjacent cysteines. As a general rule (with some notable exceptions), members of the CXC chemokines are chemotactic for neutrophils, and CC chemokines are chemotactic for monocytes and a small sub-set of lymphocytes.

The main function of chemokines is to manage the migration of leukocytes (homing) in the respective anatomical locations in inflammatory and homeostatic processes.

**Basal**: homeostatic chemokines are produced in the thymus and lymphoid tissues. Their homeostatic function in homing is best exemplified by the chemokines CCL19 and CCL21 (expressed within lymph nodes and on lymphatic endothelial cells) and their receptor CCR7 (expressed on cells destined for homing in cells to these organs). Using these ligands is possible routing antigen-presenting cells (APC) to lymph nodes during the
adaptive immune response. Among others homeostatic chemokine receptors include: CCR9, CCR10, and CXCR5, which are important as part of the cell addresses for tissue-specific homing of leukocytes. CCR9 supports the migration of leukocytes into the intestine (Similarly, effector/memory T-cell trafficking into the lamina propria of the small intestine requires the interactions of α4β7 integrin and chemokine receptor CCR9 on lymphocyte surfaces with MAdCAM-1 and CCL (CC chemokine ligand) 25 on endothelial cells of gut lamina propria venules respectively), CCR10 to the skin and CXCR5 supports the migration of B-cell to follicles of lymph nodes. As well CXCL12 (SDF-1) constitutively produced in the bone marrow promotes proliferation of progenitor B cells in the bone marrow microenvironment.

**Inflammatory:** inflammatory chemokines are produced in high concentrations during infection or injury and determine the migration of inflammatory leukocytes into the damaged area. Typical inflammatory chemokines include: CCL2, CCL3 and CCL5, CXCL1, CXCL2 and CXCL8. A typical example is CXCL8, which is a chemoattractant for neutrophils. In contrast to the homeostatic chemokine receptors, there is significantly promiscuous (redundancy) binding receptor and inflammatory chemokines. This often leads to complicated research on receptor-specific therapeutics in this area.

T-lymphocytes: the four key chemokines that are involved in the recruitment of T lymphocytes to the site of inflammation are: CCL2, CCL1, CCL22 and CCL17.
Chemokine receptors are G protein-coupled receptors containing 7 transmembrane domains that are found on the surface of leukocytes. Approximately 19 different chemokine receptors have been characterized to date, which are divided into four families depending on the type of chemokine they bind; CXCR that bind CXC chemokines, CCR that bind CC chemokines, CX3CR1 that binds the sole CX3C chemokine (CX3CL1), and XCR1 that binds the two XC chemokines (XCL1 and XCL2). They share many structural features; they are similar in size (with about 350 amino acids), have a short, acidic N-terminal end, seven helical transmembrane domains with three intracellular and three extracellular hydrophilic loops, and an intracellular C-terminus containing serine and threonine residues important for receptor regulation. The first two extracellular loops of chemokine receptors each have a conserved cysteine residue that allows formation of a disulphide bridge between these loops. G proteins are coupled to the C-terminal end of the chemokine receptor to allow intracellular signaling after receptor activation, while the N-terminal domain of the chemokine receptor determines ligand binding specificity (Murdoch and Finn, 2000).
1.2. 25 Signal transduction

Chemokine receptors associate with G-proteins to transmit cell signals following ligand binding. Activation of G proteins, by chemokine receptors, causes the subsequent activation of an enzyme known as phospholipase C (PLC). PLC cleaves a molecule called phosphatidylinositol (4,5)-bisphosphate (PIP2) into two second messenger molecules known as Inositol triphosphate (IP3) and diacylglycerol (DAG) that trigger intracellular signaling events; DAG activates another enzyme called protein kinase C (PKC), and IP3 triggers the release of calcium from intracellular stores. These events promote many signaling cascades (such as the MAP kinase pathway) that generate responses like chemotaxis, degranulation, release of superoxide anions and changes in the avidity of cell adhesion molecules called integrins within the cell harbouring the chemokine receptor (Murdoch and Finn, 2000).

1.3 Apoptosis

Apoptosis, or programmed cell death, is a process that is essential for development and homeostasis, but also contributes to diverse pathologic processes (Nicholson, 2000), ranging from cancer and atherosclerosis to rheumatic and neurodegenerative diseases. The endothelium senses and transduces signals between blood and tissue, orchestrates the trafficking of hematopoietic cells, maintains a non-thrombogenic surface permitting the flow of blood, and initiates and amplifies the inflammatory response. Situated at the
interface between blood and tissue, the endothelium is exposed to stimuli with the potential to promote or prevent apoptosis (Figure 12).

The physiological endothelial function involves a balance between pro- and anti-apoptotic signals, and perturbation of this balance may contribute to the pathogenesis of diverse vascular diseases (Mallat and Tedgui, 2000).

**Figure 12 Endothelial apoptosis in vascular disease.** Diverse stimuli have been reported to induce EC apoptosis in vitro, and endothelial apoptosis has been demonstrated in vivo in several human diseases and animal models. EC apoptosis can lead to disruption of the endothelial barrier with vascular leak, extravasation of plasma proteins, and exposure of a prothrombotic subendothelial matrix. Apoptotic EC are themselves procoagulant and proadhesive in vitro. Endothelial apoptosis thus has the potential to be an important mechanism of vascular injury and dysfunction (Winn and Harlan, 2005). Picture adapted from Journal of Thrombosis and Haemostasis, 3: 1815–1824.
1.3. 1 Characteristics of apoptosis

Apoptotic cell death was initially defined by characteristic morphologic features including condensation of cytoplasm and nucleus with cell shrinkage. Additionally, there is internucleosomal cleavage of chromatin and blebbing of the plasma membrane.

Importantly, during apoptosis the cell membrane remains intact, preventing release of cellular contents, and there is exposure of a number of surface molecules that trigger rapid engulfment by neighboring or phagocytic cells (Savill, 2000). Consequently, there is little resulting inflammation during apoptosis. In contrast, during necrotic cell death, the cell swells and the membrane is disrupted leading to release of constituents, which elicit an inflammatory response. In reality, the distinctions between apoptotic and necrotic cell death may be blurred as apoptotic cells may undergo secondary necrosis (Okada and Mak, 2004) (Figure 13).
Cellular events in apoptosis. In the process of cell death, apoptosis involves a series of biochemical events in a cell, including cell shrinkage, chromatin condensation, membrane blebbing, nuclear collapse and DNA fragmentation. Some of those events can be used to detect the occurrence of apoptosis. Picture adapted from Toxicol Pathol. 2007; 35(4): 495–516.

During the process of apoptosis, EC adherens junction proteins are degraded with disruption of barrier function \textit{in vitro} (Bannerman et al., 1998). \textit{In vivo}, this could trigger vascular leakage and promote inflammation in adjacent tissue by extravasated plasma constituents such as complement and coagulation factors. As EC are exposed to flowing blood and shear stress, apoptotic EC could detach prior to engulfment. Loss of even a small number of EC by this process could lead to vascular leakage and expose a thrombogenic subendothelial matrix. As apoptotic EC become proadhesive for platelets and leukocytes (Bombeli et al., 1999) as well as procoagulant (Bombeli et al., 1997), they could promote coagulation in situ, prior to engulfment or detachment, or in the circulation once detached.
Several gene families play a pivotal role in regulation of apoptosis in EC as in other cell types. The caspase family of cysteine proteases includes proteases that initiate apoptosis and proteases that act as executioners, ultimately resulting in the dismantling of the apoptotic cell (Riedl and Shi, 2004). The Bcl-2 family includes both pro-apoptotic proteins and anti-apoptotic proteins that largely determine whether a cell lives or dies (Cory et al., 2003). Inhibitors of apoptosis proteins (IAPs) directly bind and inhibit caspases, and are also important determinants of cell fate during apoptosis (Cory et al., 2003).

1.3. 2 Extrinsic and intrinsic apoptosis signal pathway

There are two main pathways that lead to caspase-dependent apoptosis (Figure 14). The extrinsic pathway involves stimulation of members of the tumor necrosis factor receptor (TNFR) superfamily, such CD95/Fas, TNFR or TRAILR (death receptors). The intrinsic pathway is induced by stimuli such as radiations and cytotoxic drugs and is characterized by mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial cytochrome c, which results in assembly of a caspase-activating complex between caspase-9 and APAF1 (the apoptosome).

The extrinsic pathway typically results in the recruitment and activation of caspase-8 by the Fas-associated death domain (FADD)/TNFR1-associated death domain protein (TRADD) to form a death-inducing signaling complex (DISC) that can further propagate death signals in three ways: via proteolysis of the BCL2 homology-3 (BH3)-only protein BID, which provokes translocation of truncated BID to mitochondria and consequent MOMP; by direct proteolytic activation of downstream effector caspases; or via activation of the kinase RIP (Pua et al., 2007).
In the intrinsic pathway, a range of BH3-only proteins act as sentinels for cell stress, organelle-specific damage or infection, and can be mobilized via post-translational modification (such as proteolysis or phosphorylation) or subcellular relocation to initiate MOMP. The BH3-only proteins stimulate MOMP by triggering the oligomerization of BAX and/or BAK in the outer mitochondrial membrane, thereby forming channels that permit the escape of multiple proteins from the mitochondrial intermembrane space (Galonek and Hardwick, 2006; Kroemer et al., 2007). In the context of DNA damage, stabilization of the p53 tumour-suppressor protein can result in transcriptional activation of the BH3-only proteins (such as PUMA and NOXA) that can promote MOMP via the BAX/BAK channel (Vousden and Lane, 2007). Alternatively, DNA damage can activate caspase-2 in a multinuclear complex that involves the p53-induced protein with a death domain (PIDD) and the RIP-associated protein with a death domain (RAIDD) (together known as the piddosome) (Takacs-Vellai et al., 2005). Caspase-2 may then induce MOMP and/or direct caspase activation. Several factors among the mitochondrial proteins that are released as a result of MOMP (apoptosis-inducing factor (AIF), Omi, EndoG can promote caspase-independent cell death (Nutt et al., 2005), which can also result from stimuli that cause lysosomal membrane permeabilization (LMP), resulting in the release of cathepsin proteases into the cytosol (Kroemer and Martin, 2005). Such cathepsins can also trigger MOMP, thereby stimulating the mitochondrial pathway of apoptosis.
The two main pathways lead to caspase-dependent apoptosis. The extrinsic pathway involves stimulation of members of the tumor necrosis factor receptor (TNFR) superfamily, such CD95/Fas, TNFR or TRAILR (death receptors). The intrinsic pathway is characterized by mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial cytochrome c, which results in assembly of a caspase-activating complex between caspase-9 and APAF1 (the apoptosome). Picture adapted from (Maiuri et al., 2007).

1.3. 3 Cytotoxic T lymphocytes (CTL)

Cytotoxic T lymphocytes (CTLs) provide potent protection against virus infection and intracellular pathogens. However, CTLs have lytic machinery which can be directed against self-tissues in autoimmune disorders, transplanted cells during graft rejection and
host tissues to cause graft-versus-host disease, which is one of the most serious diseases related to CTL function (Maiuri et al., 2007).

The targets of cytotoxic T lymphocytes include virus-infected cells (e.g., HIV-infected CD4+ T cells); cells infected with intracellular bacterial or protozoal parasites; allografts such as transplanted kidney, heart, lungs, and cancer cells.

### 1.3. 4 Mechanisms of cytotoxic T cell-mediated killing

A) **Cytotoxic granzymes**

CTLs have cytoplasmic granules that contain the proteins perforin and granzymes. When a CTL binds to its target, the contents of the granules are discharged by exocytosis. A dozen or more perforin molecules insert themselves into the plasma membrane of target cells forming a pore that enables granzymes to enter the cell.

Granzymes are serine proteases -the two most abundant ones are granzyme A and B. Once inside the cell, they enter the mitochondria and cleave a subunit of complex I (the NADH dehydrogenase) of the electron transport chain producing reactive oxygen species (ROS) that kill the cell; Granzyme B proceeds to cleave the precursors of caspases thus activating them to cause the cell to self-destruct by activating extrinsic apoptosis.

B) **Fas-mediated killing**

CTLs express on their surface the death activator designated Fas ligand (FasL, CD178). CTL targets express a receptor for FasL designated Fas (CD95). When cytotoxic T cells recognize (bind to) their target cells, they increase the expression of FasL at their surface.
This binds with the Fas on the surface of the target cell leading to its death by apoptosis (Figure 15).

In addition, CTLs also produce proinflammatory cytokines, such as tumour-necrosis factor (TNF) and interferon gamma (IFN-gamma) that exert cytotoxic action on target cells (Barry and Bleackley, 2002). CTL are found to be active in some autoimmune disorders such as Type I diabetes mellitus, where beta cells of the islets of Langerhans are destroyed (Barry and Bleackley, 2002)
Figure 15 Engagement of Fas (CD95) on a target cell with CTL-expressed Fas ligand (FasL; CD178) results in apoptotic death. Stimulation of the Fas receptor results in recruitment of the initiator caspase, caspase-8, through interaction with the adaptor molecule Fas-associated death domain protein (FADD) by means of death domains and death-effector domains. This results in the activation of caspase-8. Caspase-8 has different effects in different cells of the TYPE I/II SYSTEM. In type I cells, it is able to activate other members of the caspase family (such as caspase-3) directly. By contrast, in type II cells, caspase-8 activation results in cleavage of the proapoptotic Bcl2-family member Bid, and the translocation of Bid and Bax to the mitochondria. Once inserted into the mitochondrial membrane, Bid and Bax induce the release of mitochondrial cytochrome c, which results in the activation of caspase-9 through interaction with the adaptor molecule apoptotic protease-activating factor 1 (Apaf1). Then, caspase-9 is able to activate caspase-3 (Barry and Bleackley, 2002). Picture adapted from (Maiuri et al., 2007).

1.3. 5 Regulation of endothelial cell death and survival

EC apoptosis has been linked to human diseases, for examples atherosclerosis (Norata et al., 2002), allograft vasculopathy (Gao et al., 1998) (Mueller et al., 2004), ischemia-reperfusion injury (Matsushita et al., 2000) and sepsis (Koide et al., 1996). Apoptotic EC become proadhesive for platelets and procoagulant (Bombeli et al., 1997; Bombeli et al., 1999) and thus contribute to the progression and complications of the above mentioned conditions.

Inhibition of EC apoptosis is thought to be an essential mechanism to maintain vascular integrity during inflammation. Several factors which are known to promote EC survival in vitro are mentioned briefly below.
1.3. 6 Cell–matrix interactions

EC are dependent on interactions with multiple components of the extracellular matrix for their survival (Stupack and Cheresh, 2003). In particular, soluble antagonists of several integrins ($\alpha2\beta1$, $\alpha5\beta1$, $\alpha\nu\beta3$, and $\alpha\nu\beta5$) have been shown to efficiently promote EC apoptosis (Stupack and Cheresh, 2003). Disruption of integrin-mediated signaling leads to anoikis, a type of programmed cell death due to loss of cell/matrix interactions (Michel, 2003). The $\beta1$-integrins signal through focal adhesion kinase and Src family kinases, leading to downstream activation of mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3-K) and thereby promoting cell survival (Desrumaux et al., 2003).

1.3. 7 Growth and angiogenic factors

VEGF is a multi-functional protein affecting EC proliferation (Conn et al., 1990) (Kim et al., 2012), migration (Abedi and Zachary, 1997) and permeability (Keck et al., 1989). VEGF appears to play a pivotal role in the regulation of normal and abnormal angiogenesis (a complex process consisting of interrelationships of angiogenic, survival and differentiation factors and their receptors with cell adhesion and cell-matrix adhesion molecules) (Beck and D'Amore, 1997; Folkman, 1996; Risau, 1997).

Other growth factors such as fibroblast growth factor-2 (FGF-2), hepatocyte growth factor, angiopoietin-1 and insulin also provide survival signals through engagement with their cell surface receptors VEGF-R2, FGF-R, Met, Tie-2 and IGFR respectively (Munoz-Chapuli et
al., 2004). These receptors signal survival through the PI3-K/Akt pathway (Downward, 2004; Munoz-Chapuli et al., 2004). Some of the antiapoptotic molecules such as A1, Bcl-2, and IAPs can be stimulated in response to these growth factors (Munoz-Chapuli et al., 2004).

1.3. 8 Nuclear factor kappa B

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (Gilmore, 2006). NF-κB plays a key role in regulating the immune response to infection (κ light chains are critical components of immunoglobulins). Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κB has also been implicated in processes of synaptic plasticity and memory (Albensi and Mattson, 2000; Meffert et al., 2003).

NF-κB is responsible for cytokine production and cell survival. Moreover, activation of NF-κB by tumor necrosis factor (TNFα), αvβ3 integrin (Scatena et al., 1998) or other stimuli induces transcription of a number of endothelial genes with anti-apoptotic function, including FLICE-inhibitory protein (FLAR), the Bcl-2 homologue A1/Bfl1, and IAPs (Li et al., 2004) (Figure 16).
**Figure 16 Mechanism of NF-κB action.** The NF-κB heterodimer between RelA and p50 proteins is used as an example. While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and subsequent degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function. Picture adapted from Gilmore TD et al, 2006.

### 1.3. 9 cFlar

cFlar, also known as cFlip, is a death effector domain (DED)-containing family member that inhibits one of the most proximal steps of death receptor-mediated apoptosis. Two isoforms of cFlar are commonly detected in human cells: a long form (cFlarL) and a short form (cFlarS). cFlarL, a 55-kDa protein, contains two DEDs and a caspase-like domain, whereas cFlarS, a 26-kDa protein consists only of two DEDs (Peter, 2004). Both Isoforms
are recruited to the DISC, prevent procaspase-8 activation and block DR-mediated apoptosis, although through different mechanisms. cFlarL is overexpressed in a number of different tumors and its overexpression is related to TRAIL resistance (Safa et al., 2008) (Figure 17).

![Diagram of cFlar and DISC formation](Image)

**Figure 17 The effects of cFlar on Fas-mediated DISC formation.** The model depicts the Fas-mediated DISC formation and apoptotic signalling affected by cFlar. cFlar competes with procaspase 8 binding to Fadd, thus decreasing caspase 8 recruitment to the DISC and its activation, which inhibits Fas-mediated apoptosis. Pcitue adapted from resistance (Safa et al., 2008).

1.3. 10 Cytoprotective signalling pathways of cFlar

There has been report showing that Akt, a serine-threonine kinase, interacts with cFlar and cFlar in turn enhances the anti-apoptotic functions of Akt by modulating Gsk3β activity (Iyer et al., 2011). Moreover, through its effects on Gsk3β, cFlar overexpression in cancer cells induces resistance to TRAIL. Downregulation of the DNA-PK/Akt pathway is also
reported to correlate with high responsiveness to TRAIL-mediated growth inhibition and apoptosis (Kim et al., 2009).

In addition, cFlar might regulate other DR-mediated signals that may be important for tumor-promoting functions, such as proliferation, migration, inflammation or metastasis (Kataoka et al., 2000). The activation of the transcription factor NF-κB, and mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38, have been shown to be a consequence of DR triggering.

1.3. 11 cFlar as a therapeutic target treatment

Ectopic expression of cFlar variants decreases apoptosis caused by death ligands and anticancer agents (Poukkula et al., 2005), indicating that overexpression of these proteins leads to survival. Several review articles have discussed classes of agents that can specifically decrease expression of cFlar which sensitize cancer cells to TRAIL or anticancer drugs (Safa et al., 2008). These agents affect cFlar transcription, trigger cFlar degradation through the ubiquitin-proteasome system, or decrease cFlar translation. For example, pre-treatment with chemotherapeutic drugs including cisplatin or doxorubicin downregulates cFlar variants expression in various tumor cells by inhibiting its transcription and rendering cells sensitive to death receptor-triggered apoptosis (Longley et al., 2006).
1.3. 12 FOXO3

FOXO3 belongs to the O subclass of the forkhead family of transcription factors which are characterized by a distinct fork head DNA-binding domain. There are three FoxO family members in humans, FOXO1, FOXO4 and FOXO6. These transcription factors share the ability to be inhibited and translocated out of the nucleus upon phosphorylation by proteins such as AKT/PKB in the PI3K signaling pathway (aside from FOXO6, which may be constitutively nuclear) (Brunet et al., 1999). Other post-translational modifications including acetylation and methylation are seen and can result in increased or altered FOXO3a activity.

This protein likely functions as a trigger for apoptosis through upregulation of genes necessary for cell death, such as Bim and PUMA (Ekoff et al., 2007) or downregulation of anti-apoptotic proteins such as cFlar (Skurk et al., 2004). A functional requirement for FOXO3 as a regulator of Notch signaling pathway (an essential regulator of quiescence in adult stem cells) is required in the self-renewal of stem cells during muscle regeneration (Gopinath et al., 2014).

1.3. 13 PD-L1

Programmed death-ligand 1 (PD-L1) is a 40kDa type 1 transmembrane protein that plays a major role in suppressing the immune system during particular events such as pregnancy, tissue allografts, autoimmune disease and other disease states such as hepatitis.
PD-L1 binds to its receptor, PD-1, found on activated T cells, B cells, and myeloid cells, to modulate activation or inhibition. The formation of PD-1 receptor / PD-L1 ligand complex transmits an inhibitory signal which reduces the proliferation of CD8⁺ T cells at the lymph nodes.

PD-L1 also has an appreciable affinity for the costimulatory molecule CD80 (B7-1), but not CD86 (B7-2) (Butte et al., 2007). CD80's affinity for PD-L1 (1.4µM), is intermediate between its affinities for CD28 and CTLA-4 (4.0µM and 400nM, respectively). In addition, engagement of PD-L1 with its receptor PD-1 on T cells delivers a signal that inhibits TCR-mediated activation of IL-2 production and T cell proliferation. The mechanism involves inhibition of ZAP70 phosphorylation and its association with CD3ζ (Sheppard et al., 2004). PD-1 signaling attenuates PKC-θ activation loop phosphorylation (resulting from TCR signaling), necessary for the activation of transcription factors NF-κB and AP-1, and for production of IL-2.

1.3. 14 Shear stress

Laminar shear stress is a potent antiapoptotic stimulus in EC, affording protection against a number of stimuli in vitro. Turbulent flow areas are characterized by increased EC turnover rates (Davies et al., 1986), suggesting increased cell death. This view is supported by recent studies showing that ECs cultured under static conditions undergo apoptosis, whereas normal levels of shear stress are protective (Dimmeler et al., 1996; Kaiser et al., 1997). These findings suggest a mechanistic link between low shear stress, and EC apoptosis. Notably, areas of turbulent flow in the large vessels are the most susceptible to atherosclerotic plaque development. EC death by apoptosis may also participate in plaque
disruption and thrombosis. Exposure of the subendothelium to blood flow promotes platelet aggregation and vasospasm, and it was recently shown that apoptotic ECs exhibit marked procoagulant activities and become proadhesive, even for nonactivated platelets (Bombeli et al., 1997).

Several mechanisms have been also proposed to account for the antiapoptotic effects of laminar shear stress, including upregulation of superoxide dismutase (Dimmeler et al., 1999) and Akt-mediated activation of nitric oxide synthase, with subsequent inhibition of the caspase cascade (Dimmeler et al., 1999).

1. 4 The immune system

The immune system is a dynamic system of biological structures and processes within an organism that protects against disease. The immune system can be divided into two branches; the innate immune system and the adaptive immune system.

The innate immune system utilises the following four mechanisms to prevent toxins and pathogenic microbes from entering the body:

1. Anatomical. Including the skin (epidermis and dermis) and mucous membranes.
2. Physiologic. Includes thermoregulation, PH (i.e. acidity in the stomach) and chemical mediators (i.e. lysozymes, interferons).
3. Phagocytic/Endocytic. Neutrophils and macrophages can phagocytose micro-organisms
4. Inflammatory. Damage and infection cause leakage of vascular fluid into tissues which contains serum proteins, antibodies and phagocytic cells.
The innate immune system exhibits limited specificity to toxins and pathogens whereas the adaptive immune system is characterised by its ability to discriminate between a huge variety of foreign and self-antigens. In addition, activation of the innate immune system does not lead to the development of memory responses whereas activation of the adaptive immune system does.

The adaptive immune system comprises B cells that express B cell receptors (BCR) and generate antibodies and T cells that express T cell receptors (TCR) and produce cytokines and can in some cases, directly kill specific cells. Each B cell or T cell clone has a unique specificity for antigen; this specificity is generated by the rearrangement of a set of gene segments to generate antibodies (BCR shed from the surface of B cells), and TCR.

T cells are only able to recognise specific peptide epitopes, derived from self or foreign proteins, when they are presented on the surface of a cell in association with a major histocompatibility complex (MHC) molecule. T cells only generate an effective response when their TCR is engaged by MHC-peptide at the same time as they are co-stimulated by ligands on an antigen presenting cell (APC) (Malissen et al., 2014). Engagement of the TCR alone leads to anergy, deletion or tolerance. Co-stimulatory molecules can have positive or negative effect on the activation of T cells.
1.4. 1 The major histocompatibility complex

The major histocompatibility complex (MHC) is a set of cell surface molecules encoded by a large gene family which controls a major part of the immune system in all vertebrates. The major function of MHCs is to bind to peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T-cells (Janeway, 1980). It was discovered in the 50s and 60s by Peter Gorer and George Snell when they identified a group of genes that were responsible for the rejection of transplantable tumours and other tissues in mice. It was subsequently established, in both mice and humans, that MHC Class I genes encode glycoproteins that are expressed on all nucleated cells and present antigen to CD8 T cells and that the MHC class II genes encode glycoproteins that are expressed on APCs and present antigen to CD4 T cells (Figure 18).

MHC (major histocompatibility complex) class II molecules are a family of molecules normally found only on antigen-presenting cells such as dendritic cells, mononuclear phagocytes, some ECs, thymic epithelial cells and B cells. The MHC-II pathway primarily processes proteins, derived from extracellular sources, via the endosome/lysosome/phagosome compartments for presentation on the surface of professional APC.

These molecules play a critical role in the normal immune system, namely the presentation of peptides in a form that can be recognized by T cells. In particular, CD8+ T cells recognize peptides presented by class I molecules and CD4+ T cells recognize those presented by class II molecules and this is valid both for the self-MHC molecules as well
as the allo-MHC molecules. From the crystal structures of the extracellular portions of human class I and class II molecules, it is now clear that they form a ‘groove’ where the peptide to be presented is bound (Rudolph et al., 2006). The peptides presented are the result of the natural processing of cellular and serum proteins. The peptide-binding groove of the MHC molecules on each cell is thus occupied by a very diverse (several hundreds) set of different peptides. Class I molecules are mainly occupied by peptides originating from intracellular proteins, whereas those presented by class II molecules have mainly an extracellular origin; although cross-presentation of peptides of extracellular origin has been widely demonstrated in class I molecules (Bozzacco et al., 2007). It has been confirmed that the TCR recognizes a complex of two MHC helixes and a bound peptide. In the allore cognition setting in a direct pathway response, these MHC–peptide complexes recognized are from the allogeneic tissue. Recently, it has been shown that complementarity-determining region (CDR) 3α could undergo rearrangements to adapt to structurally different peptide residues. This CDR3 loop flexibility helps to explain TCR binding cross-reactivity and thus supports the conundrum of T cells responding to MHC molecules that they have not been selected to recognize (Borg et al., 2005).

As will be discussed later, MHC molecules are the main antigen recognised by allospecific T cells during transplant rejection.

1.4. 2 T cell development

T cells originate in the bone marrow (BM) as haematopoietic stem cells (HSCs). HSC’s differentiate into T lineage progenitors (TLP) which migrate into the blood stream and then into the thymus (Bhandoola et al., 2003)- it is where the T cell progenitors proliferate and
differentiate (Lind et al., 2001). Histologically, each lobe of the thymus can be divided into a central medulla and a peripheral cortex which is rounded by an outer capsule. Both play different roles in the development of T cells, including negative and positive selection of the T cell repertoire that determines the ability of the immune system to distinguish self from non-self.

Double negative (DN) thymocytes are early thymocytes negative for CD4 and CD8. A positive signal through the pre-TCR ensures the thymocytes survival as they become CD4⁺CD8⁺ double positive (DP) thymocytes which express a mature TCR (von Boehmer et al., 2003). Mature TCR molecules are dimers formed by the varying combination and interaction of the TCR proteins. The TCR is a disulfide-linked membrane-anchored heterodimer normally consisting of the highly variable alpha (α) and beta (β) chains expressed as part of a complex with the invariant CD3 chain molecules. T cells expressing this receptor are referred to as α:β (or αβ) T cells, though a minority of T cells express an alternate receptor, formed by variable gamma (γ) and delta (δ) chains, referred as γδ T cells. The huge diversity of the TCR repertoire is generated by the rearrangement of the four genes (Tcra, Tcrb, Tcrd and Tcrg), that encode these proteins, during T cell development in the thymus (Davis and Bjorkman, 1988). Rearrangement is driven by the Recombinase Activating Gene 1 and 2 (RAG1/2) proteins that create double stranded breaks at specific Recombination Signal Sequences (RSSs) that flank the TCR variable (V), diversity (D) and joining (J) gene segments. When the segments are re-joined they generate a TCR sequence that is translated into the TCR. Each T cell clone expresses a unique TCR on its cell surface (Bassing et al., 2002).
DP thymocytes with a mature TCR interact with APCs, including thymic B cells, DCs, cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). DP thymocytes are then positively selected if they interact, with sufficient affinity, with self-peptide-MHC complexes on cTECs. At this point, DP thymocytes that interact with MHC-I restricted peptides are destined to become CD8+ T cells and those that interact with MHC-II are destined to become CD4+ T cells. Positively selected thymocytes migrate to the medulla and interact with mTECs. In the medulla, DP thymocytes that interact with self-peptide-MHC with very high affinity are negatively selected (Davey et al., 1998).

The results of the thymic process of positive and negative selection is a repertoire of naïve CD4+ and CD8+ T cells that have a controlled affinity for self-peptide-MHC complexes. Crucially thymocytes that have a strong affinity for self-peptide MHC complexes are deleted and this prevents auto-immunity (Davey et al., 1998).

After release from the thymus, mature, naïve CD4+ and CD8+ T cells are found continually circling through the blood, peripheral tissues and lymph nodes. Naïve CD4+ and CD8+ T cells are characterised by the low expression of CD44 and high expression of CD62L. CD62L (L-selectin) is a cell adhesion molecule that facilitates the entry of T cells into the secondary lymphoid organs. To become activated, T cells require at least two signals, one through the TCR and one through a co-stimulatory molecule, most commonly CD28 (Harding et al., 1992). Stimulation through the TCR alone leads to T cell death or anergy (Jenkinson et al., 1987) and the induction of tolerance (Choi and Schwartz, 2007).
Activated T cells have increased expression of CD44 and chemokine and homing receptors (e.g. CCR5/7) and reduced expression of CD62L; this expression profile facilitates their migration to the periphery and sites of inflammation and infection. The type of cytokines and cell surface markers that are expressed, by the T cells, after activation are influenced by the microenvironment, the cytokines secreted by the APC and other cells in close proximity and the co-stimulatory signals received by the T cell from the APC.

In addition to the expression of molecules that contribute to activation, activated T cells also begin to express inhibitory molecules such as Programmed Death-1 (PD-1) and Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) which, when bound to their cognate antigen on APC, causes the death and inactivation of activated T cells. This feedback system regulates the strength and extent of the immune response ensuring that cytotoxic effects are limited to a small area and short time frame to minimise damage to healthy tissue.

1.4. 3 CD4+ T cells

As mentioned above, DP thymocytes that recognise MHC-II restricted peptides, presented by cTECs during thymocyte development, are destined to become CD4+ T cells; upon interaction with MHC-II in the thymus, they gradually down regulate CD8 and up regulate CD4 and migrate out of the thymus as mature naïve CD4+ T cells.

Activated CD4+ T cells can develop cytotoxic functions (Williams et al., 1996) but their primary function is to maintain and regulate B cell and CD8+ T cell responses (Janssen et al., 2003). Following antigen clearance, a small proportion of activated CD4+ T cells
survive and persist in the blood stream and lymph nodes as memory CD4+ T cells which are rapidly reactivated if the same antigen is presented again.

CD4+ T cells can be separated into four distinct subsets, Th1, Th2, Th17 and regulatory T cells (Treg) based on their function and cytokine profiles.

*Th1*

A type 1 T helper response (Th1) is generated in response to intracellular pathogens such as bacteria and viruses. It is characterised by secretion of Interferon gamma (IFNγ), IL-2 and Tumour Necrosis Factor alpha (TNFα, activation of CD8 T cells and macrophages and the production of opsonising antibodies from B cells). IFNγ increases the production of IL-12 by DC and macrophages which in turn stimulates the secretion of IFNγ by Th1 cells thus providing a feedback loop that drives the Th1 response (Mosmann and Sad, 1996).

*Th2*

A type 2 T helper (Th2) response is generated against multi-cellular organisms and is typified by the activation of B cells and secretion of IL-4, 5, 6, 9 10 and 13 (Mosmann and Sad, 1996).

*Th17*

T helper 17 cells (Th17) are found in inflammatory environments and are induced by TCR stimulation in the presence of Transforming Growth Factor-beta (TGFβ) and IL-6 (Kryczek et al., 2007). Th17 cells secrete IL-17A, IL-17F and IL-22 which induce a wide range of responses from surrounding tissue because of the ubiquitous expression of the IL-17 and IL-22 receptors (Bettelli et al., 2008).
1.4. 4 CD8+ T cells

DP thymocytes that recognise MHC-I restricted peptides, presented by cTECs during thymocyte development, gradually down regulate CD4 and up regulate CD8 and migrate out of the thymus as mature CD8 T cells. CD8 T cells are then found in the blood, periphery and lymph nodes. Upon ligation of TCR with pMHC-I on APC and sufficient co-stimulation, CD8 T cells become activated and begin to proliferate. Upon activation CD8 T cells, also known as cytotoxic or killer T cells, secrete perforins, granzymes and up-regulate Fas-Ligand (Fas-L) affecting the death of the target cell. CD8 T cell proliferation and activation is enhanced by IL-2 (Yron et al., 1980), IL-7 (Schluns et al., 2000) and IL-15 (Schluns et al., 2002). CD8 T cells spontaneously secrete Th1 cytokines IFNγ and IL-2 but can be induced to secrete Th2 cytokines in the presence of IL-4 (Sad and Mosmann, 1995).

Whether or not the source of antigen is cleared, CD8 T cells numbers fall dramatically during a phase of contraction. However, not all antigen specific CD8 T cells are lost and a small proportion survive and persist in the blood stream and lymph nodes as memory CD8 T cells which are rapidly reactivated if the same antigen is presented again (Harty and Badovinac, 2008).

1.4. 5 Regulatory T cells

CD4+ regulatory T cells (Tregs) are characterised by the expression of the forkhead/winged helix transcription factor (FoxP3) (Fontenot et al., 2005) and the α chain of IL-2 receptor
nTreg differentiate and mature in the thymus; cells are committed to the Treg lineage when they recognise self-peptide-MHC with an avidity ranging between that required for positive and negative selection. Development in the thymus may not be consistent for all thymocytes and therefore it is possible that both conventional and regulatory T cells with the same or similar specificities can develop (Fontenot et al., 2005).

The primary function of nTregs is to control self-reactive peripheral T cells that escape thymic deletion, as suggested by early studies using neonatal thymectomy or depleting anti-CD25 antibodies (Sakaguchi et al., 1995) (Kohm et al., 2006), and verified by recent investigations using Foxp3DTR mice (Kim et al., 2007).

iTregs are induced in the periphery and their primary function is similar to that of nTreg. However, iTreg differ from nTreg in that they can be induced in response to foreign antigen and can block other cells from producing IL-17 (Horwitz et al., 2008a), suggesting that they have a distinct function from nTreg, possibly in mediating non-responsiveness to environmental antigens. Induced Regulatory T (iTreg) cells (CD4+ CD25+ Foxp3+) are suppressive cells involved in tolerance. iTreg cells have been shown to suppress T cell proliferation and experimental autoimmune diseases. The development of iTregs needs a
low dose of antigen presented by resting APC and engagement of CTLA-4 (Apostolou and von Boehmer, 2004; Horwitz et al., 2008b).

1.4. 6 Cytokines

Cytokines are pluripotent mediators of the immune system, they are produced from many sources, including non-hematopoietic cells, under differential conditions. Cytokines are usually soluble although there are some that are membrane bound. They bind receptors on their target cell and induce various responses including migration, activation, proliferation, differentiation and inhibition.

\textit{IFN}\gamma

IFN\(\gamma\), a signature cytokine released by CD4 and CD8 T cells after activation, causes the up regulation of MHC-I and -II molecules in numerous cell types (Morris and Tomkins, 1989). IFN\(\gamma\) promotes anti-microbial and anti-tumour responses from macrophages; consistent with this, the neutralisation of IFN\(\gamma\) with the monoclonal antibody R46/A2 inhibits anti-viral and anti-tumour responses (Spitalny and Havell, 1984). IFN\(\gamma\) also regulates B cell functions including immunoglobulin production and class switching (Schroder et al., 2004).

\textit{TNF}\(\alpha\)

Tumor necrosis factor alpha (TNF alpha, is a cell signalling protein sectred by adipose tissue (adipokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is produced chiefly by activated macrophages, although it
can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons (Gahring et al., 1996).

The primary role of TNFα is in the regulation of immune cells. TNFα is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via IL-1- and IL-6- producing cells. Dysregulation of TNFα production has been implicated in a variety of human diseases including Alzheimer's disease (Swardfager et al., 2010), cancer (Locksley et al., 2001), major depression (Dowlati et al., 2010) and inflammatory bowel disease (IBD) (Brynskov et al., 2002).

TNFα can be produced ectopically in the setting of malignancy and parallels parathyroid hormone both in causing secondary hypercalcemia and in the cancers with which excessive production is associated.

1. 5 Transplantation

1.5. 1 Organ transplantation

Organ transplantation is the moving of an organ from one body to another or from a donor site to another location on the person’s own body, to replace the recipient’s damaged or absent organ. Organs that can be transplanted are heart, kidneys, liver, lungs, pancreas, intestine, and thymus. Tissues include bones, tendons (both referred to as musculoskeletal grafts), cornea, skin, heart valves, nerves and veins. Worldwide, kidneys are the most commonly transplanted organ, followed by the liver and the heart. Cornea and musculoskeletal grafts are the most commonly transplanted tissues.
1.5. 2 Types of transplant

Autografts are the transplant of tissue to the same person. Sometimes this is done with surplus tissue, tissue that can regenerate, or tissues more desperately needed elsewhere in the body (e.g. include skin grafts).

An allograft is a transplant of an organ or tissue between members of the same species. Most human tissues and organ transplants are allografts. Due to the genetic difference between the organ and the recipient, the recipient's immune system will identify the organ as foreign and attempt to destroy it, causing transplant rejection.

Xenografts are cells or sections of tissue that are removed from one species and grafted on or into a different species. Grafts of this type are sometimes used to provide temporary protection from infection for burn victims, as well as in the process of cancer research. In most cases, the xenograft is treated before the grafting takes place, which helps to reduce the chances of the host body rejecting the harvested tissue.

To date no xenotransplantation trials have been entirely successful due to the many obstacles arising from the response of the recipient’s immune system. This response, which is generally more extreme than in allotransplantations, ultimately results in rejection of the xenograft, and can in some cases result in the immediate death of the recipient.
1.5. 3 Transplant rejection

Transplant rejection occurs when the transplanted (donor) tissue is rejected by the recipient immune system under an adaptive immune response. This is normally via combination of cellular immunity (mediated by killer T cells inducing apoptosis of target cells), humoral immunity (mediated by activated B cells secreting antibody molecules) and components of the innate immune response (phagocytes and soluble immune proteins). Different types of transplanted tissues tend to favour different balances of rejection mechanisms. Dendritic cells (DCs), which are the primary antigen-presenting cells (APCs) of the donor tissue migrate to the recipient's peripheral lymphoid tissue (lymphoid follicles and lymph nodes), and present the donor's self-peptides to the recipient's lymphocytes (immune cells residing in lymphoid tissues).

The recipient's helper T cells coordinate specific immunity directed at the donor's self-peptides (indirect allorecognition, see below) or at the donor's Major histocompatibility complex molecules (direct allorecognition, see below), or at both.

1.5. 4 Allorecognition

Allorecognition can proceed via several mechanisms (Figure 19): direct allorecognition, whereby T cells recognize determinants on the intact donor MHC molecules displayed on the surface of transplanted cells (Warrens et al., 1994); indirect allorecognition in which donor MHC molecules or polymorphic proteins are processed and presented as peptides by self-MHC molecules - in a similar fashion to conventional antigen processing (Lechler
and Batchelor, 1982); and a recently described third mechanism termed *semi-direct* allorecognition where trafficking recipient dendritic cells (DC) acquire intact donor MHC:peptide complexes from cells of the graft enabling them to then be able to stimulate antigen-specific immune responses (Herrera et al., 2004).

### 1.5. 5 Histocompatibility antigens

The protein products of major histocompatibility complex (MHC) genes, expressed on the surface of all nucleated cells, are responsible for the immune response to allogeneic tissues. Of all the genes included in this region, two highly variable groups are central in allorecognition. These are the class I and class II molecules. Class I molecules are known as human leukocyte antigens (HLA)-A, -B and -C in humans and H2-K, -D, -L in mice and are constitutively expressed on most nucleated cells. Class II molecules are known as HLA-DR, -DP and -DQ in humans and H2-A and -E in mice and are constitutively expressed only by bone marrow-derived APCs, such as macrophages, DC, B lymphocytes and by thymic epithelial cells. The convention is to identify the genes in Roman letters (e.g. HLA-DRB or H2-D) and the encoded proteins in corresponding Greek symbols (e.g. HLA-DR β or H2-Aβ) (Figure 18).
Figure 18 The major histocompatibility complex (MHC) in mice and humans. MHC genes are encoded on chromosomes 6 and 17 in humans in humans and mice, respectively. T cells recognize antigen via the variable regions of the clonotypic T cell antigen receptor molecule (TCR). CD8+ T cells recognize peptide antigen as 8-10-residue-long peptides embedded into the antigen-binding groove of MHC class I molecules (the products of gene loci H2-K, -D, and -L in mice; HLA-A, -B and -C in humans). CD4+ T cells recognize peptide fragments of 13 residues or longer embedded into the open-ended antigen-binding groove of MHC class II molecules (the products of gene loci H2 I-A and I-E in mice; HLA-DR, -DQ and -DP in humans). Picture adapted from http://pathmicro.med.sc.edu/ghaffar/mhc2000.htm

1.5.6 Minor histocompatibility antigens

A different set of polymorphic non-MHC proteins have been identified that are important in provoking transplant rejection, they were defined by Snell and colleagues as mHAg, as the rejection reactions they induced in mice were slower (Barth et al., 1956). In principle, any protein that has polymorphisms within a species can become mHAg. Peptides from these proteins are presented to T cells in an MHC class I or class II restricted manner. The number of possible mHAgs in transplants performed between genetically unrelated, MHC-matched individuals is very large. However, the reactions seem to be restricted to a few
epitopes, thus dubbed immunodominant. The molecular basis for this phenomenon is incompletely understood, although it has recently been shown that both the duration of individual mHAg presentation and the avidity of T-cell antigen recognition influence the magnitude of the cytotoxic response that ensues (Simpson et al., 2002).

The frequency of T cells responding to these antigens in non-transplanted individuals is very small and can only be measured \textit{in vitro} after \textit{in vivo} immunization or repeated stimulations, as opposed to direct pathway responses. When alloreponses of mHAgs have been measured, the cells that respond to these antigens are generally CD8$^+$ T cells, implying that most mHAgs are peptides bound to self-MHC class I molecules. However, peptides bound to self-MHC class II molecules can also participate in the response to MHC-identical grafts.

1.5. 7 Direct alloreognition

Direct alloreognition was long believed to be the only mechanism by which allogeneic antigens could be recognized in the donor graft (Figure 19). When measured, this response to allogeneic MHC molecules is of high frequency (Baker et al., 2001). Two non-mutually exclusive theories regarding the molecular mechanisms of this high frequency have been proposed, namely the ‘high determinant density’ and ‘multiple binary complex’ models which differ in the importance they allot to the presence of peptide in the allogeneic MHC–peptide complex.

Firstly, it has been proposed that alloreactive T cells are directly able to recognize the exposed polymorphic residues on allogeneic MHC, thus consigning the bound peptide to
secondary importance. This model predicts that if every MHC molecule on a cell surface can serve as a ligand for an allospecific T cell then the antigen density on the cell surface would be extremely high, in marked contrast with the density of a specific peptide plus MHC. The high ligand density available for stimulating alloreactive T cells implies that receptors of much lower affinities would be able to respond to the foreign MHC, leading to a high frequency of alloreactivity. This hypothesis is supported by demonstration that blocking the TCR-contacting regions of allo-MHC using synthetic peptides (Schneck et al., 1989) or site-specific mutations inhibits specific alloresponses (Villadangos et al., 1994), presumably through inhibition of TCR–MHC contact (Lombardi et al., 1991). Additionally, alloreactivity in the absence of peptide has previously been shown (Smith et al., 1997).

Second, the multiple binary complex model proposes that recognition of peptide bound by allogeneic MHC is of primary importance to direct allore cognition in a manner akin to conventional self-restricted responses (Sherman and Chattopadhyay, 1993). Multiple different bound peptides, in combination with one allogeneic MHC gene product, may produce determinants recognized by different cross-reactive T cells. Although the peptide is likely to be naturally processed and derived from a serum or cellular protein, the set of peptides bound by an allogeneic MHC molecule is often substantially different from that bound by the self-MHC homologue because of sequence variation in the peptide-binding groove. This model predicts that if each bound peptide is an essential component of the determinant recognized by alloreactive T cells, each peptide–allo-MHC complex will be recognized by a different alloreactive T cell and a single MHC incompatibility can stimulate a wide diversity of T cells. This hypothesis is supported by the mutant-transfected T2-I-A<sup>b</sup> cell line that is unable to process antigen and is incapable of
stimulating allospecific responses and in which transfection with stable peptide–MHC class II complex restores the ability to stimulate alloreponses (Weber et al., 1995). Similarly, alloreactive CD8+ T cells have been shown to be specific for a self-peptide presented by foreign class I molecules, with no evidence of peptide-independent components. Furthermore, displacement of endogenous peptides from allogeneic antigen-presenting cells (APCs) by incubation with exogenous peptides leads to loss of allore cognition by allospecific T cells (Eckels et al., 1988). Another study looked at the peptide-complex recognition ability of 12 cytotoxic allogeneic T-cell clones and for all of them the allore cognition was peptide specific whether the allogeneic MHC molecules were expressed on normal cells or antigen-processing-deficient cells (Smith et al., 1997).

The vigorous nature of the direct alloreponse and its immediacy in comparison with the indirect pathway (see below) is the result of direct recognition of intact MHC by T cells without the need for processing and presentation by self-MHC. Mechanistically, it is likely that direct allore cognition can proceed via both mechanisms discussed above, the overall contribution of each being related to the site and magnitude of the differences in MHC molecules between responder and stimulator cells. Specifically, where the allogeneic MHC is structurally very disparate from responder MHC, the alloreponse may be directed against residues on the MHC itself (high determinant density pattern of recognition), whereas where self and foreign MHC are closely matched, the focus of the alloreactivity may be directed towards epitopes of endogenous peptides that are displayed by stimulator but not by responder MHC molecules (multiple binary complex pattern) (Lechler et al., 1990).
1.5. 8 Indirect allore cognition

The indirect pathway refers to the recognition of processed peptides of allogeneic histocompatibility antigens or donor-derived polymorphic proteins presented by self (recipient’s) MHC molecules (Lechler and Batchelor, 1982) and therefore differs from the direct pathway by the requirement for antigen processing (Figure 19) and the smaller size of precursor allospecific T cell frequency. There is considerable evidence for the involvement of this pathway in chronic allograft rejection (Dalchau et al., 1992) including studies of human recipients of heart, kidney and liver allografts with in vitro detection of indirect response showing a strong correlation with episodes of clinical rejection (Vella et al., 1997).

Alloantigens shed from a graft are, in general, processed as exogenous antigens and therefore presented by APCs in association with self-MHC class II. Therefore, the response to alloantigen presented by the indirect pathway is dominated by CD4+ T cells. While there is considerable amplification of the rejection response through the generation of multiple epitopes via processing of alloantigens, the natural corollary is that responses to the indirect pathway are slower compared with those to the direct pathway. It is also likely that the indirect response is responsible for long-term responses to engrafted tissues once passenger (donor) APC, and by inference direct responses, are exhausted.

The importance of the indirect pathway is suggested by demonstrations that immunization of animals with peptides of allogeneic MHC (by definition able to elicit only indirect rather than direct responses) results in vigorous allograft rejection (Fangmann et al., 1993),
whereas intrathymic injection of similar peptides down-modulates the indirect response sufficiently to prolonged survival of subsequent allografts of the same MHC type (Sayegh et al., 1993). Similarly, in the antibody response to transplanted tissues, B-cell function is dependent on T-cell help from CD4+ T cells stimulated through the indirect, rather than the direct response (Steele et al., 1996).

### 1.5.9 Semi-direct allore cognition

Recently, a number of publications have shown that intact cell surface molecules, including MHC, can be transferred between cells of the immune system and that MHC-recipient cells become able to stimulate T-cell responses as a result (Herrera et al., 2004) (Figure 19c). Although the mechanism of this transfer is likely to involve cell-to-cell contact (Game et al., 2005), other mechanisms such as release and uptake of small vesicles (exosomes) have also been implicated (Morelli et al., 2004).

Traditional descriptions of cross-talk between the direct and indirect pathways (e.g. that indirect pathway CD4+ T cells can both amplify and diminish direct pathway CD8+ T-cell responses) have relied on a four-cell, unlinked model, whereby CD8+ T cells are stimulated through the direct pathway by donor cells, while helper or regulatory CD4+ T cells are recruited through interaction with recipient DC presenting allogeneic MHC through the indirect pathway.

The description of MHC transfer helps to resolve the paradox that the four-cell hypothesis is non-compliant with the dogma that CD4+ and CD8+ T cells are recruited (and linked) by the same APC by proposing an alternative method of alloantigen presentation. This ‘semi-direct’ pathway of allore cognition (Smyth et al., 2006), whereby recipient APCs acquire
allogeneic MHC:peptide complex through MHC transfer (and stimulate CD8$^+$ T cells through the direct pathway) as well as peptides of allogeneic histocompatibility antigens (which are processed and recruit CD4$^+$ T cells through the indirect pathway) links direct and indirect allore cognition through a single APC and also provides a mechanism for the observed cross-talk between them.

Figure 19 Direct, indirect and semi-direct pathways of allore cognition. (A) Direct pathway. Recognition of intact foreign major histocompatibility complex (MHC) on donor antigen-presenting cell (APC) primes CD4 and CD8 recipient T cells. CD4 cells then provide T-cell help for the effector function of CD8 cells. (B) Indirect pathway. The indirect pathway involves presentation of processed allogeneic MHC shed from foreign cells through cell necrosis and apoptosis. Recipient APCs present the processed peptides in the context of self-MHC class II to CD4 T cells. (C) Semi-direct pathway. Cell-to-cell contact between donor and recipient APC may transfer intact membrane components including intact allo-MHC (a). Likewise, donor APC can release small vesicles, known as ‘endosomes’ containing intact MHC (b), which fuse with the membrane of recipient APCs (c). The recipient APC, now chimaeric for MHC, stimulate direct pathway CD4 and CD8 responses through intact foreign MHC and indirect responses through processing and presentation of peptides of foreign MHC acquired from necrotic and apoptotic cell material. Given that the same APC stimulates both CD4 and CD8 cells, linked help can occur. Picture adapted from Tissue Antigens 07/2007; 69(6):545-56. DOI:10.1111/j.1399-0039.2007.00834.x.
1.5. 10 GvHD

The *in vivo* correlate of an immune response to an mHAg is transplant rejection, or in MHC-matched individuals, GvHD (Simpson, 1998). GvHD is a series of manifestations and symptoms that appear after bone marrow transplantation (BMT) and results from an immune response of the immunocompetent cells of the donor against the tissues of the recipient. The effector immune responses are specifically described later on. Notably, even though mHAgs are named minor, and the frequency of responders to these antigens is very low, after transplantation, a single immunodominant mHAg can induce GvHD. Minor HLA antigens important in transplantation have been described from different cellular origins.

(a) Encoded by sex chromosomes: The most thoroughly studied are a set of proteins encoded on the male-specific Y chromosome that are known collectively as H-Y antigens. The absence of Y-chromosome-specific gene products in females induces responses to male antigens. In fact, these responses are very frequent (37–50%) in women with previous male pregnancies (Piper et al., 2007), whereas male anti-female responses are not seen (because both males and females express X-chromosome-derived genes). To date, the number of H-Y epitopes described in humans that are important in transplantation is 10 (Goulmy, 2006). These are restricted by either class I or class II molecules and originate in six different loci of the Y chromosome (DFFRY, SMCY, TMSB4Y, UTY, DBY and RPS4Y1).

(b) Encoded by autosomes: Non-Y-linked mHAgs have also been shown by T cells from patients with GvHD after BMT between HLA identical individuals. The first example
identified in humans was named ‘HA’ after the patient. Recognition of this peptide was restricted by class I molecules. In the interim, other antigens have been identified for humans (HA-1, -2, -3, -8, HB-1, ACC-1, etc.); their cellular origin is varied: Mysoin 1G, LBC oncogene, BCL2A1, and some not yet identified genes (Goulmy, 2006) are examples. 
(c) Encoded by mitochondrial DNA (mtDNA): Tracking of an mHAg to the small mitochondrial genome from the studies of a maternally transmitted transplantation antigen informed that such peptides could become histocompatibility antigens. Cytotoxic T lymphocytes (CTL) were used to test candidate peptides derived from polymorphic regions of the enzyme mt-ND1. A simple amino acid difference in the peptide was found to account for immunogenicity. Subsequently, additional mitochondrial genes in mouse and rat have been found to encode mH peptides, and several are presented to T cells by non-classical, MHC class I molecules (Bhuyan et al., 1997). 

1.5. 11 Types of immune responses in transplantation:

Cellular immunity

T lymphocytes occupy a central role in the rejection response to allogeneic tissues, with depletion or suppression of their function being instrumental in the prolongation of transplant survival. Immunological memory and specificity, hallmarks of T-cell involvement, are both features of allograft rejection as re-exposure to the same alloantigens (re-transplant from the same or genetically identical donor) elicits an accelerated and heightened immune response (second set rejection) than on first encounter (first set rejection), whereas re-transplantation from a third party (unrelated) donor shows only first set rejection.
Humoral immunity

Developed through an earlier primary exposure that primed specific immunity to the non-self-antigen, a transplant recipient can have specific antibody cross-reacting with the donor tissue upon the transplant event, a secondary exposure. This is typical after earlier mismatching among A/B/O blood types during blood transfusion. At this secondary exposure, these cross-reactive antibody molecules interact with aspects of innate immunity-soluble immune proteins called complement and innate immune cells called phagocytes, which inflames and destroys the transplanted tissue.

Opsonization

The IgG's Fc region also enables opsonization by a phagocyte, a process by which the Fc receptor on the phagocyte such as neutrophils in blood and macrophages in tissues-binds the antibody molecule's FC stalk, and the phagocyte exhibits enhanced uptake of the antigen, attached to the antibody molecule's Fab region.

Complement cascade

When the paratope of Ig class gamma (IgG) binds its matching epitope, IgG's Fc region conformationally shifts and can host a complement protein, initiating the complement cascade that terminates by punching a hole in a cell membrane. With many holes so punched, fluid rushes into the cell and ruptures it.

Cell debris can be recognized as damage associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), on membranes of phagocytes, which thereupon secrete proinflammatory cytokines, recruiting more
phagocytes to traffic to the area by sensing the concentration gradient of the secreted cytokines (chemotaxis).

### 1.5. 12 Rejection detection

The histological pathology for rejection can be observed from tissue biopsy by signs (1) of infiltrating T cells, perhaps accompanied by infiltrating eosinophils, plasma cells, and neutrophils, (2) structural compromise of tissue anatomy, varying by tissue type transplanted, and (3) injury to blood vessels.

### 1.5. 13 Immunotherapy in transplantation

To date, immunotherapies to cure, prevent, or delay disease onset in humans has been inefficient. For instance, options for patients with type 1 diabetes to restore normoglycemia are limited to daily insulin injection, combined kidney-pancreas or experimental islet transplantation. However, the important correlation between loss of islet-graft function and frequencies of circulating autoreactive islet-specific T cells observed in transplanted patients, in particular when the donor and the recipient share the same HLA class I haplotypes, points to the importance of recurrent autoimmunity in islet-graft failure (Pinkse et al., 2005) (Hilbrands et al., 2009). Along the same line, it appears that, in case of autotransplantation, islet grafts are much less affected, suggesting that persistent attacks from autoimmunity, alloimmunity and possibly the toxicity of immunosuppressive treatments have a major responsibility in graft loss (Blondet et al., 2007).
1.6 The Biology of CD31

Platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as (CD31) is a member of the immunoglobulin (Ig)-superfamily of cell adhesion molecules. It is expressed on most cells of the hematopoietic lineage including platelets, monocytes, neutrophils, and lymphocyte subsets (Newman and Newman, 2003; Newton-Nash and Newman, 1999; Newton et al., 1997). CD31 is also highly expressed on EC, where it is a major constituent of the EC intercellular junction in confluent vascular beds (Delgado et al., 2011; Muller et al., 1989; Newman et al., 1990).

1.6.1 Structure and function of CD31

CD31 is a type I transmembrane glycoprotein that consists of an extracellular region composed of six Ig-like homology domains, a 19-residue transmembrane domain, and a 118 residue cytoplasmic tail (Newman and Newman, 2003). The biological properties of CD31 in cellular adhesion and signaling have been mapped to specific regions of the CD31 molecule.

Extracellular Ig-homology domain 1 contains residues important for mediating homophilic CD31/CD31 interactions (Sun et al., 2000). Most heterophilic binding interactions are thought to be mediated by amino acid residues located in Ig-homology domains 5 and 6 (Figure 20). The only heterophilic binding partner of CD31 that has thus far been shown to be physiologically relevant is the neutrophil-specific antigen CD177 (NB1) (Sachs et al., 2007). Other perhaps more controversial heterophilic binding partners of CD31 include
glycosaminoglycans (GAG) (DeLisser et al., 1993b), and CD38 on lymphocytes (Deaglio et al., 1998).

The cytoplasmic tail of CD31 contains residues that serve as potential sites for palmitoylation, phosphorylation, and the docking of cytosolic signaling molecules (Newman and Newman, 2003). The best characterized feature of the CD31 cytosolic domain is two Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) that encompass Tyr663 and Tyr686 of human CD31 (Figure 20), which when phosphorylated, recruit Src homology 2 (SH2) domain-containing proteins, the best characterized of which is the SH2 domain-containing protein tyrosine phosphatase SHP-2 (Newman and Newman, 2003).
The general description of the roles of phosphotyrosine and SHP-2 will be discussed below.

**Figure 20 Structure of CD31.** CD31 is a 130 kDa type I transmembrane glycoprotein belonging to the Ig-like superfamily of adhesion molecules. The biological functions of CD31 have been mapped to specific regions of the CD31 molecule. Residues important for mediating homophilic and heterophilic binding interactions are located within Ig-domain 1 and Ig-domains 5 & 6, respectively. Localization of CD31 to membrane microdomains occurs after palmitoylation of residue Cys595. Two ITIMs encompass residues Y663 and Y686 within the cytoplasmic tail and are able to serve as docking sites for cytosolic signaling molecules when the tyrosines become phosphorylated.

### 1.6.2 Phosphotyrosine (Tyr (P))

Phosphorylation of proteins on tyrosine residues provides a key cellular control mechanism for intracellular signaling processes that regulate cell growth, proliferation, adhesion, differentiation, and metabolism (Cantley et al., 1991; Fischer et al., 1991). The level of tyrosine phosphorylation of cellular proteins is controlled by the coordinated actions of protein-tyrosine kinases and protein-tyrosine phosphatases. Signal transmission by tyrosine
phosphorylation is mediated by the binding of sequence-specific Src homology-2 (SH2) domains present on cytosolic signaling molecules to phosphotyrosine Tyr(P) sites on activated receptors (Mayer et al., 1988; Sadowski et al., 1986; Tyers et al., 1988). These highly conserved protein modules play an important role in mediating protein-protein interactions and can regulate many facets of the signaling process (Koch et al., 1991) (Pawson and Gish, 1992). The association of SH2-containing proteins with Tyr(P) sites on activated receptors can elicit biochemical changes within the cell, including regulating catalytic activity (Sugimoto et al., 1994), directing subcellular localization (Sabe et al., 1994), and enhancing tyrosine phosphorylation (Rotin et al., 1992) to potentiate downstream signaling events.

1.6. 3 SHP-2

SHP-2 (also known as PTP1D, PTP2C, SHPTP2, or Syp) is a family member of the SH2 domain containing phosphatases (SHPs), which are characterized by two SH2 domains at the N-terminus and a phosphatase domain at the C-terminus. SHP-2 was found to bind directly to a variety of receptor tyrosine kinases (RTKs) in response to stimulation by growth factors or cytokines (Feng et al., 1993; Lechleider et al., 1993). It is ubiquitously expressed (Neel, 1993) (Pawson, 1994) in contrast to its close relative, SHP-1, which is primarily detected in hematopoietic cells.

Multiple reverse-genetic studies suggest that SHP-2 is a required positive component of growth factor and cytokine signal transduction pathways. Microinjection of mutant SHP-2 mRNA molecules has revealed that SHP-2 is required for Xenopus mesoderm induction and completion of gastrulation (O'Reilly and Neel, 1998). Corkscrew (csw), the Drosophila
SHP-2 homolog, is needed for RTK signaling involved in early development (Allard et al., 1996). More recently, genetic evidence has been presented that SHP-2 enhances signaling from the epidermal growth factor receptor (EGFR) in mouse growth and development (Chen et al., 2000; Qu et al., 1999). Gene-targeted deletion of SHP-2 led to early mouse embryonic lethality, which is a common outcome for biologically important cell signaling components (Saxton et al., 1997).

A variety of studies indicate that SHP-2 positively regulates the activation of MAPK (mitogen activated protein kinase) or phosphatidylinositol 3-kinase (PI3K) signaling by growth factors and cytokines (Bennett et al., 1996; Deb et al., 1998). However, the important physiological functions of SHP-2 may not solely result from its involvement in mediating MAP kinase pathway activation. For example, SHP-2 has been demonstrated to play an important role in the regulation of cell spreading and cell migration (Oh et al., 1999; Yu et al., 1998).

Other SH2 domain-containing proteins that have been reported to associate with phosphorylated CD31 ITIMs, include members of the Src family of tyrosine kinases (SFK) (Matsumura et al., 1997) (Osawa et al., 1997), SHP-1 (Hua et al. 1998; Henshall et al. 2001), SH2 domain-containing inositol 5’-phosphatase (SHIP) and phospholipase Cγ1 (PLCγ1) (Pumphrey et al., 1999). Another residue in the CD31 cytoplasmic domain that is subject to post-translational modification is Cys595, which, when palmitoylated, can target CD31 to membrane microdomains where it can act as a regulator of cell signaling and apoptosis (Sardjono et al., 2006).
1.6. 4 Isoform-specific functions of CD31

The CD31 gene consists of 16 exons, with the cytoplasmic domain being encoded from the end of exon 9 through to exon 16 (Kirschbaum et al., 1994). Alternative splicing of the CD31 cytoplasmic and transmembrane domains results in the production of numerous CD31 isoforms, including a soluble form (Goldberger et al., 1994) and various isoforms that lack one or more cytoplasmic exons (Baldwin et al., 1994; Kirschbaum et al., 1994; Sheibani et al., 2000).

In humans, full length CD31 is predominantly the isoform expressed in all cells (Wang et al. 2003b), whereas CD31 mRNA in mice tends to undergo more extensive alternative splicing with Δ14,15 (loss of exons 14 and 15) being the predominant isoform expressed in most cells (Sheibani and Frazier, 1999). In particular, isoform switching of CD31 has provided the functional diversity in signalling mechanism because only CD31 isoforms that contain ITIMs encoded by exons 13 and 14 are able to efficiently recruit and activate SHP-2 (Wang and Sheibani 2006; Dimaio and Sheibani 2008; Bergom et al. 2008). This has cell-specific consequences, as expression in heterologous Madin-Darby canine kidney (MDCK) cells of a mouse CD31 isoform containing exon 14, as opposed to one lacking exon 14, led to activation of mitogen activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK), and the small GTPases Rac1 and Rap1, resulting in loss of cell-cell contacts, de-stabilization of adherens junctions, and a change in the subcellular localization of cadherins and catenins (Sheibani et al. 2000; Wang and Sheibani 2006). These effects have been proposed to explain the more migratory phenotype of CD31-expressing EC during angiogenesis, as exon 14-positive CD31 isoforms were found to be
preferentially expressed early in vascular development, and replaced later by isoforms lacking exon 14 (Sheibani et al., 1997; Sheibani et al., 2000).

**Figure 21** Cytoplasmic domain of CD31. The maroon-coloured exons 12, 13, 14 and 15 have been shown to be alternatively spliced during development. Of these exons, they have shown the following: exons 13 and 14 encode ITIM tyrosines that mediate the differential binding and activity of the phosphatase SHP-2; exon 15 encodes the binding site for tyrosine-phosphorylated β-catenin (β-cat), STAT3 and STAT5. Tyrosine residue Y686 encoded by exon 14 mediates binding of a kinase capable of tyrosine phosphorylating the bound STAT family member; and the phosphorylation state of serine residue S674, encoded by exon 13, appears to modulate γ-catenin (γ-cat) binding. Picture adapted from (Ilan et al., 2000).
1.6. 5 Of mice and men: clinical relevance of CD31 functions in the immune system

The immunoregulatory role of CD31 and its interactions has also been associated with vast number of 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\(^{\text{low}}\) 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), 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).

Genetic association studies tested for a correlation between genetic variants of CD31 and diseases such as graft-versus-host disease (GVHD) and the severity/complication of atherosclerosis has been highlighted. For instance, 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) 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).
Lastly, it was found that 11 different single-nucleotide polymorphisms (SNPs) exist within the gene encoding human CD31, but thus far only 3 have been associated with disease (Novinska et al., 2006). Three common polymorphisms (Val125Leu, Asn563Ser, and Gly670Arg) are found to locate on CD31 gene disease (Novinska et al., 2006; Pumphrey et al., 1999). The polymorphisms of CD31 have been deemed to be associated with myocardial infarction or coronary artery disease (CAD) (Shalia et al., 2010). Nevertheless, the functional significance of these SNPs has yet to be explored (Figure 22).
Figure 22 A. Amino acid sequence of the full-length human CD31 cytoplasmic domain (●) and of 2 predicted products of alternatively spliced CD31 mRNA species in which exons 14 and/or 15 are deleted and exon 16 is translated in an alternative reading frame (○). Perfectly conserved tyrosine residues are yellow-filled, perfectly conserved serine residues are orange-filled, and a free cytoplasmic cysteine residue near the membrane is red-filled. B, cDNA and corresponding predicted amino acid sequences around the sites at which human CD31 mRNA undergoes alternative splicing, resulting in removal of exon 14 and/or 15. Dotted lines identify splice junctions, and stop codons are boxed. Picture adapted from (Bergom et al., 2008).
1.6. 6 CD31 contribution to leukocyte transendothelial migration

CD31 was first cloned and characterized in 1990 (Newman et al., 1990; Stockinger et al., 1990). Many of the early studies of the biological functions of CD31 were focused on its pro-inflammatory role in leukocyte diapedesis. The first indications that CD31 helped to promote leukocyte transendothelial migration were demonstrated in two reports showing that CD31-specific antibodies blocked both leukocyte transmigration across endothelial monolayers in vitro (Muller et al., 1993; Vaporciyan et al., 1993) and leukocyte accumulation at sites of inflammation in vivo (Vaporciyan et al., 1993). These studies set the stage for a large body of literature further investigating the mechanism by which CD31 promotes leukocyte transmigration. As such, many of the established pro-inflammatory functions of CD31 (Table 1) center around its ability to support leukocyte emigration out of the vasculature and into inflammatory sites (Figure 23).
**Figure 23 Role for CD31 in leukocyte transendothelial migration.** 1. CD31 promotes chemokine-directed migration of leukocytes by enhancing actin polymerization and cycling. (See 1.2.7). 2. The binding interaction between CD177 on neutrophils and CD31 on ECs is the only known heterotypic binding interaction that has been shown to be important for CD31-mediated leukocyte transmigration. 3. Homophilic binding interactions between CD31 on leukocytes and ECs are thought to be essential for leukocyte diapedesis as blocking these interactions prevents diapedesis. 4. Downstream of homophilic binding interactions, CD31 is able to activate integrins on leukocytes that are essential for adhesion to ECs and passage through the basement membrane. Proposed mechanisms of CD31-mediated integrin activation on leukocytes include, but are not limited to, the activation of PI3K and the GTPase Rap1 (Reedquist et al., 2000). Picture adapted from (Privratsky et al., 2010).
### Table 1 Proinflammatory function of CD31

<table>
<thead>
<tr>
<th>Proinflammatory functions of CD31</th>
<th>Function</th>
<th>Mouse strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmigration of monocytes across cultured endothelial monolayers</td>
<td>Antibody blockade of CD31 prevents transmigration through unstimulated and cytokine-stimulated monolayers but does not prevent chemotaxis</td>
<td>N/A</td>
<td>(Muller et al. 1993)</td>
</tr>
<tr>
<td>Glycogen-induced peritonitis in rats</td>
<td>Antibody-blockade of CD31 prevented accumulation of neutrophils into the peritoneal cavity</td>
<td>N/A</td>
<td>(Vaporciyan et al. 1993)</td>
</tr>
<tr>
<td>Endotoxin-induced keratitis</td>
<td>Antibody blockade of CD31 inhibited neutrophil recruitment and prevented endotoxin-induced increases in stromal thickness and haze</td>
<td>BALB/c</td>
<td>(Khatri et al. 2002)</td>
</tr>
<tr>
<td>Leukocyte transmigration across endothelial monolayers</td>
<td>CD31 is part of the LBRC, a surface connected membrane compartment, that promotes both paracellular and transcellular migration</td>
<td>N/A</td>
<td>(Mamdouh et al. 2003; Mamdouh et al. 2008; Mamdouh et al. 2009; Dasgupta et al. 2009)</td>
</tr>
<tr>
<td>EAE</td>
<td>Short-term administration of chimeric soluble CD31 fused to human IgG-Fc (PECAM-Fc) decreased migration of lymphocytes across brain endothelial monolayers and diminished the severity of EAE when administered at the onset of symptoms, though chronically high levels of PECAM-Fc hastened onset of EAE</td>
<td>SJ/L</td>
<td>(Reinke et al. 2007)</td>
</tr>
<tr>
<td>Collagen antibody-induced arthritis</td>
<td>PECAM-Fc treatment reduced inflammation and attenuated bone and cartilage destruction in symptomatic mice</td>
<td>DBA-1</td>
<td>(Dasgupta et al. 2010)</td>
</tr>
</tbody>
</table>

CD31 has been linked with leukocyte diapedesis during both early and late stages of the adhesion cascade (Nourshargh et al., 2006; Woodfin et al., 2009). CD31 on leukocytes is reported to promote chemokine-mediated directional migration of leukocytes to inflammatory sites (Wu et al., 2005). Chemokine gradients serve to direct leukocytes to
their destination by activating integrins on the leukocyte surface and by promoting actin cycling and polymerization events at localized sites within the cell (Baggiolini, 1998). Consequently, leukocytes that express CD31 are better able to be recruited to the site of inflammation.

After leukocytes firmly adhere to the area of the endothelium to which they are recruited, they then squeeze through endothelial junctions and traverse the perivascular basement membrane to enter the inflamed tissue, both of which are processes that CD31 is known to promote. One of the well-established mechanisms by which CD31 promotes these processes is through homophilic CD31/CD31 adhesive interactions between leukocytes and EC as they traverse the endothelial cell-cell junction (Liao et al., 1995; Muller et al., 1993). Though GAGs and the integrin αvβ3 expressed on EC have been reported as heterotypic ligands for leukocyte CD31 (Buckley et al., 1996; DeLisser et al., 1993a), their physiological relevance remains in question. Perhaps, it is interesting to note that the cytoplasmic tail of CD31, is an important integrin adhesion amplifier, propagates signals that induce T cell adhesion via β1 (VLA-4) and β2 (LFA-1) integrins (Reedquist et al., 2000). They identified the small GTPase, Rap1, as a critical mediator in regulating ligand-induced cell adhesion and suggest that Rap1 may play a more general role in coordinating adhesion-dependent signals during leukocyte migration and extravasation.

In addition, antibodies directed against CD31 blocked accumulation of neutrophils in (1) the peritoneum following glycogen-induced peritonitis in mice and rats, (2) the lung following IgG immune complex deposition in rats, and (3) human skin grafts transplanted onto immunodeficient mice (Vaporciyan et al., 1993). Additionally, CD31 blocking reagents attenuated disease progression in a model of endotoxin-induced keratitis (Khatri
et al., 2002); decreased disease burden in dextran sulfate sodium (DSS)-induced colitis (Rijcken et al., 2007), which is a murine model of inflammatory bowel disease; significantly reduced ischemia-reperfusion injury in rats by preventing the accumulation of neutrophils in the myocardium following ischemic injury (Gumina et al., 1996); attenuated the severity of experimental autoimmune encephalitis (EAE) following short-term administration (Reinke et al., 2007) and significantly eliminated cartilage and bone destruction in collagen antibody-induced arthritis (Dasgupta et al., 2010).

Notably, there is growing evidence to indicate that leukocytes transmigration by CD31 is dependent on the genetic background of the mice. Briefly, mice on the C57BL/6 background appeared under-responsive to CD31 blockage or genetic deletion in a number of inflammatory models as compared with several other mouse strains FVB/n, SJL and Swiss Webster mice (Schenkel et al., 2004). The reason for these findings is at present unclear.

1.6. 7 Regulation of T lymphocyte trafficking by CD31

*In vitro* studies showed that blocking 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 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.
Furthermore, 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).

In addition, another study looked at by our group has also showed that CD31-mediated signals attenuate T cell chemokinesis both in vitro and in vivo (Kishore et al., 2012). This effect selectively affects activated/memory T lymphocytes, in which CD31 are clustered on the cell membrane where it segregates to the leading edge. This study provides evidence that this molecular segregation, which does not occur in naïve T lymphocytes, might lead to cis-CD31 engagement on the same membrane and subsequent interference with the chemokine-induced PI3K/AKT signaling pathway. Therefore, CD31-mediated modulation of memory T cell chemokinesis is a key mechanism by which this molecule contributes to the homeostatic regulation of effector T cell immunity.

1.6. 8 Anti-inflammatory roles of CD31

CD31 also plays a role in dampening inflammation as shown in a variety of clinically-relevant acute and chronic inflammatory conditions in C57BL/6 mice (summarized in Table 1), including collagen-induced arthritis (Tada et al., 2003; Wong et al., 2005), late-stage autoimmunity (Wilkinson et al., 2002), autoimmune encephalitis (Graesser et al., 2002), lipopolysaccharide (LPS)-induced endotoxic shock (Maas and Huijing, 2005) Carrithers et al. 2005), atherogenic diet-induced steatohepatitis (Goel et al., 2007), and
atherosclerosis (Goel et al., 2008). CD31 is thought to exert its anti-inflammatory effects by three main mechanisms, including:

(1) raising the threshold for leukocyte activation as a consequence of its function as an inhibitory receptor (Newton-Nash and Newman, 1999; Wilkinson et al., 2002),
(2) helping to maintain and restore the vascular barrier (Carrithers et al., 2005; Maas et al., 2005) and
(3) dampening production of pro-inflammatory cytokines (Carrithers et al., 2005; Tada et al., 2003). These three mechanisms will be discussed below.

1.6.9 The inhibitory role of CD31 in leukocyte activation

Evidence suggests the ability of CD31 to raise the threshold for leukocyte activation through its cytoplasmic ITIMs as indicated by the evidence that T cell activation was inhibited through attenuation of calcium mobilization from intracellular stores (Newton-Nash and Newman, 1999). It was later revealed that reduction of calcium mobilization in B cells by CD31 cross-linking required the CD31 ITIMs and the presence of SHP-2 (Newman et al. 2001). These findings demonstrated the importance of the immunoreceptor tyrosine-based inhibition motifs of CD31. In support of CD31 as an inhibitory receptor in lymphocytes, CD31 KO mice exhibit aberrant proliferation and activation of B cells, which correlates with development of autoimmune disease in older mice (Wilkinson et al. 2002). Genetic deletion of CD31 has been associated with exacerbation of T cell-mediated autoimmunity by two main lines of in vivo investigation. First, under conditions of immunological stress, lack of CD31 affects the extent of T-cell-mediated inflammation in mice. 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 (Tada et al., 2003; Wong et al., 2005).

Recently, our group has reported that tumor and allograft rejection are significantly enhanced in CD31-deficient mice, which are also resistant to tolerance induction (Ma et al 2010). I 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. I further showed 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 (Ma et al 2010).

Moreover, the novel role for CD31 in regulating CD4+ T cell homeostasis has been observed in a model of systemic Salmonella infection - which induces high levels of T cell activation and depends upon CD4+ T cells for resolution. Infection of CD31-deficient (CD31KO) mice demonstrates that these mice fail to control infection effectively. During infection, CD31KO mice have diminished numbers of total CD4+ T cells and IFN-γ-secreting Th1 cells. This is despite a higher proportion of CD31KO CD4+ T cells exhibiting an activated phenotype, and an undiminished capacity to prime normally and polarize to Th1. Reduced numbers of T cells reflected the increased propensity of naive and activated CD31KO T cells to undergo apoptosis after infection compared to wild-type (WT) T cells. Using adoptive transfer experiments, they show that loss of CD31 on CD4+ T cells alone is sufficient to account for the defective CD31KO T cell accumulation. These data are consistent with CD31 helping to control T cell activation as in its absence T cells...
have a greater propensity to become activated, resulting in increased susceptibility to become apoptotic. The impact of CD31 loss on T cell homeostasis becomes most pronounced during severe, inflammatory and immunological stresses such as those caused by systemic *Salmonella* infection (Ross et al., 2011).

### 1.6. 10 CD31 and dendritic cells

Recently, CD31 has been shown to act as a key coinhibitory receptor on stimulated dendritic cells, favoring the development of tolerogenic functions and finally resulting in T-cell tolerance (Clement et al, 2014). It was showed that the disruption of CD31 signaling favored the immunogenic maturation and migration of resident DCs to the draining lymph nodes. In contrast, sustaining the CD31/SHP-1 signaling during DC maturation resulted in reduced NF-κB nuclear translocation, expression of costimulatory molecules, and production of immunogenic cytokines (e.g., IL-12, IL-6), whereas the expression of TGF-β and IL-10 were increased. More importantly, CD31-conditioned DCs purified from the draining lymph nodes of ovalbumin-immunized mice favored the generation of antigen-specific regulatory T cells (CD25+ forkhead box P3+) at the expense of effector (IFN-γ+) cells upon co-culture with naive ovalbumin-specific CD4+ T lymphocytes ex vivo. Finally, the adoptive transfer of CD31-conditioned myelin oligodendrocyte glycoprotein-loaded DCs carried immune tolerance against the subsequent development of MOG-induced experimental autoimmune encephalomyelitis *in vivo*. The key co-inhibitory role exerted by CD31 on DCs highlighted by this study may have important implications both in settings where the immunogenic function of DCs is desirable, such as infection and cancer, and in settings where tolerance-driving DCs are preferred, such as autoimmune diseases and transplantation.
Likewise, in mast cells, CD31 suppresses their activation, which prevents systemic and local IgE-dependent anaphylactic reactions when animals are challenged with allergic stimuli (Wong et al. 2002). In macrophages, ligation of CD31 with a CD38-Fc fusion protein (a reported heterotypic ligand for CD31 on lymphocytes) was reported to negatively regulate Toll-like receptor (TLR) 4 signaling, likely through ITIM/SHP-2 interactions (Rui et al., 2007). While in B cells, CD31KO mice exhibit a hyperresponsive B-cell phenotype, increased numbers of B-1 cells, reduced B-2 cells, and develop autoantibodies. In addition, CD31 KO B cells display hyperproliferative responses to lipopolysaccharide and anti-IgM stimulation and showed enhanced kinetics in their intracellular Ca$^{++}$ response following IgM cross-linking. CD31 KO mice showed increased serum levels of IgM with elevated IgG isotypes and IgA antidinitrophenol antibody in response to the T-independent antigen, dinitrophenol-Ficoll. And CD31 KO mice developed antinuclear antibodies and lupuslike autoimmune disease with age (Wilkinson et al., 2002).

Taken together, these studies provide compelling evidence that CD31 is able to negatively regulate pro-inflammatory activation in lymphocytes, mast cells, and macrophages, likely through ITIM-mediated inhibitory signaling (Figure 24).
**Figure 24 The CD31 cytoplasmic tail contains ITIM tyrosines**, which when phosphorylated, can serve as docking sites for cytosolic signaling molecules. Most times, ITIMs recruit phosphatases to counteract kinases that become activated by ITAMs. The best characterized phosphatase that is recruited by CD31 is SHP-2. CD31/SHP-2 interactions have been proposed to inhibit activation of B cell, T cell, mast cell, and macrophage functions.

### 1.6. 11 CD31 dampens cytokine production

Studies by Carrithers, et al. demonstrated that CD31 helps to suppress the production of pro-inflammatory cytokines following endotoxin exposure (Carrithers et al., 2005; Maas et al., 2005). It was subsequently demonstrated that CD31 also suppresses cytokine production in two other mouse models of inflammation, namely nonalcoholic steatohepatitis (Goel et al., 2007) and collagen-induced arthritis (Tada et al., 2003). In the latter study, lymphocytes expressing CD31 produced lower levels of the pro-inflammatory
cytokine, Fin, following stimulation with collagen than did CD31 KO lymphocytes (Tada et al. 2003). The mechanism by which CD31 regulates cytokine production is still not clear. Rui et al. 2007 reported that CD31 ligation in T cells with a CD38 fusion protein was able to inhibit activation of the JNK, NF-κB, and IRF-3 pathways, which was correlated with dampened cytokine production.

1.6. 12 Cytoprotective effect of CD31

There are studies and reports showing that the anti-inflammatory effect of CD31 in endotoxic shock and autoimmunity is dependent on its expression by EC, but not leukocytes (Carrithers et al., 2005). However the exact mechanism accounting for this effect is not known.

Engagement of CD31 and induction of AKT phosphorylation to prevent apoptosis have been described in several cell types (Bird et al., 1999; Poggi et al., 2010). AKT activation represents a positive, CD31-ITIM mediated signaling.

CD31 has been demonstrated as a specific and potent inhibitor of mitochondrial-dependent apoptosis as shown by the fact that murine EC and human T lymphocytes lacking CD31 were far more sensitive than their CD31expressing counterparts to multiple death signals that stimulate Bax, a multi-domain, proapoptotic member of the Bcl-2 family that plays a central role in mitochondrial dysfunction-dependent apoptosis (Gao et al., 2003). It was found that CD31 markedly suppressed Bax overexpression-induced cytochrome c release, caspase activation, and nuclear fragmentation. Amino acid substitutions within CD31’s extracellular homophilic binding domain, or within its cytoplasmic ITIM, completely abolished CD31—mediated cytoprotection. Taken together, these data implicate CD31 as a
novel and potent suppressor of Bax-mediated apoptosis and suggest that members of the immunoglobulin gene (Ig) superfamily, like cell surface integrins, may also transmit survival signals into blood and vascular cells (Gao et al., 2003).

Interestingly, it has also been shown that ligation of CD31, which drives CD14+CD34+ (peripheral blood mononuclear cells) transendothelial migration, leads to an increase in Bcl-2 A1 and Bcl-X intracellular content, and to protection from starvation-induced apoptosis. This event is dependent on the engagement of phosphatidylinositol-3 kinase and activation of AKT/PKB that is known to contribute to Bcl-2 and Bcl-X induction. These data point to a critical role of the endothelium in preventing the apoptotic program triggered by starvation, possibly inducing a prolonged survival of antigen presenting cell precursors, in order to allow recirculation of these cells and localization to the site of priming of T lymphocytes (Ferrero et al., 2003).

At a molecular level, CD31 expression was correlated with enhanced phosphorylation of STAT3 in both EC and splenocytes from mice (Carrithers et al., 2005). It was proposed that binding of SHP-2 to the CD31 ITIMs sequesters SHP-2 away from STAT3, which prevents SHP-2-mediated STAT3 dephosphorylation and prolongs activation of STAT3 (Carrithers et al., 2005). Consequently, EC expressing CD31 are postulated to have more STAT3 mediated anti-inflammatory signaling. This mechanism bears further examination in mice, however, since the predominant isoforms of CD31 that are expressed in murine tissues, including EC, lack exon 14 (contains the second cytoplasmic ITIM) (Sheibani et al., 1997), and thus are not likely to be able to efficiently recruit SHP-2 (Wang and Sheibani, 2006).
Alternatively, Cepinskas, et al. reported that CD31 engagement, induced by either antibody-mediated cross-linking or leukocyte transmigration, resulted in decreased levels of NF-κB in the nuclei of EC. This suggested that the inhibition of NF-κB translocation to the nucleus by CD31 may initiate a negative feedback loop that prevents excessive leukocyte recruitment to sites of inflammation by dampening the NF-κB-dependent expression of pro-inflammatory adhesion molecules on the EC surface (Cepinskas et al., 2003). However, further investigations reported inconsistent findings to show correlation between NF-κB and CD31 in anti-inflammation (Privratsky et al 2010 and 2010). Similarly, Ma et al 2012 suggested that the CD31-mediated signals is induced by upregulation of extracellular-signal-regulated kinase (Erk) activity, independent of NF-κB (Ma et al., 2010).

Interestingly, even though engagement of endothelial CD31 during leukocyte transmigration does not appear to inhibit NF-κB activity, it likely does send inhibitory signals to prevent excessive endothelial activation. Couty, et al. demonstrated that CD31 ligation with monoclonal antibodies counteracted ICAM-1 ligation-induced endothelial activation and cytoskeletal rearrangement, which is thought to promote junctional opening and leukocyte transit (Couty et al., 2007).

All in all, CD31 is likely to be protective in EC during inflammation due to its ability to (1) inhibit cytokine production, (2) maintain vascular integrity, and/or (3) inhibit pro-inflammatory signaling.
1.6. 13 CD31 helps to maintain vascular barrier function

Furthermore, report by (Ilan et al, 1999) showed that CD31 can act as a dynamic modulator of vascular EC behaviour/reservoir for tyrosine phosphorylated β-catenin. The ability of CD31 to prevent β-catenin nuclear translocation and to enable cell border localization in stably transfected human colon cancer cell line SW280 cells was reported. The results suggested a mechanism by which ECs regulate changes in the free cytoplasmic pools of β-catenin and maintain their stability. Specifically, CD31 is involved in controlling the localization and levels of tyrosine phosphorylated β-catenin by both recruiting it to the plasma membrane and by its presumed SHP-2-mediated dephosphorylation (Ilan et al, 1999) (Figure 25). SW480 cells are not only defective in their APC protein, but also lack E-cadherin (Vermeulen et al., 1995). It is not surprising, therefore, that upon transient transfection, N-cadherin or alpha-catenin were able to prevent β-catenin nuclear translocation (Simcha et al., 1998). In parallel, the protein tyrosine phosphatase SHP-2 was found to coimmunoprecipitate with CD31 in ECs and in platelets (Jackson et al., 1997), a molecular interaction that resembles the CD31/β-catenin/SHP-2 interaction in the case of ECs. This also further suggested the importance of ITIM motif (Tyr 663 and 686) for SHP-2 interaction although the precise role of β-catenin tyrosine phosphorylation on EC adhesion and permeability is still to be determined (Jackson et al, 1997).

The complex organization of the cadherin-catenin-cytoskeleton is thought to function not only as an adhesion compartment but also to transfer signals to, or between cells (Ben-Ze'ev and Geiger, 1998). One way by which catenin-based signalling can be modulated is
by controlling the cytoplasmic levels of catenins. Indeed, in addition to their roles as structural proteins in adherens junction complexes, both β-catenin and plakoglobin can translocate to the nucleus and initiate gene expression. Therefore, β-catenin cytoplasmic levels are tightly controlled by being incorporated into junctional complexes and by complexing with APC, GSK-3β and axin (Ikeda et al, 1998), which direct β-catenin to proteosome degradation (Barth et al., 1997).

**Figure 25 Working model of the dynamic CD31/b-catenin/SHP-2 interactions.** CD31 is a sink/reservoir for tyrosine phosphorylated b-catenin. Tyrosine phosphorylation of b-catenin (b-cat) occurs upon VEGF stimulation, c-src activity or as a result of ECM-cell interactions. The tyrosine phosphorylated b-catenin then binds to the cytoplasmic domain of CD31, sequestering it and keeping it available for subsequent dephosphorylation by SHP-2. CD31 is a modulator of b-catenin tyrosine phosphorylation. During EC attachment, spreading, migration and differentiation there is modulation of the phosphorylation state of the CD31 ITAM domain by protein tyrosine kinases and protein tyrosine phosphatases (PTK and PTP), thus regulating the binding of the tyrosine phosphatase SHP-2. Bound, activated SHP-2 may then de-phosphorylate the CD31- bound tyrosine phosphorylated b-catenin. Diagram adapted from (Ilan et al., 1999)
1. 7 Aims of this PhD project

Specific aims of this study are summarized below:

**Aim 1.** To establish the contribution of CD31-activated signals to cytoprotection of EC exposed to extrinsic pro-apoptotic signals including cytokines, cytotoxic T cells and complement-binding antibodies.

**Aim 2.** To define signalling pathways and intracellular mediators of CD31-mediated pro-survival activity and the contribution of intracellular CD31 domains (ITIMS) to the activation of these pathways.

**Aim 3.** To investigate the role of CD31 in maintaining endothelial barrier integrity during adaptive immune responses.
Chapter 2 Materials and Methods
## 2. 1 Media

### Table 2. Media, use and composition

<table>
<thead>
<tr>
<th>Medium</th>
<th>Use</th>
<th>Composition</th>
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<tbody>
<tr>
<td>EC culture medium</td>
<td><em>in vitro</em> cultures</td>
<td>ECs were serially sub-cultured at 37°C with 5% CO2 in DMEM (Life Technologies) supplemented with 2 mM glutamine (Life Technologies), 100 μg/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 10 mM 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), and 75 μg/ml EC growth supplement (Sigma-Aldrich) in 2% gelatin-coated (Sigma-Aldrich) tissue culture flasks (BD Biosciences).</td>
</tr>
<tr>
<td>T cell culture medium</td>
<td><em>in vitro</em> cultures</td>
<td>RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Globepharm), 2 mM glutamine (Gibco), 50 μg/mL penicillin (Gibco), 50 μg/mL streptomycin (Gibco), and 50 μM 2-mercaptoethanol (Gibco). CD8+ T cells were subsequently isolated by immunomagnetic negative selection (Mitenyi Biotech) prior to use.</td>
</tr>
<tr>
<td>Freezing Medium</td>
<td>Storing cells in liquid nitrogen</td>
<td>8 ml complete medium, 1 ml DMSO, 1 ml 20% FCS</td>
</tr>
<tr>
<td>PBS</td>
<td>Resuspending of cells/reagents for <em>in vivo</em> use</td>
<td>NaCl, 137 mmol/L; KCl, 2.7 mmol/L; Na₂HPO₄, 8 mmol/L; KH₂PO₄, 2 mmol/L; pH 7.3. Oxoid, Hampshire, UK.</td>
</tr>
<tr>
<td>RBC lysis buffer (Qiagen, Hilden, Germany)</td>
<td>Depletion of red blood cells before counting/FACS</td>
<td>Unspecified concentrations of NaCl, EDTA and Sodium Bicarbonate</td>
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</table>
2. 2 Mice

All mice in this study were used between the ages of 4-11 weeks and purchased from Charles River. CD31KO mice were bred in house. All the in vivo experiments were conducted under the Home office regulation following approval by the Queen Mary University of London Ethics Committee. The mice used in this project were housed under Special Pathogen-Free conditions in the Biological Services Unit in accordance with Home Office guidelines as defined in the project licence.

Table 3. Mice, strain, phenotype and primary reference

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>Wild type, Thy1.2⁺</td>
<td>Charles River</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>CD31 KO</td>
<td>(Duncan et al., 1999b)</td>
</tr>
<tr>
<td>Balb c</td>
<td>Wild type</td>
<td>Charles River</td>
</tr>
</tbody>
</table>

Generation of CD31KO mice

A 129/J mouse genomic library was screened with a CD31 cDNA probe, and a phage clone containing exons 6–8 was isolated. A targeting vector was designed to disrupt exon 7 by inserting a pGK1neo resistance expression cassette in reverse orientation of CD31.
transcription. The targeting vector (20 μg) was linearized with KpnI and electroporated into 5 × 10⁶ E14K embryonic stem (ES) cells (derived from 129/Ola mice) maintained on a layer of mitomycin C-treated embryonic fibroblasts in DMEM, supplemented with leukemia-inhibitory factor, 15% FCS, L-glutamine, and 2-ME. The electroporated cells were subsequently cultured in 300 μg/ml G418 for 8 days. Homologous recombinants were identified by PCR using the following primers: 5′-AGG TAA GGA CCT ACA GGT GTG TTC-3′ plus 3′-CTT CCT CGT GCT TTA CGG TAT C-5′, yielding a mutant band of 1 kb. PCR conditions were as follows: 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C for 30 cycles. Colonies positive for PCR were genotyped by Southern blotting analysis using a PCR-amplified flanking probe and a neomycin-specific probe.

Chimeric mice were produced by microinjection of targeted ES cells into 3.5-day C57BL/6J blastocysts that were subsequently transferred to CD1 pseudopregnant foster mothers. Chimeric males were mated with C57BL/6J females. Germline transmission of the mutant allele was verified by Southern blotting analysis of tail DNA from F₁ offspring with agouti coat color. Both of the two targeted ES clones contributed to the germline transmission of the mutation. F₂ offspring from heterozygous intercrosses were genotyped by Southern blotting analysis and analyzed by flow-cytometric analysis of surface expression of CD31 on the surface of leukocytes. Mutant mice derived from the two targeted ES cell clones showed the same phenotype.

2. 3 Reagents and antibodies

The reagents used were: recombinant murine TNF alpha (PeproTech), AKT inhibitor (AKT Inhibitor X; Calbiochem) and ERK inhibitor (PD98059; Cell Signalling). The
antibodies used for western blots were: anti-P-AKT (ser-473) (1:1000), anti-AKT (1:1000), anti-PERK1/2 (1:1000), anti-ERK1/2 (1:1000; Cell Signaling); anti- PY20 (1:500), anti-SHP2 (1:500; Abcam); anti-FLIP-L (1:500), anti-PECAM1 (1:500), anti-b-actin (1:1000; Santa Cruz Biotechnology). The IgG2a, kappa, and Rat IgG2a K Isotype Control Purified (clone eBR2a) were purchased from eBioscience. As a blocking reagent, rat anti-mouse CD31 Antibody clone 390 (eBioscience) was used at a concentration of 10 μg/mL. To stimulate signaling, CD31 molecules were ligated with polyclonal rabbit anti-mouse CD31 (5 μg/mL) (ab28364; Abcam) plus goat–anti-rabbit Ig (2.5 μg/mL) (Thermo Scientific).

2. 4 Cell cultures

Murine ECs were purified from WT and CD31 KO mouse lung tissue as described (Lidington EA 2001) in 2% gelatin-coated (Sigma-Aldrich) tissue culture flasks (BD Biosciences). At confluence, ECs were detached from the culture flasks using trypsin/EDTA (Life Technologies) and passaged. In all experiments, ECs were used between passages 3 and 4. HY-specific CD8+ T cells were generated by stimulation of T cells isolated from the lymph nodes of C57BL/6 female mice with splenocytes isolated from syngeneic male mice and interleukin-2 (20 U/mL; Roche) for 10 days in RPMI 1640 medium plus supplements as listed above.

SV40 T-transformed insulinoma cells derived from NOD mice, MIN6, were kindly provided by Professor Marco Falasca and grown in DMEM culture medium containing 25 mM glucose (Life Technologies) supplemented with 15% FBS, 2 mM glutamine, 100 ug/ml penicillin and 100 ug g/ml streptomycin.
2. 5 Flow cytometry

Cells were prepared for Flow Cytometric analysis as follows: Cells were depleted of red blood cells using 2ml of red cell lysis (Qiagen) buffer incubated for 15mins at room temperature. Cells were suspended in FACS buffer (PBS, 1% BSA, 0.01% sodium azide), stained with the appropriate concentration of fluorescence-conjugated antibodies, isotype control antibodies or CFSE according to the manufacturer’s instructions, fixed in fix buffer (PBS, 4% PFA, 1%FCS) and analyzed by a FACS ARIA (Becton Dickinson).

The following antibodies were used: PE- conjugated anti-mouse CD95 (APO-1/Fas; BD Biosciences), anti-mouse CD274/PDL1 (BD Biosciences) and rat anti-mouse CD31 antibody clone 390 (eBioscience). The IgG2a, kappa, and Rat IgG2a K Isotype Control Purified (clone eBR2a) were purchased from eBioscience. All flow cytometry antibodies were used at 1:100 dilutions unless otherwise specified. Acquired samples were analyzed using FlowJo 7.6 software (TreeStar Inc).

2. 6 Complement lysis assay

As a source of complement and complement-fixing xenoantibodies, human serum was obtained after sedimentation of blood of healthy donors. For separating serum from the blood cells, sterile tubes without any anticoagulants were used and the tubes were left in a standing position for about 20-30 minutes before centrifugation at 1500g (20°C) for 10 minutes. Serum was then extracted and frozen at -80°C. In cell lysis assays, the human
serum was diluted 1:10 in RPMI and then added to murine EC monolayers, which were then incubated at 37°C.

2. 7 TdT-mediated dUTP nick-end labeling (TUNEL) assay

dUTP nick-end labeling (TUNEL) in situ cell death detection kit Fluorescein (Roche Diagnostics). DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) has been used for the incorporation of labelled nucleotides to DNA strand breaks in situ. The tailing reaction using TdT, which was also described as ISEL (in situ end labelling) technique, has several advantages in comparison to the in situ nick translation (ISNT) using DNA polymerase:

- Label intensity of apoptotic cells is higher with TUNEL compared to ISNT, resulting in an increased sensitivity.
- Kinetics of nucleotide incorporation is very rapid with TUNEL compared ISNT, resulting in an increased sensitivity.
- Tunel preferentially labels apoptosis in comparison to necrosis, thereby discriminating apoptosis from necrosis and from primary DNA strand breaks induced by antitumor drugs or radiation.

Briefly, cells were fixed with 4% paraformaldehyde for 1 h and permeabilized with 0.2% Triton X-100 in phosphate buffered saline for 10 min before incubating in TUNEL reaction mixture. Cells were subsequently stained with 4, 6-diamidino-2-phenylindole and visualized under a fluorescence microscope with an (x40) objective. Eight representative areas were randomly selected. At least (500 cells) 4, 6-diamidino-2-phenylindole-positive
cells were scored. The percentage of apoptotic cells was determined by dividing the number of TUNEL-positive cells by the total number of cells in the corresponding area.

2. 8 Gene expression analysis by quantitative real-time polymerase-chain reaction (qRT-PCR)

Total RNA was extracted with Trizol (Life Technologies), purified using the PureLink RNA mini-kit (Life Technologies) and assessed for quality and quantity using absorption measurements. RNA with an RNA integrity number of ≥7.6 was used for analysis using pathway-focused RT-PCR array systems for apoptosis (RT2 Profiler PCR Array Mouse Apoptosis; SABiosciences) using an Applied Biosystems 7500 Fast Real-Time PCR machine. Ct values were gathered using 7500 Fast System SDS Software. Comparative data analysis was performed via the ΔΔCt method using the PCR Array Data analysis Web Portal (http://www.SABiosciences.com/pcarraydataanalysis.php) to determine relative expression differences between the comparison groups. Changes of mRNA abundances by 2-fold and higher with a p value <0.05 were considered significantly different between the comparison groups as shown. (see supplementary 2).

Confirmation studies and relative quantification of additional genes was performed using QuantiFast SYBR Green RTPCR Kit (SABiosciences) with 100 ng RNA/reaction and following the manufacturer’s protocol. Specific primers for qRT-PCR were designed with the help of online tools (Primer 3Plus) using at least one exon junction-binding site per primer pair. Sequences of the qRT-PCR primers are as follows: CD95 (5’-ATG CTG GGC ATC TGG ACC CT-3’ and 5’-CAA CAT CAG ATA AAT TTA TTG CCA C-3’); c-FLAR (5’-GAC CCT TGTGCT TCC CTA-3’ and 5’-GTT AAT CAC ATG GAA CAA
TTT CC-3'); Caspase 7 (5'- CTACCGCCGTTGGGAAACGATGGCAGA-3'and 5'- CGAAGGGCCATACCTGTCACTTTATC-3'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'- AGA ACG GGA AGC TTG TCA TCA-3' and 5'- GAC CTTGCC CAC AGC CTT G-3'). Thermal cycling profile for amplification was: 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 54°C for 1 min. Amplification was 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. To ensure the amplification specificity, melting curve program was set as follows: 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec, right after the PCR cycles.

2. 9 Immunoprecipitation

Target antigens are immunoprecipitated from complex solutions, cell lysates, the goal being to isolate and eventually detect and measure a specific protein (i.e., the antigen of the specific antibody). The basic protocol for performing an IP is diagrammed below, where the order (sequence) of steps can be done in two different ways.

In one sequence (left), an antibody (monoclonal or polyclonal) against a specific protein is pre-immobilized onto an insoluble support, such as agarose or magnetic beads, and then incubated with a cell lysate containing the target protein. During the incubation period, gentle agitation of the lysate allows the target antigen to bind to the immobilized antibody. The immobilized immune complexes are then collected from the lysate, eluted from the support and analyzed based on the nature of the target antigen.

Alternatively (right), free, nonbound antibody is allowed to form immune complexes in the lysate and then the complexes are retrieved by the beads. While the pre-immobilized
antibody approach is more commonly used for IP, using free antibody to form immune complexes is beneficial if the target protein is present in low concentrations, the antibody has a weak binding affinity for the antigen or the binding kinetics of the antibody to the antigen are slow (Figure 26).

Figure 26 Diagram of immunoprecipitation (IP) using either pre-immobilized or free antibodies. Elution at the final step typically involves heating the beads in sample loading buffer for polyacrylamide gel electrophoresis (SDS-PAGE), which results in denaturing the proteins and irreparably damaging the beads. Adapted from (Bjorck and Kronvall, 1984).
Most immunoprecipitations are performed with Protein A, G or Protein A/G, which is an engineered recombinant protein combining four Protein A and two Protein G antibody binding sites. Protein A and G both show high affinity for antibodies of multiple, but not necessarily identical, subclasses and Ig species, while Protein A/G binds all of the subtypes to which Protein A and G individually bind.

Immobilized Protein A, G and A/G (hereafter collectively called "Protein A/G") are effective tools for attaching antibodies to a beaded support for IP applications. Innovations in manufacturing of prepared Protein A/G resins have yielded commercially available supports that have very high binding capacities, enabling excellent immunoprecipitation results to be obtained with very small volumes of beads. Binding capacities of 30 to 50mg Ig per mL resin with very low nonspecific binding are possible with these affinity resins (table 4).
Table 4 Species immunoglobulin Protein G binding. The affinities of immunoglobulins for Protein G vary between different species. Although Protein G is recommended for most species, it is not recommended for detection of mouse IgA, IgM, or IgD.

The steps involve 1) Formation of antigen antibody complex: mix cell lysate with antibody. 2) Immunoprecipitations: add protein G agarose. 3) Removal of non-specific bindings. Snap off column tip, place in microfuge tubes. Spin and wash extensively. 4) Elution of the immunoprecipitated proteins, inverted broken tip serves as column closure (Figure 27).
Figure 27 Schematic of procedure of Protein-G IP. The matrix bound protein (via the specific antibody) can then be separated from the mixture by centrifugation. The matrices commonly used are agarose bound Protein A, G, or L, or anti-Ig bound to agarose. The Protein G Immunoprecipitation protocol is especially designed to allow maximal recovery of immunoprecipitates. The process is performed in mini-spin columns, instead of in microcentrifuge tubes, which enables convenient washing of the antigen-antibody bound beads. Picture adapted from Sigma, Immunoprecipitation.
2. 10 Western blotting

Whole-cell lysates were lysed in Nonidet P-40 lysis buffer (50 mM hepes 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). Membranes were blocked for 2 hours at room temperature in 5% Milk / TBS-T, incubated overnight at 4°C with the primary antibodies listed above in Reagents and Antibodies and subsequently with HRP conjugated secondary antibody (1:5,000 Amersham Bioscience). Films were then scanned and the intensity of the bands was quantified using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA).

2. 11 Caspase activity assays

The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in purified enzyme preparations or cultures of adherent or suspension cells. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. The Caspase-Glo® 3/7 Reagent is optimized for caspase activity, luciferase activity and cell lysis. Addition of the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal. The Caspase-Glo®
3/7 Assay is designed for use with multiwell plate formats, making it ideal for automated high-throughput screening of caspase activity or apoptosis (Figure 28).

Cells were first seeded in 96-well plates and incubated with or without inflammatory stimulus, then Caspase-Glo® 3/7 Reagent (Promega) was added according to manufacturer’s protocol. The samples were incubated for 15 min in the dark and then analysed by measuring the fluorescence with an excitation wavelength of 488 nm and an emission wavelength range of 530 nm (Molecular Devices).

Figure 28 Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light. Picture adapted from https://www.promega.co.uk/resources/protocols/technical-bulletins/101/caspase-glo-37-assay-protocol/
2. 12 Preparation of CD31 recombinant constructs

The murine CD31 plasmid is a gift from Prof Chris Buckley. cDNA of CD31 fused in-frame with GFP at the C-terminus (figure 29). Figure 30 shows the 8 amino acid spacer used to attach GFP to the cytoplasmic tail of CD31. It was then used as template to generate tyrosine to phenylalanine (Y to F) mutations at the amino acids residues 663 and 686 (Y663F or Y686F, respectively) using the QuickChange site-directed mutagenesis kit, Stratagene (Figure 31). Mutagenesis of the experiment was performed by his team. The diagram for recombinant lentiviral expression vector pWPT-CD31 is shown in figure 32.
Figure 29 WT CD31 cloned into pcDNA3 using Hind3 and Not1. This pcDNA3.1 vector is designed for high-level, constitutive expression in a variety of mammalian cell lines. Picture adapted from addgene.
Figure 30. 8aa Spacer used to attach GFP to the cytoplasmic tail of CD31. This picture shows an 8-amino-acid linker provides a spacer between the proteins.

Figure 31 Recombinant PCR generation of new CD31 mutants. To generate new point mutation the CD31-pCDNA3 plasmid was used as a template (Figure 31). In brief, step 1) specific primer pairs incorporating the codon change were used (full length in black and mutation primer in red), resulting in 2 PCR products. The same full length primers were then used in reaction 3 to produce a full length mutated insert (step #2). Finally, in (step
restriction digests were performed to allow the directional cloning of the mutated insert into the CD31-pcDNA construct, resulting in a new CD31 point mutant.

This construct was further subcloned into PWPT-GFP vector for retroviral infection.

Figure 32 pWPT expressing plasmid. Wild-type (WT), Y663F, and Y686F CD31 cDNAs were then sub-cloned into the pWPT lentiviral expression vector at MluI and SalI restriction sites and checked by sequencing. Picture adapted from https://www.addgene.org/12255/
2. 13 Lentivirus preparation for gene silencing and overexpression

Bacterial glycerol stocks containing sh-RNA plasmid clones targeting cFlar cloned in the pLKO.1 vector were purchased from Sigma. Lentivirus packaging and envelope vectors, pMDLg/pRRE, pRSV-Rev and pENV were purchased from Addgene.

Lentivirus packaging and envelope vectors, R8.91 and MD2G were purchased from Addgene. HEK293T-cells were grown in 10 x 10cm cell culture dishes to 70% confluence and transfected with the above listed plasmids using the calcium phosphate method. The supernatant were harvested 48 and 72 hours after transfection and hundred-fold concentrated in an ultracentrifuge. Aliquots were stored at -80°C.

2. 14 Lentiviral transduction of ECs and MIN6 cells

CD31KO ECs and MIN6 cells were seeded in six-well plates and cultured in DMEM to 60–70% confluent. Lentivirus was added to the cells in the presence of 5 μg/ml polybrene, and the six-well plate centrifuged at 2,300 rpm for 90 min at room temperature, followed by 8h incubation at 37°C with 5% CO₂. Virus was removed 24h later; T-cells washed twice with PBS and incubated for 24h in complete DMEM. Expression of GFP enabled tracking and sorting of infected cells by flow cytometry.
2. 15 Insulin production

This assay is a Sandwich ELISA based, sequentially, on: 1) Antigen coating. Prepare an antigen solution at the appropriate concentration in PBS. Note: If problems with non-specific binding occur, an additional blocking step (30 min. 5% BSA-PBS) may be required. (Vogt, R.F., et al., J. Immunol. Meth., 101, 43 (1987)). 2) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-tittered amount of a monoclonal mouse anti-mouse insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, 3) wash away of unbound materials from samples, 4) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 5) wash away of free enzyme conjugates, and 6) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3’,5,5’-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

Insulin secretion was measured in serum free DMEM with no glucose, supplemented with 2 mM L-glutamine and 25 mM Hepes, pH 7.4. MIN6 cells were plated in 24-well culture plates at 2-5 × 10^5 cells/well overnight. Cells were then washed twice and preincubated in DMEM containing 2.8 mM glucose for 30 min at 37 °C. After the preincubation period, cells were washed thoroughly with PBS and incubated at 25 mM for 1 h at 37°C. Aliquots
(450 μl) were taken at the end of the incubation period and stored at -20 °C until assayed by EZRMI-13K | Rat/Mouse Insulin ELISA, Merck Millipore.

2. 16 Induction of diabetes

I am indebted to Dr. Liang Ma for completing this protocol. To induce the type 1 diabetic mice, mice were given a single intraperitoneal injection of streptozotin at dose of 170 mg/kg (sigma-Aldrich) freshly dissolved in 0.05 M citrate buffer. I have chosen a single large dose of STZ capable of eliminating endogenous insulin production in order to rigorously evaluate islet graft function.

Mouse blood glucose values were used to determine diabetes induction. Glucose was determined in blood samples obtained from the tail vein of mice and using blood glucose and ketone monitoring system (FreeStyle Optium, Abbott Laboratories Limited, UK) under the nonfasting status. The measurement was taken twice a week. Type 1 diabetic mice showing ≥ 300 mg/dL of blood glucose levels after STZ injection were used.

2. 17 Transplantation of pancreatic cells in mice

I am indebted to Dr. Liang Ma for completing this protocol. Mouse islet subcapsule transplantation is widely used in diabetes-related studies. Reliable and reproducible transplantation is essential to the success of these types of investigations.
The islets produce insulin, which actively regulate the level of glucose in the blood. For the purpose of the experiment, the blood glucose tracings under syngeneic and allogeneic islet transplantations will be monitored continually.

_Packaging islets for transplantation_

1. Transfer predetermined amount of islets for each recipient into 1.5 ml eppendorf tubes individually and let the islets settle down to the bottom.
2. Collect the islets into PE-50 tubing at about the half way of full length of the tubing (15~20 cm) with a microsyringe, and then fold the PE-50 tubing at about half way leaving all islets in one end (Fig. 33).
3. Insert the folded PE-50 tubing into a 200 μl pipet tip, then, put into a 15ml centrifuge tube and centrifuge at 2500 rpm for 10 min at 4oC (Fig.33).
4. After centrifugation, attach PE-50 tubing to the microsyringe again, remove the pipet tip and cut a cant at about 1 cm apart from the islet pellet.

_Islet transplantation_

5. Anesthetize the diabetic mouse with Nembutal (i.p., ~0.05ml per mouse) and shave the left flank of the mouse. Then, swab the shaved area with iodophors.
6. Make a small incision through skin and muscle of the left back side of the animal.
7. Expose the kidney outside the body using two saline-wetted cotton-tipped applicators. Apply a slight pressure to both sides of the incision, raise or pop the kidney out of the abdominal cavity. Keep the kidney moist by applying saline with a cotton-tipped swab.
8. Using a 25G syringe needle, make a small scratch on the upper pole of the kidney, creating a nick in the kidney capsule (Fig. 34).

9. Insert the “L”-type glass rod into the hole in the capsule and carefully move it under the capsule to make a small pouch (Fig. 34).

10. Slightly lift the capsule with the glass rod and carefully insert the islet-containing PE-50 tubing into the pouch. Then, release the islet-pellet with the aid of microsyringe. Once all islets are inside the pouch slowly remove the tubing and quickly seal the pouch with a cautery.

**Figure 33 Packaging islets into transplantation tubing.** Packaging islets into transplantation tubing. Given amount of islets for each mouse were kept with an eppendorff tube. After the islets settled down, they were collected into PE-50 tubing with the aid of microsyringe. The islet-containing tubing was folded and inserted into a 200 μl pipet tip, then, put into a 15 ml conical centrifuge tube. An islet pellet was formed after centrifugation. The islet pellet containing tubing was mounted onto the microsyringe for transplantation. Picture adapted from http://www.nature.com/protocolexchange/protocols/2060/#/procedure
Figure 34 Illustration of renal subcapsule pouch making and islet transplantation. The corresponding step-wise flow chat is described as follows: punch a hole on the top pole of the kidney → insert the glass rod and make a subcapsular pouch → insert the islet-containing tubing with the aid of the glass rod → move the tubing back and forth as indicated by arrows → release islet into the bottom of the pouch with the aid of the microsyringe. The recipient mice were anesthetized with pentobarbital sodium at 50 mg/kg and were placed in anatomical microscope. Then total 5x10^5 MIN-6 beta cells transfected with CD31 molecule or its control molecule pWPT were collected and transplanted into the capsule of left kidney of the recipient mice. 100 μl of Vetersgesic Multidose (0.3 mg/ml, Alstoe Animal Health) were given for post-operative analgesia. All animal experiments were performed in accordance with protocols approved by the Home Office and our institutional Animal Care and Use Committee. Picture adapted from http://www.nature.com/protocolexchange/protocols/2060#/procedure

2. 18 Immunohistochemistry

Kidney tissues were placed in 10% neutral-buffered formaldehyde immediately after mice were sacrificed. Paraffin embedded tissue dissections were used for identification of indicated proteins. Samples were briefly deparaffinised and rehydrated sections were subject to antigen retrieval via boiling in sodium citrate buffer. Sections were then incubated in blocking solution (TBS, 3% bovine serum albumin) and sequentially incubated with the following primary antibodies: Insulin (ab7842, dilution 1:200; Abcam);
CD3 (ab5690, 1:100; Abcam); CD31 (ab28364, 1:150; Abcam). Sections were then labelled with biotinylated secondary antibodies. The sections were counterstained with haematoxylin (Sigma).

2. 19 Mixed lymphocyte reaction

T cells were isolated from draining, non-draining lymph nodes and spleens collected from CD31 WT/ pWPT-transplanted and naive BALB/C mice at the day of sacrifice. Live cells were isolated by layering harvested cells on Ficoll and centrifuging for 20 mins at 400g. Live cells were lifted from the top layer of Ficoll and washed once with PBS, before labelled with 5 μm (final concentration) of 5-(and 6)-CFSE (Molecular Probes Europe BV, Leiden, The Netherlands) in PBS for 10 min at 37°C. T cells (2x10⁴/well) were then stimulated with mitomycin-treated C57BL/6-derived splenocytes (2x10⁵/well). Cells were harvested 5 days later and CFSE dilution – indicator of cell division was assessed by flow cytometry.

Noted, CFSE will bind to free amines in aqueous conditions and thus reduce the remaining CFSE concentration. To avoid the loss of CFSE to amino acids in the labeling media, PBS is the recommended diluent for CFSE prior to adding to cells. Cells are uniformly suspended in PBS with serum, and the CFSE/PBS stock is immediately mixed rapidly with the cells and allowed to incubate for the optimal amount of time.
2. Measurement of transendothelial resistance

In this assay, EC are seeded on one side of the membrane, while medium to be tested for chemotactic activity is placed on the opposing side. The membranes are available with different pore sizes to accommodate different cell types and with several choices of ECM coatings for use. Costar and R&D Systems as product lines of disposable transmembrane inserts for 24 well tissue culture plates (Hulkower and Herber, 2011).

WT and CD31 KO ECs were treated with 300 U/ml IFN-γ for 48 h to induce MHC molecule expression. EC (5 × 10⁴/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 (Figure 35).

![Figure 35. Trans Epithelial electric resistance (TEER) measurement.](image)

Prior to Trans Epithelial electric resistance (TEER) measurements, the well contents were replaced with fresh EC media to remove nonadherent ECs in the upper chamber. TER was measured with an Epithelial Voltohmmeter with a chopstick-type electrode (World
Precision Instruments, Sarasota, FL) stabilized at 148 ± 12 Ω (Figure 36). In Ab-crosslinking experiments, the Abs were added at the indicated concentrations immediately after the first TER measurement (time 0).

![Image of precision instrument](image)

**Figure 36 Automated tissue resistance measuring system (EVOM epithelial voltammeter).** The EVOM epithelial voltammeter is designed to perform routine TEER (Trans Epithelial Electric Resistance) measurement in tissue culture research. It dramatically increases the speed and accuracy of monitoring tissue culture growth on permeable substrates. It also provides a standard 24-well plate examination in only 10 minutes Membrane Voltage Range: ±199.9 mV. Picture adapted from [http://www.wpiinc.com/](http://www.wpiinc.com/)

### 2.21 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% CO$_2$ in EC media to form a monolayer. EC monolayers were then fixed with 4% buffered paraformaldehyde (Sigma-Aldrich) for 30 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 Vectorshield (Vector Laboratories) mounting medium.
for fluorescence with DAPI (Vector Laboratories) on glass slides. The slides were analyzed with wide-field fluorescence microscopy.

2. 22 Quantification of stress fibers

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, I 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. I 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 $\geq$ 2000). The number of stress fibers at high intensity was quantified.
2. 23 Statistical analysis

Results are expressed as mean ± standard deviations (SD) or standard error of the mean (SEM), as indicated. The Student’s t-test analysis of variance test were used. All reported p-values are two-sided. P-value of less than 0.05 was regarded as significant.
Chapter 3. CD31-mediated protection from extrinsic apoptosis induces immune privilege
3. 1 Result

3.1. 1 CD31 interactions protect from TNFα- and T-cell-, but not complement-induced cell death.

Despite a large body of indirect evidence suggesting that CD31 signals contribute to endothelial viability, a direct role of CD31 signals in endothelial cytoprotection has been demonstrated only following exposure to cytotoxic drugs (Bergom et al., 2006). In a series of experiments, I compared the susceptibility of wild-type (WT) or CD31-deficient murine primary microvascular ECs to apoptosis induced by canonical cell-extrinsic apoptosis-inducing stimuli including TNFα and antigen-specific CTLs, and complement-induced necrosis.

CD31 KO ECs exposed to TNFα underwent a significantly higher rate of apoptosis compared to WT ECs (from WT: 10% + 5%) (KO: 40% + 5%) (Figure 37 A-B). Importantly, antibody blockade of CD31 increased the rate of TNFα-induced cell death in sub-confluent ECs (C), confirming that CD31 homophilic interactions between ECs are necessary to confer resistance to TNFα-induced apoptosis.

Lack of CD31 has been associated with enhanced T cell-mediated autoimmunity (Ma et al 2010); However whether loss of CD31-mediated cytoprotection contributes to this effect is unknown. I therefore sought to assess the protective effect of CD31 expression by the endothelium from T cell-mediated cytotoxicity. CD8+ HY (male)-specific H2-Db-restricted WT T cells were co-incubated with IFN-γ-treated ECs derived from WT and
CD31-deficient female mice (H-2b background) pulsed with the HY-derived Uty peptide (Millrain et al., 2005). As a control non-peptide-pulsed ECs were used. As shown in (D-E), CTL-mediated killing was much increased in CD31-deficient antigenic ECs, indicating that CD31 expression can protect ECs from CTL mediated cytolysis, in line with previous observations (Ma et al., 2010).

Complement-mediated cell lysis is another means of cell elimination during inflammatory responses. To establish whether CD31-mediated cytoprotection extended to mechanisms of cell death other than apoptosis, a comparison experiment was conducted on confluent murine ECs challenged with xenogeneic human serum as a source of complement-fixing antibodies (xenospecific IgM) and complement for up to 9 hours. As complement-induced cytolysis is largely independent of caspase activation (Scott et al., 2014), EC survival was assessed by trypan blue exclusion analysis, revealing similar levels of cell death of WT and CD31KO (F).

Collectively, these data indicate that CD31 is only effective for cytoprotection of ECs against apoptosis induced by TNFα and CTL-mediated killing, but fails to prevent complement-induced necrosis.
Figure 37 CD31 protects from extrinsic apoptosis. WT and CD31KO ECs were exposed to TNFα (50ng/ml) for 6 hours and apoptosis was measured by TUNEL assay (A-B). Alternatively, WT ECs were pre-treated with either a blocking anti-CD31 or an isotype control antibody prior to exposure to TNFα (C). D-E: Female-derived murine ECs (pretreated with IFN-gamma for 48 hours to upregulate MHC molecule expression) were incubated with HY-specific CTLs (1:5 EC/CTL ratio, 6 hours), and EC death was measured by TUNEL assay and trypan blue (TpB) exclusion assay. ECs were also cultured in 10% human serum as a source of antibody and complement (F), and cell death was measured by TpB exclusion assay. Representative images are shown in panels A and D (scale bar 100µm). The mean percentage of apoptotic (B, C, E) and necrotic (F) cells in 3 independent experiments (± SD) is shown. *p<0.05, ***p<0.001, ****p<0.0001.
3.1.2 CD31 is required for the activation of signaling cascades involved in EC cytoprotection from the extrinsic pathway of apoptosis.

The antiapoptotic signaling pathways engaged by CD31 are to date contentious. PI3K/Akt activation correlated with CD31 pro-survival activity in a number of studies (Cerovic et al., 2014a; Cerovic et al., 2014b; Martin et al., 2014). However, this pathway was found not to be required for protection from mitochondria-dependent apoptosis (Gao et al., 2003). As a first step to investigate the molecular pathways involved in CD31-dependent cytoprotection, I assessed whether CD31 is induced to signal upon TNFR engagement. Analysis of CD31 molecules immunoprecipitated from WT ECs either untreated or exposed to TNFα showed increased phosphorylation and recruitment of the SHP-2 phosphatase, indicating that TNFR signaling activates CD31 (Figure 38, panel A).

It has recently been shown that, in T cells, CD31-mediated protection from activation induced cell death, which is largely Fas-dependent, is associated with Erk 1 and 2, but not NFkB activation (Ma et al., 2010). I therefore sought to assess whether the Akt/Erk pathway was induced in my system. I found low constitutive levels of Erk activation in WT and CD31-deficient ECs, which significantly increased in WT but not CD31-deficient endothelium upon TNFα stimulation (B-D) suggesting that CD31 engagement is required for Erk activation downstream of TNFR signaling. Akt activation was induced following exposure of ECs to TNFα in WT, but not CD31 KO endothelium (D). The key role of these mediators in EC protection from cell-extrinsic apoptosis was confirmed by experiments in which ECs were incubated with TNFα following exposure to selective Akt and ERK
inhibitors, which led to significantly increased apoptosis (E-F). Altogether, these data suggest that EC resistance to extrinsic apoptotic stimuli correlates with CD31-mediated enhancement of Erk activation and induction of the Akt pathway.

Given the above observations and previous literature (Gao et al., 2003), it seems that CD31 engages distinct pro-survival pathways in response to extrinsic or intrinsic apoptotic stimuli. CD31 is required to promote a pro-survival transcriptional program downstream of TNFα signals.

Programmed cell death has been shown to be regulated by a coordinated transcriptional program, in which the quantitative balance of pro- and anti-apoptotic gene transcription can determine the ultimate outcome of inflammatory stimuli (Bao et al., 2010). I profiled the expression of apoptosis-related genes by WT and CD31-deficient ECs following exposure to TNFα by RT2 profiler apoptosis array as described in Methods. (The mRNA expression levels were normalized to untreated control cells).

Among the 84 genes analyzed, a total of 19 genes were differentially regulated in either WT or CD31KO ECs, of which 13 genes in WT, 16 in CD31KO, 3 uniquely in WT, 6 uniquely in CD31KO, 10 in both WT and CD31KO, whilst no significant differences in gene expression were observed when comparing ‘resting’ WT and CD31KO ECs (G-H).

Based on pairwise comparisons of TNFα-treated WT and CD31KO ECs (G&H), the TNF-family Death Receptor CD95/Fas, the executioner Caspase member Caspase 7 and the anti-apoptotic gene cFlar (CASP8 and FADD-like apoptosis regulator), also designated as cFlar, were selected for further investigation (circled). C-E: The mean percentage of
apoptotic cells in 3 independent experiments (± SD) is shown. *p<0.05, **p<0.01, ***p<0.001.
Summary of differentially regulated genes in WT/CD31KO ECs stimulated with TNFα compared with untreated counterpart ECs.

NB: Fold-change greater than 2 are indicated in red; fold-change values less than 0.5 are indicated in blue.
✓ Indicates genes chosen for further analysis
* Fas upregulation was significantly higher in CD31KO ECs – see Gene Array Datasets

(A) WT EC +/- TNFα

(B) CD31 KO EC +/- TNFα
Figure 38 Anti-apoptotic pathways activated by CD31 in ECs (A-H). A: Immunoprecipitation of CD31 molecules from ECs exposed to TNF alpha for 20 minutes followed by immunoblotting with an anti-phospho-tyrosine antibody and an anti-SHP2 antibody. Erk and Akt activation in WT and CD31 KO ECs exposed to TNFα (time-points indicated) is shown in panels B-C and B, D, respectively. Actin, Erk and Akt are used to normalize Western blots. E: WT ECs were treated with an Akt inhibitor (3 μM) or with a MEK 1/2 inhibitor (10 μM) for 4 hours at 37°C prior to incubation with TNFα. EC apoptosis was measured after 6 hours by TUNEL assay. F: Akt activation in WT ECs pre-treated with a blocking anti-CD31 or an Isotype-control antibody and exposed to TNFα for 30 minutes. G: Strategy for gene selection. Summary of differentially regulated genes in WT and CD31KO ECs stimulated with TNFα compared with untreated counterpart ECs. Fold-change greater than 2 are indicated in red; fold-change values less than 0.5 are indicated in blue. (✔) Indicates genes chosen for further analysis/ * Fas upregulation was significantly higher in CD31KO ECs – see Gene Array.). H: Apoptosis-related gene profile of WT and CD31KO ECs before and after exposure to TNFα (See supplementary 2).
3.1. 3 CD31 modulates the pro-apoptotic transcriptional programme downstream of death receptor signalling.

Based on pairwise comparisons of TNFα-treated WT and CD31KO ECs, the TNF family Death Receptor CD95/Fas, the executioner Caspase member Caspase 7 and the anti-apoptotic gene cFlar (CASP8 and FADD-like apoptosis regulator), also designated as cFLIP (Flice-like inhibitory protein), were identified as those genes, which are transcriptionally repressed (Fas, Caspase 7) or transcribed de novo (cFlar) in WT but not CD31 KO ECs in response to TNFα.

The differential regulation of Fas, Caspase 7 and cFlar was further confirmed by qRT-PCR, flow cytometry and Caspase 3/7 Assay (Figure 39A-E; 40 A-B). Overall these suggest that the CD95 mediated cytotoxicity of T cells may be downregulated by the expression of CD31.

Next, I compared the expression of PDL-1, a negative co-stimulator of T cells not included in the RT2 profiler, known to be expressed by the endothelium (Tanimoto et al., 2008) and observed no differences between WT and CD31-deficient ECs (F). There is no significant difference between the levels of Spi6 in WT v CD31KO EC treated with TNF (G).
Figure 39 CD31 modulates the pro-apoptotic transcriptional programme downstream of death receptor signalling. A-C: Expression of Fas mRNA and surface receptor by WT and CD31KO ECs either untreated or exposed to TNFα (6 hours) quantified by qRT-PCR (A) and flow cytometry (B-C). D-E: Expression of Caspase 7 mRNA and Caspase 3/7 activity by WT and CD31KO ECs either untreated or exposed to TNFα as measured by qRT-PCR (D) and ApoTox- Glo Triplex caspase 3/7 Reagent (E). F: Surface expression of the inhibitory co-receptor PDL1 by untreated or TNFα-treated WT and CD31KO ECs. G: Expression of Spi6 mRNA by WT and CD31KO ECs either untreated or exposed to TNFα was quantified by qRT-PCR A, C-E, G: Cumulative data from 3 independent experiments (± SD) are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
3.1.4 The anti-apoptotic gene cFlar is instrumental to CD31-mediated cytoprotection.

cFlar is an anti-apoptotic protein with significant homology to caspase-8 (Irmler et al., 1997). A substitution of two amino acids in the region of cFlar that corresponds to the catalytic active site of caspase-8 renders it incapable of proteolysis. Upon CD95/Fas ligand binding, the adapter protein FADD is recruited to the Fas receptor via an interaction between the death domain (DD) of each protein, an interaction that initiates the extrinsic apoptotic cascade (Bao et al., 2010). By competitively binding to FADD and blocking the assembly of a functional death signaling complex, cFlar has been proposed to inhibit the activation of upstream initiator caspases, including caspase-8 (Nagata et al., 1998). cFlar has previously been shown to be induced downstream of Akt and to be required for EC protection from LPS-induced apoptosis (Hartmann et al., 1998) as well as Fas-induced cell death (Mine et al., 1998). I therefore further investigated the involvement of this gene in the cytoprotective effect of CD31. qRT-PCR and immunoblotting experiments confirmed the lack of cFlar gene transcription and protein synthesis in CD31KO ECs upon TNFα stimulus (Figure 40A-D). cFlar knockdown by shRNA in WT ECs enhanced their sensitivity to TNFα-induced apoptosis and this could not be rescued by CD31 antibody stimulation (E) (Ma et al., 2010), indicating that cFlar acts downstream of CD31 in mediating its prosurvival activity. Importantly, inhibition of Akt and Erk activation prevented cFlar expression by WT ECs exposed to TNFα (F), suggesting that these pathways, which require CD31 expression for activation downstream of TNFR signaling, directly mediate the CD31 pro-survival activity via the induction of cFlar (G-H) show that TNFα stimulates more nuclear localization of Foxp3 in CD31KO EC.
An important implication of these observations is that, rather than directly regulating gene expression, CD31 is required to counteract ‘ad hoc’ the genetic reprogramming initiated by TNFα.
Figure 40 The anti-apoptotic gene cFlar is instrumental to CD31-mediated cytoprotection. mRNA was isolated from CD31KO or WT ECs either untreated or stimulated with TNFα (50ng/ml) for 6 hours. A-B: cFlar mRNA quantification (A) and protein expression (B) by WT and CD31KO ECs after incubation with TNFα. C-D: cFlar expression by ECs following sh-RNA knockdown was evaluated by qRTPCR (C) and immunoblotting (D). (E): Apoptosis of ECs exposed to TNFα following cFlar knockdown measured by TUNEL assay. In some cFlar-silenced cells, CD31 was stimulated by antibody ligation. (F): WT ECs were treated with an Akt inhibitor (3 μM) or a MEK1/2 inhibitor (10 μM) prior to incubation with TNFα. Expression of cFlar was measured after 6 hours. G-H): EC cultures were fixed and FoxO3a intracellular localization was assessed by immunofluorescence staining. Nuclei were stained with DAPI. Representative images of nuclear translocation of Foxp3 are shown in panel G. Scale bar: 50μm. Panel H measures the quantification of immunofluorescence staining patterns for endogenous FOXO3 (n: 100 cells per experiment). The mean value of data in 3 independent experiments (± SD) is shown in panels A, C, E and H. **p<0.01, ***p<0.001, ****p<0.0001.
3.1.5 Both intracellular ITIM motifs are required for CD31 anti-apoptotic activity

Both ITIM-mediated signals and homophilic binding have previously been shown to be required for efficient CD31 protection from the intrinsic apoptotic pathway (Gao et al., 2003). To investigate whether these signaling domains are also required for CD31-mediated inhibition of the extrinsic pathway, I generated two CD31 gene constructs with mutation leading to the loss-of-function amino acid substitutions Y663F and Y686F in the ITIMs, which were lentivirally transduced into CD31 KO ECs (CD31Y663F, CD31Y686F ECs; Figure 41a). As a control, ECs transduced with a wild-type CD31 gene construct (WT) or an empty plasmid (Mock) was used. I subsequently tested whether these mutants had maintained the antiapoptotic activities of the WT CD31. I hypothesized that CD31 mutants fail to be cytoprotective against inflammatory challenges. First, I observed that both Y663F and Y686F mutant CD31 receptors fail to protect ECs from TNFα- and CTL-mediated cytolysis as measured by TUNEL assay (B-D). Further, both CD31Y663F and CD31Y686F ECs exhibited impaired Erk and Akt activation compared to CD31 WT ECs (E-G).

Finally, I observed that neither mutant CD31 was able to suppress TNFα-induced expression of CD95/Fas (H-I), transcription and activity of caspase 7 (J-K) or promote the expression of cFlar (L). Overall these data suggest that both CD31 ITIMs are required for the CD31-mediated reprogramming of TNFα-induced transcription that is necessary for EC cytoprotection.
Figure 41. Both CD31 ITIMs are required for its pro-survival activity. A: Surface expression of CD31Y663F and CD31Y686F molecules following transduction of CD31KO ECs. As a control, WT CD31 and empty plasmid (Mock) constructs were used. B-C: Apoptosis of Mock, WT CD31 and empty plasmid (Mock) constructs were used. B-C: Apoptosis of Mock, WT CD31 and empty plasmid (Mock) constructs were used. D: Complement-mediated lysis of the indicated EC populations. E-G: Erk and Akt activation in ECs transduced with the indicated constructs following exposure to TNFα. H-L: CD95/Fas surface expression (H-I), caspase expression and activity (J-K) and cFlar protein expression (L) in the indicated EC populations, either untreated or exposed to TNFα (6 hours) are shown. B-D, F, G, I-K: the mean value of data measured in 3 independent experiments (± SD) is shown **p<0.01, ***p<0.001, ****p<0.0001.
3.1.6 Transfer of CD31 activity in pancreatic beta cells recapitulates constitutive cytoprotection of the endothelium and confers immune privilege against T cell alloresponses.

Besides CD31 activity, a plethora of signals can sustain EC survival in vivo, including integrin-mediated cell adhesion to basement membrane, adhesion to adjacent cells mediated by VE-cadherin, growth factors, and survival signals derived from pericytes (Santiago et al., 2012). As most of these signals selectively prevent mitochondrial apoptosis (Bao et al., 2010) and many also apply to cells of non-endothelial lineage, I sought to establish whether CD31 anti-apoptotic activity, which is endothelium- and leukocyte-selective, is not only necessary, but also sufficient for maintaining cell survival during activation of the extrinsic pathway of cell death. To address this question, I first tested whether transfer of CD31 expression would endow CD31-negative cells with resistance to extrinsic apoptosis. CD31-, H2b-expressing MIN6 (Tanaka et al., 1998) pancreatic beta cells were transduced with WT CD31 gene or the empty vector (Mock).

The efficiency of gene transfer is shown in Figure. 42A, and did not affect insulin production in response to glucose stimulation (B). Transduction of CD31 gene in MIN6 cells recapitulated all the CD31-mediated molecular and cellular events which I observed in ECs upon exposure to TNFα, including resistance to TNFα-induced cell death (C-D) and CTL-mediated cytolysis (E-F). Interestingly, unlike what I had observed in CTL-mediated EC killing (F), apoptosis of CD31-negative MIN6 cells exposed to allospecific CTLs represented only a proportion of the overall cell death, which was completely prevented only by inhibition of both death receptor (DR) signalling and lytic granule release (G), indicating that perforin-induced osmotic lysis might be contributing to MIN6 cell death.
However, the ratio of Mock-transduced and CD31-expressing MIN6 cell death after exposure to CTLs measured by TUNEL and TpB was very similar (3.8±1.09 by TUNEL vs 3.6±1.32 by TpB), suggesting that CD31-mediated cytoprotective signals are equally effective in preventing cell death induced by DR and perforin. In line with these observations, FoxO3, which has been implicated in osmotic lysis, localized to the nucleus only in Mock-transduced, but not in CD31-expressing MIN6 cells after incubation with CTLs (H). Similar to our observations in ECs (Figure 37C), antibody-blockade of CD31 in transduced MIN6 cells significantly increased their susceptibility to CTL-induced death (TpB) to the levels observed when mock-transduced cells were used as targets (I).
Figure 42 CD31-transduced pancreatic beta cells are protected from extrinsic apoptosis. A: Surface expression of CD31 by Mock- and CD31-transfected MIN6 cells. B: Insulin secretion by Mock- and CD31-transfected MIN6 - starved in DMEM containing 2.8 mM glucose for 30 min at 37 °C - 1 hour after reconstitution with 25 mM glucose. C-F: Cell death of Mock- and CD31-transfected MIN6 either exposed to TNF alpha (50ng/ml, 6 hours, C-D) or co-cultured with allospecific CTLs (6 hours, E-F) as measured by TUNEL and TpB assay. Min 6 cells were harvested and then subjected to TUNEL assay to determine the apoptotic cells. Representative images for green positive stained apoptotic cells are shown in the panels C and E (scale bar 100um). G: Apoptosis of Mock- and CD31-transfected MIN6 cells cultured with allospecific CTLs (6 hours) and pre-treated with a Death Receptor Ligand (DRL, 5 uM) inhibitor and/or Concanamycin A (5 ug/ml)
for 1 hour). H: FoxO3a intracellular localization in MIN6 cells was assessed by immunofluorescence staining following co-culture with CTLs (1:5 Min6/CTL ratio, 6 hours). Quantification of immunofluorescence staining patterns for endogenous FOXO3 is shown (n: 100 cells per experiment). I: Apoptosis of CD31-transfected MIN6 cells cultured with CTLs (6 hours) following treatment with a blocking anti-CD31 antibody or Isotype Control. Mock-transduced cells are included for comparison. Panels B, D, F, G, H, I show the mean percentage of apoptotic cells (± SD) measured in 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.1. 7 Anti-apoptotic pathways activated in CD31-transduced pancreatic beta cells.

The results here demonstrated that the efficiency of gene transfer are associated with activation of the Erk/Akt pathway (Figure 43 A), downregulation of Fas (B), Caspase 7 expression and activity (C-D), and upregulation of cFlar expression (E).
Figure 43 Anti-apoptotic pathways activated in CD31-transduced pancreatic beta cells. A-E: Erk and Akt activation (A), Fas surface expression (B), caspase 7 expression and activity (C-D) and cFlar protein expression (E) by Mock- and CD31-transfected MIN6 cells exposed to TNFα for 6 hours. F: Nuclear localization of FoxO3 in MIN6 cells exposed to TNFα for 6 hours. Quantification of immunofluorescence staining patterns for endogenous FOXO3 is shown (n: 100 cells per experiment). C, D, F: The mean value of data measured in 3 independent experiments (± SD) is shown. **p<0.01
3.1. 8 In vivo model of Streptozotocin induced diabetes

To establish whether CD31 expression is sufficient to prevent the induction of cell extrinsic apoptosis - as in CTL-mediated allograft rejection - in physiologic settings, I chose to assess rejection of CD31-expressing pancreatic beta cells grafted in a fully allogeneic recipient in vivo model. In solid organ transplantation, with the exception of preformed antibody-triggered, complement-mediated cell lysis in hyperacute responses, damage of the endothelium is relatively limited compared with that of other epithelial components, and the vasculopathy occurring during chronic rejection features abnormal perivascular responses leading to endothelial dysfunction, rather than direct loss of ECs (Takeshita et al., 1998).

Mock- or CD31-transduced MIN6 cells were transplanted under the renal capsule of BALB/c mice rendered diabetic by injection of streptozotocin 3 weeks earlier. As a control, WT MIN6 cells were transplanted in syngeneic C57BL/6 diabetic mice, leading to complete normalization of glycaemia Figure. 44. As shown in (A-B), diabetes persisted in recipients of WT MIN6 cells, indicative of transplant rejection. In contrast, in recipients of CD31-expressing MIN6 glycaemia returned to normal for the duration of the experiment.

Moreover, the time course of islet graft being rejected is measured by the conformity of histology between donor and receipt. Healthy MIN6 islets were detected in the kidney of these recipients; while the rejection of the min6 mock took place as MIN 6 cells were destroyed, suggesting that these cells had become resistant to rejection (C-D).
Furthermore, an analysis of anti-H-2D alloresponses revealed that these were conserved in both animals that received WT or CD31-expressing MIN6 cells (E-F), suggesting that CD31 transduction led to the induction of immune privilege, rather than tolerance. Interestingly, cellular resistance to immune-mediated cell death strictly correlates with CD31 expression. Those have previously described that CD31 limits the contraction phase of T cell responses and reduces cytotoxic killing of antigen-presenting cells (Ma et al., 2010).
Figure 44 CD31-expressing pancreatic beta cells become resistant to T cell mediated allograft rejection. The function of Mock-transduced and CD31 MIN6 islets was evaluated by measuring blood glucose levels in grafted syngeneic (C57BL/6, A) or allogeneic (Balb/C, B) mice rendered diabetic by STZ injection. Panels C-D show representative images of a kidney that received either mock- or CD31-transduced MIN6 beta cells, and stained to detect insulin production. Magnified sections of the grafts
processed as indicated within each images are shown on the right-hand side (Scale bar 10micron). At the time of sacrifice, T-cells were isolated from the spleen and lymph nodes of recipients and labelled with CFSE. Cell division in response to an ex-vivo re-challenge with C57BL/6 splenocytes by T-cells from Naïve mice, recipients of mock-transduced or CD31 MIN6 cells was measured by flow cytometry. Representative histograms are shown in panel E. The mean % of divided T-cells (± SD) measured from at least 5 recipients is shown in panel F. **p<0.01, ***p<0.001.
3.2 Discussion

Maintenance of vascular integrity during inflammation is a major challenge for the cooperation between the immune and the vascular systems. During acute inflammation, ECs can become themselves targets of innate and adaptive cytotoxic mechanisms, such as cytokines including TNFα and cytotoxic T-lymphocytes (CTLs). Upon immune attack, ECs can negatively regulate CTL responses by expressing co-inhibitory receptors such as Herpes simplex entry mediator (Kaibori et al., 2006) and programmed cell death ligand-1 (Yanagida et al, 2006). How ECs maintain vascular integrity during inflammation has been the subject of intense investigation.

Traditionally, two major cell death pathways—termed the extrinsic and intrinsic pathways of apoptosis exist in mammalian cells (Hengartner, 2000). The intrinsic apoptotic pathway is characterized by permeabilisation of the mitochondria and release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi-protein complex known as the apoptosome and initiates activation of the caspase cascade through caspase 9. On the other hand, the extrinsic pathway is initiated by engagement and aggregation of tumor necrosis factor (TNF) family death receptors (such as CD95/Fas) which, through a series of death domain—containing adaptor molecules, recruit and directly activate cytosolic caspase 8, which in turn converts procaspase 3 to caspase 3 - the central executioner of the apoptotic process. Although ECs express death receptors on their surface, TNFα and Fas ligand do not efficiently activate apoptosis in ECs (Santiago et al, 2006). This resistance may be in part due to the expression of the caspase 8-antagonist cFlar (Hartmann et al, 1998).
The involvement of CD31 signaling in EC protection from the intrinsic pathway of apoptosis has been established and characterized (Bergom et al., 2005).

In contrast, a potential role for CD31 in EC protection from the extrinsic pathway has not been investigated. My study provides evidence that the Ig-family receptor CD31, selectively expressed by ECs at high density, becomes activated upon the delivery of pro-apoptotic immune stimuli and it is both necessary and sufficient to prevent EC death as a consequence of these stimuli. Importantly, I also show that the apoptotic stimuli themselves induce CD31 phosphorylation and activation, which in turns counteracts the pro-apoptotic gene reprogramming initiated by such stimuli. Thus, rather than directly interfering with proapoptotic signaling pathways, CD31 signals prevent ‘ad hoc’ the genetic reprogramming initiated by these pathways by redirecting the transcription factor FoxO3 to the cytoplasm via Akt-mediated phosphorylation. This is a previously unknown pathway for the acquisition of immune privilege. The concept of immune privilege refers to the observation that tissue grafts placed in certain anatomical sites, including the brain and eye, can survive for extended periods of time (Frangipane et al., 1977). Immune privilege is thought to reflect an evolutionary adaptation to protect vital structures from damage by inflammatory responses directed against pathogens. It is now currently viewed as the ability by the tissue itself to downmodulate T cell effector function during cognate recognition of self and alloantigens (Sonoda et al., 1995). For example, expression of Fas ligand by cells in the anterior chamber of the eye and by the Sertoli cells in the testis preserve their integrity against immune attack by inducing death of CD95 (Fas)-expressing T lymphocytes (Bellgrau et al., 1995; Griffith et al., 1995). Privileged tissues also feature other protective mechanisms, such as tight endothelial barrier, downregulation of MHC
molecules and production of anti-inflammatory cytokines such as TGF alpha (Streilein et al., 1997).

The ability of CD31 expression to protect MIN6 cells from CTL-mediated killing appears to affect both DR-induced apoptosis and perforin-mediated lysis – the latter event not playing a significant role in EC cytolysis. The molecular mechanism of this effect is at present unclear, and it does not involve induction of the Serpin inhibitor Spi6. Notably, IL-4-induced phosphorylation of another FoxO family member, FoxO1, has been shown to prevent EC cytolysis by upregulating claudin-5 expression and junction tightness (Dalmasso et al., 2014).

Interestingly, cellular resistance to immune-mediated cell death strictly correlates with CD31 expression in other CD31-expressing cells. These have previously described that CD31 limits the contraction phase of T cell responses and reduces cytotoxic killing of antigen-presenting cells (Ma et al., 2010). Selective expression of CD31 by endothelium and dendritic cells may serve the purpose of preserving their integrity and function during immune inflammation: thus, the endothelium can mediate the development of inflammatory infiltrates without being damaged by migrating activated leukocytes; resistance to activation-induced cell death permits the maintenance of T cell memory; and antigen presenting cells can serially activate multiple cognate T-cells without themselves being killed at the first encounter.

Furthermore, it has been known that the homophilic binding is required for efficient CD31 border localization (Wu et al, 2001) and also influences the subcellular localization of CD31. For example, Lys89Ala variant of CD31 (which lacks homophilic binding capacity)
failed to inhibit apoptosis (Newton et al., 1997), may shed light in the extracellular domains of CD31 that play an important cytoprotective role. Consistent with my observations, I saw increased cell death in EC treated with CD31 blocking antibody in Figure 37. Similarly, the mutations in the CD31 cytoplasmic domain are capable of abolishing its antiapoptotic functions, indicating that phosphorylation of the two tandem tyrosine residues (Y663 and Y686) within the ITIM motif is important for protection from cell-intrinsic apoptosis (Gao et al., 2003). Collectively these data support the notion that CD31-CD31 interactions, either in trans between cells, or in cis within the plane of the membrane, are required to support transmission of a signal to the cytoplasmic domain that interferes with one or more antiapoptotic pathways.

There is still a great controversy as to whether CD31 engagement is association with A1 expression (Evans et al., 2001; Noble et al., 1999). In endothelial cells, A1 is induced in response to proinflammatory stimuli, protects them against TNF- and ceramide-mediated apoptosis and prevents endothelial cell activation through inhibition of NF-κB and nuclear factor for activated T cells (NFAT); or up-regulates antiapoptotic, or down-regulates proapoptotic, members of the Bcl-2 family most likely to influence cell death. My data provide new evidence that CD31 may modulate the expression of pro/anti-apoptotic genes in favour of cell survival by interfering with proapoptotic signaling induced by other mediators/receptors. In particular using qRT-PCR array, I found that CD31-induced transcriptional events contribute to the prosurvival phenotype of CD31 expressing cells, such as the expression of the caspase 8-antagonist cFlar. It is also known that the activation of the antiapoptotic Akt pathway is required for cFlar-mediated EC resistance to extrinsic apoptotic signals, including TNFα and Fas ligand (Miner and Croft, 1998).
In order to determine whether CD31 is both necessary and sufficient to mediate cell survival against immune attack I chose to transduce this molecule in CD31-negative pancreatic beta cells and assess their survival following transplantation in an allogeneic diabetic recipient.

Type 1 diabetes results from selective and progressive destruction of insulin-producing cells by autoreactive CD8\(^+\) T cells. The direct cell–cell contact initiated by T-cell receptors recognizing a β-cell–specific antigenic peptide presented by the major histocompatibility complex (MHC) class I (MHC-I) at the surface of the target cell appears to be critical for β-cell destruction in type 1 diabetes. CD8\(^+\) T-cells appear to be the most directly damaging cell type. β-cell death occurs by several molecular pathways leading to apoptosis. Pathways triggered by the contents of the cytolytic granules of CD8\(^+\) T-cells, cell death receptors such as Fas, and proinflammatory cytokines, including tumor necrosis factor, interleukin-1, and interferon-γ (Hamilton-Williams et al., 2003), have been implicated. The blood glucose tracing were monitored throughout the syngeneic and allogeneic islet transplantations.

In this condition, islet transplantation offers a promising approach for restoring endogenous insulin secretion (Kleijwegt et al., 2011). The success of this strategy is still jeopardised by transplant rejection and/or recurrence of autoimmunity. To address this issue, other strategies to protect or improve insulin secretion of the transplanted β cells, for example using encapsulated islets, are currently under investigation. Our \textit{in vivo} data show that CD31 gene transfer is sufficient to recapitulate the cytoprotective mechanisms in CD31-negative pancreatic beta cells, which become resistant to immune-mediated rejection when grafted in fully allogeneic recipients. The result is an unexpected and novel
observation, with potential therapeutic impact. My findings set the basis to develop transplantation of genetically immunoprotected islets using viral vector transduction of CD31 without affecting their function, as a new therapeutic approach to this condition.
Chapter 4. CD31 Exhibits Multiple Roles in Regulating T lymphocyte Trafficking In Vivo
4. 1 Results

4.1. 1 CD31-deficient naive T cells display defective homing to secondary lymphoid tissue

In physiologic conditions, naive T lymphocytes continuously recirculate through secondary lymphoid tissue (Butcher and Picker, 1996). Although CD31 is expressed on both high endothelial venules in the lymph nodes (Pfeiffer et al., 2008) and sinusoids in the spleen (Pusztaszeri et al., 2006), the contribution of this molecule to naive 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 naive T cells to migrate to lymph nodes and spleen. Naive 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. T cells were then labeled with PKH26 and injected i.v. (10\(^7\)/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 Figure 45, CD31\(^{-/-}\) CD4\(^+\) (A-B) and CD8\(^+\) (C-D) naive T cells localization to both LNs and spleen was partially but significantly impaired, suggesting that CD31-mediated interactions contribute to naive T cell homing to secondary lymphoid tissue.
To assess the functional effects of reduced migration of CD31$^{-/-}$ T cells in the secondary lymphoid organs, naive 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 (Marelli-Berg et al., 2004). T cell localization and division were assessed 72 h later by flow cytometry. As it is shown in (E), although significantly fewer CD31$^{-/-}$ 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 previously described (Ma et al., 2010), leading to similar numbers of activated T cells compared with their WT counterpart.
Figure 45 CD31 promotes naive T cell migration to secondary lymphoid tissue. Naive T cells (CD44\textsuperscript{low}) isolated by cell sorting from lymph nodes of C57BL/6 mice were labeled with PKH26 and injected intravenously (10\textsuperscript{7}) into WT and CD31\textsuperscript{−/−} mice. The localization of CD4\textsuperscript{+} (A, B) and CD8\textsuperscript{+} (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\textsuperscript{−/−} T cells derived from female mice (10\textsuperscript{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 adapted from Ma et, al 2012.
4.1.2 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 (Graesser et al., 2002). However, the absence of spontaneous T cell inflammation and signs of endothelial leakage in CD31−/− mice (Duncan et al., 1999b) suggest that other mechanisms might be in place which underlie the enhanced T cell infiltration in these models. I therefore sought to study the contribution of CD31-mediated interactions on effector memory T cell trafficking.

Effector memory HY (male Ag)-specific T cells were generated by immunization of female WT and CD31−/− mice with syngeneic WT male splenocytes, as previously described (Ma et al., 2010).

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 (10⁷/mouse), and T cell localization to several tissues was assessed 16 h later by wide-field microscopy. Compared with their WT counterpart (Figure 46A) CD31−/− T cells displayed significantly impaired ability to access nonlymphoid, nonantigenic, noninflamed tissues, 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 tissues (Graesser et al., 2002), I 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 tissues of CD31−/− recipients was also severely impaired (B).

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 46 CD31 is required for constitutive memory T cell trafficking. (A) HY-specific H2-A^b^-restricted WT (PKH26-labeled) and CD31^-^- 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^-^- 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 (Jarmin et al., 2008). 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). Figure adapted from Ma et al, 2012.
4.1. 3 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 (Manes and Pober, 2011). To investigate the contribution of CD31-mediated interactions of memory T cell recruitment to antigenic sites in inflammatory conditions, I first compared the recruitment of HY-specific CD4\(^+\) WT and CD31 KO 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 i.p. injection of IFN-\(\gamma\) (600 U) 48 h prior to adoptive transfer (Marelli-Berg et al., 2004). 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-\(\gamma\)-treated male, but not female mice (Marelli-Berg et al., 2004). PKH26-labeled WT or CD31 KO 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\(^+\) (Figure. 47A) and CD8\(^+\) (Figure. 47B) T cells were recruited in the peritoneal lavage of WT male recipients. Migration of HY-specific CD31 KO T cells to the peritoneal cavity of IFN-\(\gamma\)–treated WT male mice was significantly decreased, although the enhancing effect of Ag recognition was not completely abrogated. As expected, few WT and CD31 KO HY-specific T cells migrated to the peritoneal cavity of IFN-\(\gamma\)–treated female mice. These data suggest that CD31 expression by T cells enhances, but is not required for migration of specific effector CD4\(^+\) and CD8\(^+\) T cells to nonlymphoid Ag-rich tissue.
Figure 47 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 CD31KO T cells (10⁷/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) (David et al., 2009) 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. Figure adapted from Ma et al. 2012.
4.1.4 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. 48 A&B). 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 (C).

Similar observations were made when CD31-deficient T cells were injected into CD31−/− recipients, suggesting that the effect of CD31 loss by the endothelium is dominant over the lack of CD31 expression by T cells.
Figure 48 Selective loss of endothelial CD31 leads to enhanced Ag-driven T cell migration. PKH26-labeled WT HY-specific T cells (10⁷/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. Figure adapted from Ma et al, 2012.
4.1. 5 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 CD31-mediated interactions 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 (Duncan et al., 1999b; Marelli-Berg et al., 2004). I 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, I 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 Figure 49A, MHC molecule triggering led to a quick and similar reduction in resistance (i.e., increased permeability) by both WT 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 (B); 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 C, 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 (D), suggesting that this effect is specific to MHC molecule signaling (i.e., to migrating T cells).
Figure 49. CD31 interactions promote the recovery of endothelial integrity following increased permeability induced by MHC molecule triggering. (A) WT and CD31−/− ECs (6 × 10^4/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 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 EC that underwent MHC molecule ligation and those treated with isotype control and secondary Ab. (C) Monolayers of WT EC were stimulated with 1 μg/ml mouse–anti-mouse H-2Ld/H-2Db (BD Biosciences) or isotype control and 0.5 μg/ml goat–anti-mouse IgG (BioLegend) with and without coligation of CD31 molecules (rabbit–anti-mouse CD31 5 μg/ml plus goat–anti-rabbit Ig 2.5 μg/ml). (D) WT EC underwent MHC class 1 molecule stimulation in the presence or absence of ICAM-1 ligation (rat–anti-mouse CD54 5 μg/ml plus goat anti-rat Ig 2.5 μg/ml; BioLegend). TER was measured with a transepithelial voltmeter stabilized at 148 ± 12 Ω at the indicated time points. *p < 0.05, **p < 0.01.
As endothelial contractility leading to decreased endothelial resistance is associated with cytoskeletal rearrangements, particularly with F-actin polymerization and stress fiber formation, I further analyzed EC cytoskeletal rearrangements following MHC triggering with or without CD31 ligation. As it is shown in Figure 50A, 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 (B). Overall, these data suggest that CD31-mediated signals serve to reestablish endothelial integrity following cognate recognition by migrating T cells.
Figure 50. Effects of MHC class I and CD31 molecule triggering on stress fiber assembly. IFN-γ–treated monolayers of WT EC were stimulated with 1 μg/ml mouse–anti-mouse H-2Ld/H-2Db or isotype control and 0.5 μg/ml goat–anti-mouse IgG with and without coligation of CD31 molecules (rabbit–anti-mouse CD31 [5 μg/ml] plus goat anti-rabbit Ig [2.5 μg/ml]). (A) EC were fixed and stained with rhodamine-phalloidin. In the image depicting EC undergoing MHC and CD31 coligation, enlargements of stress fiber confluence in cell-cell contact areas (demarcated) are shown. Quantification of fluorescence intensity across the lines of the corresponding cells in was performed using ImageJ software. The graph on the right side of each image shows fluorescence intensity peaks of stress fibers observed in each EC population. Only peaks >2000 FI were counted. The average of three independent experiments is shown in (B). Scale bar, 20 mm. *p < 0.05, ***p < 0.001.
4.2 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 ECs 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 tissues. This effect might have been masked in mice constitutively lacking CD31 expression, which display normal colonization of lymphoid tissues, by compensatory mechanisms (Duncan et al., 1999b) provided by the redundant and complex network of molecules involved in lymphocyte TEM (Nourshargh and Marelli-Berg, 2005). In addition, enhanced Ag-induced T cell division consequent to the loss of CD31-mediated interactions with dendritic cells (Ma et al., 2010) 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 (Bogen et al., 1994).

In line with these studies, I 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 (Manes et al., 2010).

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 (Graesser et al., 2002; Tada et al., 2003) 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 (Jindra et al., 2008a). Similarly, exposure of EC to anti-MHC class I Abs promoted proliferation through the mTOR pathway (Jindra et al., 2008b). MHC class II engagement in brain ECs was shown to be directly coupled to IL-6 production via a cAMP/PKA-dependent intracellular pathway (Adamson et al., 1999; Etienne et al., 1999).

In this study, I 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 cytoskeleton reorganization (Boulday et al., 2004).

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 (Biswas et al., 2006a). Other junctional molecules have been proposed to interfere with increased endothelial permeability induced by inflammatory mediators. For example, the junction adhesion molecule JAM-C has been shown to modulate endothelial contractility and VE-cadherin–mediated interendothelial adhesion (Orlova et al., 2006) in response to histamine and VEGF stimulation.

I 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 (Marelli-Berg et al., 2004) has been shown to sustain the development of specific T cell infiltrates and pathology in a number of experimental models of disease (Savinov et al., 2003), including experimental autoimmune encephalomyelitis (in which severity is increased by CD31 deficiency) (Graesser et al., 2002). Cognate recognition of the endothelium selectively enhances T cell TEM (Marelli-Berg et al., 2004), a process in which CD31 interactions are integrated. I propose that in this instance, interendothelial CD31 interactions serve to facilitate the re-establishment of endothelial continuity following lymphocyte TEM (Figure 51).

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
(Ma et al., 2010) 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.

Figure 51 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. Picture adapted from jcs.biologists.org/content/126/11/2343
5. Summary

CD31 is an important, yet neglected, immune inhibitory receptor (Newman, 1999; Ravetch and Lanier, 2000). Because of its extracellular Ig-like domains and localization in the lateral junctions between ECs in the vasculature (Newman et al., 1990), CD31 was initially viewed as a putative cell-adhesion molecule, and the ability of CD31-targeting antibodies or recombinant proteins (Muller et al., 1993) to inhibit leukocyte transmigration has long supported this perception. However, the absence of CD31 does not reduce the transmigration of blood leukocytes (Duncan et al., 1999b) but, rather, promotes CD4+ T-cell recruitment at inflammatory sites (Tada et al., 2003). Several subsequent experimental studies have demonstrated that CD31 plays important inhibitory signaling roles in effector-adaptive immune cells, such as T and B lymphocytes (Newton-Nash and Newman, 1999). More recent studies by us have also suggested that the homophilic engagement of CD31 between DCs and cognate T cells may establish the threshold for T-cell activation and tolerance (Ma et al., 2010). Importantly, the selective expression of CD31 by endothelium and dendritic cells may serve the purpose of preserving their integrity and function during immune inflammation.

In summary, I am the first to identify that extrinsic apoptotic stimuli (TNFa) can themselves induce CD31 phosphorylation, which in turn counteracts the pro-apoptotic gene reprogramming initiated by such stimuli. cFlar is a new component of the CD31 protection pathway. Thus, the benefit of this system is that the endothelium can mediate the development of inflammatory infiltrates without being damaged by migrating activated leukocytes; resistance to activation-induced cell death permits the maintenance of T cell memory; and antigen presenting cells can serially activate multiple cognate T cells without
themselves being killed at the first encounter; thus redirecting killing to CD31-negative targets, such as parenchymal cells, without affecting antigen presentation by DCs and extravasation (Fedele et al., 2004).

Intriguingly, aberrant expression of CD31 has been described in some tumors and correlates with adverse prognosis (Bergom et al., 2005), leading to the hypothesis that CD31 expression by non-endothelial tumors may facilitate metastasis via its ability to homophilically bind to endothelial CD31 and/or by conferring resistance to chemo- and radiotherapy (Bergom et al., 2005). Based on the data presented here, I propose that pro-survival signals generated by CD31 interactions might also be harnessed by tumors to acquire immune privilege. A summary of the potential cytoprotective mechanisms and pathways by endothelial CD31 is shown below.
Figure 52 CD31 induced prosurvival pathway. (1) Upon death receptors (DR) triggering with their cognate ligands (DR-L) the death-inducing signalling complex forms and initiates the extrinsic pathway of apoptosis; also the transcription factor FoxO3 is induced to translocate in the nucleus where it promotes transcription of pro-apoptotic genes and represses transcription of anti-apoptotic ones, including cFlar. (2) DR signals also induce phosphorylation of the intracellular tail of CD31 and recruitment of SHP-2. (3) Events that is required for the downstream phosphorylation and activation of the AKT/Erk pathways. (4) Upon CD31-dependent activation, AKT phosphorylates FoxO3 in the nucleus and promotes its re-localization to the cytoplasm thereby blocking pro-apoptotic transcriptional programmes and de-repressing transcriptional inhibition of cFlar, which (5) becomes available to counteract the extrinsic apoptotic pathway by directly blocking assembly of the Disc Complex.

Meanwhile, in chapter 4, I show that MHC class I molecule triggering induces temporary increase of endothelial permeability, which is restored upon CD31 activation. CD31 carries out this function by preventing and/or reverting β-catenin and VE-cadherin phosphorylation induced by MHC signals thus preventing their degradation (Biswas et al., 2006b), re-establishing the anchorage complex stability and, ultimately, junction integrity.
Despite compelling evidence, implicating CD31 in the maintenance or re-establishment of endothelial barrier function - particularly during T cell-mediated inflammation - the specific mechanisms by which CD31 exerts this role are unclear. The localization of CD31 at endothelial cell–cell junctions via diffusion trapping (Sun et al., 2000) suggests that homophilic interactions and inter-endothelial adhesion might be required for CD31-mediated barrier protection. In support of this hypothesis, primary human arterial endothelial expressing a homophilic binding-crippled mutant form of CD31 were unable to efficiently establish or restore the vascular barrier, whereas cells expressing variant forms of PECAM-1 lacking the cytoplasmic ITIMs exhibited normal to near-normal barrier function, both at steady-state and following thrombin stimulation (Privratsky JR et al 2011).

Alternatively, CD31 has been suggested to maintain vascular integrity by modulating β-catenin phosphorylation through ITIM-mediated recruitment of SHP-2 thus facilitating VE-cadherin complex stability (Biswas et al., 2006b).

In line with this report, our data show that signalling events required for junction stability are initiated upon CD31 activation by MHC triggering, and that the protective role of CD31 is lost upon loss-of function mutation of one of its cytoplasmic ITIMs (unpublished data).

These alternative mechanisms are not necessarily mutually exclusive. The impact of CD31 activity on junction stability has been shown to be stimulus- and vascular bed-specific, at least in the regulation of endothelial barrier function during leukocyte extravasation.
For example, CD31-mediated interactions play a major role in regulating T cell extravasation in vivo (Ma et al. 2010). In contrast, investigations of leukocyte migration through CD31−/− endothelium in inflammatory conditions in vivo did not reveal enhanced extravasation or vascular leaking as a result of lack of endothelial CD31 expression (Duncan et al., 1999a; Thompson et al., 2001). The differential impact of CD31 activity on lymphocyte and leukocyte extravasation might reflect a differential ability of distinct cell types and stimuli to activate CD31 signaling.

Thus, in our system CD31 signals are also set in motion by MHC ligation, suggesting that the effects of CD31 on junction integrity can only become apparent upon the delivery of CD31-activating stimuli following interaction of T cells with the endothelium via MHC molecules, ITIM-mediated recruitment of SHP-2 by EC PECAM-1 has been shown not to be required for leukocyte transmigration through cell monolayers (Dasgupta et al., 2009; O'Brien et al., 2003). It is conceivable that CD31 signalling activity might be a dominant mechanism following stimuli (histamine, MHC triggering) which induce its activation, while its intercellular adhesion function might be more relevant to processes not involving CD31 phosphorylation. It is also possible that the adhesive and or signaling properties of CD31 might differentially contribute to barrier integrity depending on its availability and/or distribution on the endothelial surface. Indeed, CD31 redistributes away from the intercellular junction following TNFα and IFNγ stimuli suggesting that under these conditions CD31 function might require its activation by other stimuli rather than hemophilic engagement (Romer et al., 1995).
Finally, our own observations suggest that the permeability of distinct vascular beds is differentially affected by lack of CD31, possibly reflecting the heterogeneity of junction organization and strength in different vessels.

The physiological relevance of the specific effect of CD31 on MHC-induced vascular permeability remains to be established. A large body of studies indicates a prominent protective role for CD31 signals during T cell-mediated inflammation, including the maintenance of vascular integrity. Our previous and current observations suggest that this role of CD31 specifically applies to T cell recruitment by antigen-presenting endothelium. Although this mechanism has been repeatedly observed and characterized in human and mice, its relative contribution to overall T cell trafficking during physiologic inflammation is not known. However, several reports have highlighted a significant association between genetic variants of CD31 and increased graft versus host disease (GVHD) (Behar et al., 1996; El-Chennawi et al., 2006; Grumet et al., 2001). And the severity and complications of atherosclerosis (Elrayess et al., 2004; Fornasa et al., 2012; Listi et al., 2004; Listi et al., 2007), which are both conditions defined by a T cell-mediated pathogenic component. Although the effects of these SNPs on CD31-mediated adhesion or signalling 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; Listi et al., 2007). The summary diagram for a model of CD31 mediated regulation of T cell immunity is showed in figure 53.
Figure 53 summary diagram for a model of CD31 mediated regulation of T cell immunity. Using CD31-deficient mice, I 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.
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 53. 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 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.
7.0 Supplementary

Supplementary 1

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(Continued)
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</table>
Supplementary 2

The volcano plot used is constructed by plotting the negative log of the p-value on the y-axis (base 10). This results in data points with low p-values (highly significant) appearing toward the top of the plot. The x-axis is the log of the fold change between the two conditions (treatment and untreated). The log of the fold-change is used so that changes in both directions (up and down) appear equidistant from the centre. Plotting points in this way results in two regions of interest in the plot: those points that are found toward the top of the plot that are far to either the left- or the right-hand side. These represent values that display large magnitude fold changes (hence being left- or right- of centre) as well as high statistical significance (hence being toward the top). In brief, the volcano plots showing the expression of apoptosis-related genes by WT and CD31-deficient ECs following exposure to TNFα by employing the RT2 profiler apoptosis array as described in Methods. mRNA expression levels were normalized to untreated control cells.
Chapter 8 References


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