Integrins in immunity

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Summary

A successful immune response depends on the capacity of immune cells to travel from one location in the body to another – these cells are rapid migrators, travelling at speeds of μm/minute. Their ability to penetrate into tissues and to make contacts with other cells depends chiefly on the β2 integrin known as LFA-1. For this reason, we describe the control of its activity in some detail. For the non-immunologist, the fine details of an immune response often seem difficult to fathom. However, the behaviour of immune cells, known as leukocytes (Box 1), is subject to the same biological rules as many other cell types, and this holds true particularly for the functioning of the integrins on these cells. In this Commentary, we highlight, from a cell-biology point of view, the integrin-mediated immune-cell migration and cell-cell interactions that occur during the course of an immune response.

Key words: LFA-1, T cells, Migration

Introduction

Immune cells are collectively termed leukocytes and the key players are T and B lymphocytes (also called T cells and B cells; Box 1), dendritic cells (DCs), neutrophils and monocytes that differentiate into tissue macrophages. Leukocytes, especially T cells, migrate rapidly, and a successful immune response depends on their capacity to quickly access lymph nodes (LNs) and sites of injury or infection elsewhere in the body.

LNs collect foreign antigen that drains into them from the body's tissues and thus act as the central immune-response centres or 'information hubs' (Lammermann and Sixt, 2008). It is in LNs that T cells can screen antigen-presenting cells (APCs) (Box 1), such as DCs or B cells, and it is this event that initiates a standard immune response. As a wound might occur anywhere in the body, lymphocytes migrate continuously through the widely distributed LNs where they spend ~24 hours before exiting into the lymphatic circulation and back to the blood (Cyster, 2005; von Andrian and Mempel, 2003). This timing allows maximal exposure to any foreign antigen that is trapped on APCs in the LN. Neutrophils and monocytes circulate in the blood until they are directed into wounded tissue by stimulants that are generated locally. Here they act as an 'early warning system' for the lymphocytes that have been stimulated in nearby LNs. These lymphocytes follow later and help to create an immune microenvironment within the wounded tissue that can be very similar to that of a LN. Although the myeloid cells do not normally circulate through LNs, they do so during infections. In fact, in some infections, large numbers of neutrophils enter LNs via the lymph and are found in the T cell and DC regions of the node (Abadie et al., 2005; Chtanova et al., 2008). The present view is that they may well have a greater role in antigen delivery to APCs than previously recognised.

These functions of leukocytes depend greatly, but not exclusively, on the β2 (CD18, ITB2) class of integrins. This class of four αβ heterodimers consists of: leukocyte function-associated antigen 1 (LFA-1; CD11a/CD18, αLβ2 integrin, ITAL antigen), Mac-1 (CD11b/CD18, αMβ2 integrin, ITAM antigen), p150,95 (CD11c/CD18, αXβ2 integrin, CR4, ITAX antigen), αdβ2 (CD11d/CD18, ITAD antigen). Leukocytes also express β1 integrins (CD29), and α4β1 (CD49d/CD29, ITA4 antigen) is particularly important in immune responses.

In this Commentary we will highlight the role of integrins in leukocyte function from a cell-biology point of view. We have concentrated on LFA-1 as it has a dominant role in leukocyte migration across blood-vessel walls into LNs and tissues; LFA-1 is also a key participant in the immunological synapse (IS; see Box 1) that forms between a lymphocyte and its target cell. Integrins on leukocytes are not constitutively active, but have their activity controlled by signalling through other membrane receptors that are active in an immune response. This results in 'on-the-spot' activation of integrins precisely where their function is needed. We discuss this 'inside-out' signalling that converts integrins from an inactive to an active conformation, and the 'outside-in' signalling that describes the signalling directed by integrins themselves once they are active. In general, the principles that underlie integrin function in an immune context are similar to those that determine the behaviour of integrins in other tissue environments.

Control of integrin activity

The β2 integrins are normally in an inactive or non-ligand-binding conformation when the leukocytes are circulating in the blood. Binding to other receptors on leukocytes, such as the chemokine receptors, antigen-specific T-cell receptors (TCRs) or B-cell receptors (BCRs) (see Box 1), triggers the inside-out signalling that activates the integrins (see next section). The end result of this signalling is a conformational change of the cytoplasmic and transmembrane domains of the integrin, which is relayed to the ectodomain, altering its ability to bind ligand (Kinashi, 2005). In recent years, electron microscopic and crystallographic studies have identified three predominant β2-integrin conformations that are thought to reflect different stages of activation (Luo et al., 2007; Xiong et al., 2001) (Fig. 1). These are the bent form, the extended form with a closed ligand-binding head that is of intermediate affinity (Box 1) for ligand, and the extended form in which the hybrid domain has swung out, enabling the ligand-binding I domain of the α-subunit to bind with higher affinity. These β2 conformations can be distinguished by their interactions with specific antibodies,

Box 1. Immuno-speak for cell biologists

Affinity
The strength of binding between an individual integrin and its ligand. This can be altered by conformational changes in the integrin structure.

Avidity
A measure of the overall strength of binding of clustered integrins.

Antigen-presenting cell (APC)
A general term for all cells that display antigenic peptides in complex with major histocompatibility complex (MHC) molecules. The professional APCs are the dendritic cells, B cells and, occasionally, macrophages. They present antigen to lymphocytes.

High endothelial venule (HEV)
Specialised vasculature that is found only in lymph nodes.

Immunological synapse (IS)
The zone of contact between a lymphocyte or natural killer (NK) cell and target cell. On the lymphocyte side, the IS receptors are organised to resemble a 'bull's eye' with the TCR or BCR in the middle [the central supramolecular activating cluster (cSMAC)] surrounded by a ring of LFA-1 [the peripheral (p)SMAC], with an outermost ring [the distal (d)SMAC] where the phosphatase CD45 is found.

Immunoreceptor tyrosine-based activation motif (ITAM)
A double YxxL motif that is separated by a 7-12 amino-acid linker. These motifs are often found in immune-cell signalling molecules such as the CD3 ξ-chain of the TCRs, the γ-chain of the Fc receptor (and certain other receptors) and DAP12 proteins. Once phosphorylated, often by a Src kinase, the tyrosine residues act as a docking site for SH2 domains in other signalling kinases.

Leukocytes
Collective term for all immune cells such as T and B cells, dendritic cells, neutrophils, eosinophils, NK cells and monocytes/macrophages.

Lymphocytes
Refers only to T lymphocytes (T cells) and B lymphocytes (B cells).

T-cell receptors (TCRs) and B-cell receptors (BCRs)
The antigen-specific receptors, or immunoreceptors, on T cells and B cells, which recognise a wide array of different peptides that have been processed and displayed by APCs.

which react with the different epitopes that become exposed (Fig. 1).

Chemokine-receptor signalling leads to extension of the bent 'low affinity' form of LFA-1, and this happens in less than 0.4 seconds (Shamri et al., 2005). The extended form of LFA-1 is proposed to be in an intermediate state of affinity simply because of the greater accessibility of the ligand-binding I domain compared with the bent form (Kinashi, 2006; Luo et al., 2007). The interaction of intermediate-affinity LFA-1 with its ligand intercellular adhesion molecule 1 (ICAM1) leads to the high-affinity conformation that stabilises adhesion, but there has been no clear indication as to how this switch happens. However, there is now increasing evidence that mechanical forces can drive allosteric alteration of integrin conformation (Katsumi et al., 2004) and, for leukocytes, the shear of blood flow would be the major supplier of this force (Alon and Dustin, 2007). Puklin-Faucher and colleagues showed that when

force is applied to ligand-bound αvβ3 integrin, there is a nanosecond transition to the high-affinity conformation (Puklin-Faucher et al., 2006). The same theoretical considerations apply to LFA-1 when it is bound to ICAM1 and to other leukocyte integrins such as α4β1 integrin bound to vascular-cell adhesion molecule 1 (VCAM1) or fibronectin (Astrof et al., 2006).

It has only been in recent years that details have emerged of the signalling pathways that are involved in the rapid conversion of the inactive conformation of integrin, which is expressed on circulating resting lymphocytes, to the active conformation that enables lymphocytes to bind to target cells. It is not clear how exclusive the dynamic aspect of the regulation of inside-out signalling is to the activation of integrins on leukocytes – where it has been most extensively investigated. The next section will outline what is presently known about inside-out signalling with emphasis on signalling in leukocytes.

Activation of integrins by inside-out signalling

As mentioned above, in the absence of stimulation, leukocytes circulating in the blood exhibit an overall low level of integrin activity. Stimulation by an agonist such as a chemokine leads to an increase in integrin activation. The small GTPase Rap1 is a key integrin regulator, and constitutively active mutants of Rap1 (such as Rap1V12) increase the affinity and avidity (Box 1) of LFA-1 at the lymphocyte membrane (Katagiri et al., 2000; Sebzda et al., 2002). Impaired activation of Rap1 has been seen in some patients with the rare disorder leukocyte adhesion deficiency III (LAD-III) (Kinashi et al., 2004). The leukocytes of the patients express normal levels of β1, β2 and β3 integrins, but have faulty inside-out signalling (Alon et al., 2003; Kuijpers et al., 1997; McDowall et al., 2003). The LAD-III syndrome demonstrates the importance of integrin activation through this route. The patients suffer from severe and often life-threatening infections, because their leukocytes cannot migrate into tissues and their myeloid cells cannot use integrins to engulf pathogens (see below).

A major route that leads to the activation of Rap1 has recently been proposed (Han et al., 2006). As for other GTPases, the GDP-GTP exchange cycle of Rap1 is regulated by guanine-exchange factors (GEFs). Certain Rap GEFs are activated by the second messengers Ca^{2+} and diacylglycerol (DAG), and one of the key Rap1 regulators in haematopoietic cells is CalDAG-GEF1 (also known as RasGRP2) (Fig. 2). *Rasgrp2*$^{-/-}$ mice have impaired integrin-mediated adhesion in platelets and neutrophils and are considered to be a model for human LAD-III (Bergmeier et al., 2007; Crittenden et al., 2004). This might be so because several LAD-III patients have a mutation in CalDAG-GEF1 that has been reported to affect splicing (Pasvolsky et al., 2007). These findings implicate this Rap1 GEF in the inside-out signalling of leukocyte integrins.

Another route to Rap1 activation is through protein kinase C (PKC), which – similar to CalDAG-GEF1 – is also responsive to Ca^{2+} and DAG (Han et al., 2006). In platelets, rapid chemokine induction of Rap1 occurs via CalDAG-GEF1, whereas more sustained activation is via PKC (Cifuni et al., 2008). There also seems to be fast versus slow routes to Rap1 activation in lymphocytes, as chemokines activate Rap1 within seconds, which lasts a few minutes, whereas a more sustained level of Rap1 activation occurs through the TCR (Katagiri et al., 2002; Shimonaka et al., 2003). Thus, there may be similarities between lymphocytes and platelets in the choice of pathways that lead to Rap1 activation that results in integrin activation. A downstream effector of PKC that leads to Rap1 activation is proposed to be protein kinase D1
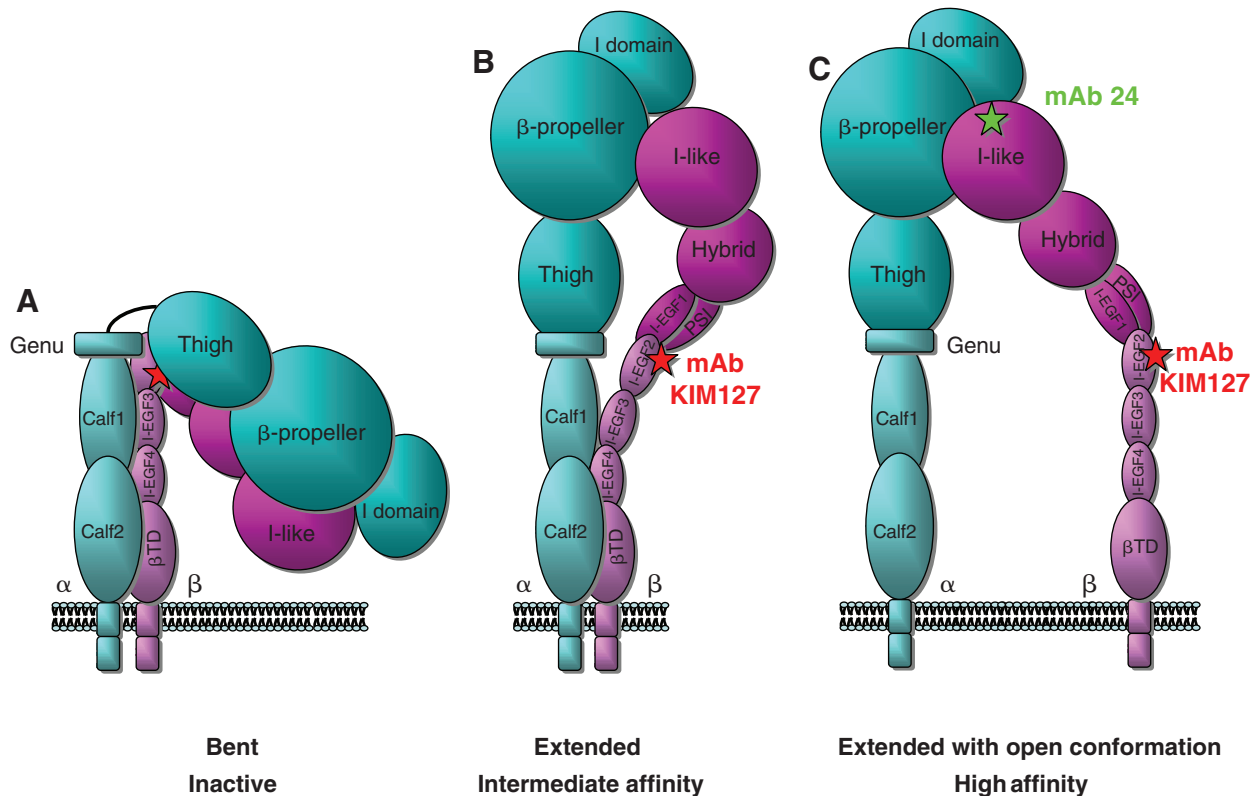
Fig. 1. The three conformations of a β2 (ITGB2) integrin. Integrins are heterodimeric glycoproteins comprising non-covalently linked α- and β-subunits. Each subunit consists of a large extracellular region, a single hydrophobic transmembrane domain and a short cytoplasmic tail. The extracellular region of the α-subunit comprises an N-terminal seven-bladed β-propeller domain followed by three β-sandwich domains (termed thigh, calf 1 and calf 2). Nine of the 18 α-subunits (including the four β2-family integrins) also contain an I domain, which is inserted in the upper face of the β-propeller. The β-subunit has an N-terminal cysteine-rich PSI (plexin-semaphorin-integrin) domain, a β-sandwich hybrid domain, a β I-like domain, four integrin EGF-like repeats (I-EGF1 to I-EGF1-4) and a β-tail domain (βTD). In the tertiary structure, the I domain is inserted in the hybrid domain. When present, the α I domain is the exclusive site of ligand binding. (A) Bent – inactive. Integrins are bent between I-EGF1 and I-EGF2 in the β-subunit and at a small Ca^{2+}-binding loop, known as the 'genu', between the thigh and calf1 domains in the α-subunit. Thus, the inactive integrin is in a V shape with the ligand-binding I domain close to the membrane. There is close association between the α- and β-subunits in the membrane-proximal region. (B) Extended – intermediate affinity. Inside-out signalling extends the integrin in a 'switchblade-like' motion, orientating the I domain away from the membrane for optimal ligand binding. This epitope for the monoclonal antibody (mAb) KIM127, which is located on I-EGF2 and obscured in the bent formation, becomes exposed. The KIM127 epitope thus serves as a marker for the extended β2 integrin. (C) Extended with open conformation – high affinity. Local conformational changes within the α and β I domains, potentially generated by shear force, result in the hybrid domain swinging out and the subunit separating at the genu. This remodelling of the I domain ligand-binding site forms the epitope for mAb 24 and causes increased affinity for ligand.

(KPCD1; also known as PKD1, PKCμ), which forms a complex independently of its kinase activity, together with Rap1 and the β1-integrin cytoplasmic domain, redistributing Rap1 to the membrane (Medeiros et al., 2005).

However PKC signalling does not always lead to Rap-1 activation and Rap1 might not be involved in the activation of all classes of integrins. Ghandour et al. report that LFA-1 activation is dependent on CalDAG-GEF1 and Rap1, whereas α4β1 integrin appears to be activated via a Rap1-independent PKC-mediated pathway (Ghandour et al., 2007). A conflicting report shows that the Rap1 inhibitor GTPase-activating protein (GAP)-signal-induced proliferation-associated protein 1 (SPA1) blocks both LFA-1- and α4β1-integrin-dependent adhesion (Shimonaka et al., 2003).

A further set of proteins can bring Rap1 to the membrane and cause its activation. The Rap1-interacting adaptor molecule (RIAM) is expressed by T cells and activates Rap1 through TCR signalling (Lafuente et al., 2004). This pathway involves the adaptor adhesion- and degranulation-promoting adaptor protein (ADAP) and its binding partner SRC-kinase-associated protein of 55 kDa (SKAP55), which interact with RIAM (Kliche et al., 2006; Menasche et al., 2007).

The activation of Rap1 leads to the redistribution of integrins from the rear to the front of the cell. Associated with Rap1 are the adaptor protein RAPL (regulator of cell adhesion and polarization enriched in lymphoid tissues) and Mst1 (mammalian sterile 20-like kinase1) that are colocalised with LFA-1 in vesicular compartments (Katagiri et al., 2006; Katagiri et al., 2003). The implication is that the RAPL-Mst1 complex has a regulatory function in the intracellular transport of LFA-1.

A final step involves the recruitment of talin by Rap1, which then binds to integrin causing the conformational shift that leads to an increase in affinity (Campbell and Ginsberg, 2004) (see also Legate and Fässler, 2009, in this issue). The recruitment of talin and the ensuing integrin activation has become more complex with the identification of kindlin-3 (URP2, Mig2B) as a haematopoietic-specific activator of integrins (Moser et al., 2008). Kindlin-3 belongs to an adhesion plaque protein family of three members, each with distinctive tissue distributions: kindlin-1 (FERM1),
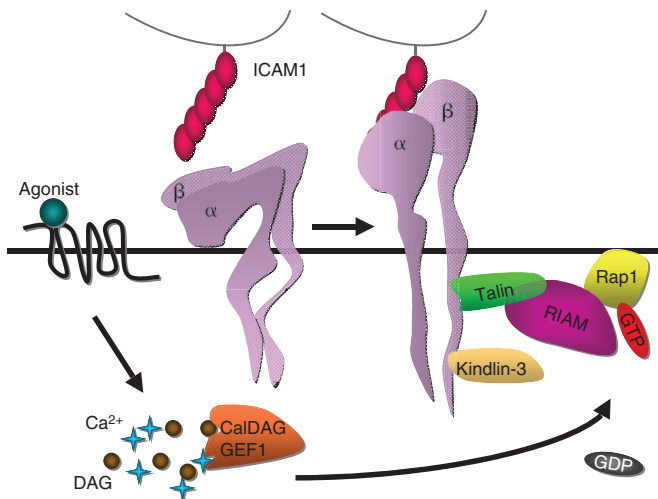
Fig. 2. A model of inside-out signalling from agonist to integrin. For integrins to become activated, they need to be triggered by the inside-out signalling cascade. The key target of this pathway is the GTPase Rap1. Agonist signalling leads to an increase in Ca^{2+} and DAG that activates the Rap1 GEF CalDAG-GEF1, which is a major Rap1 activator in haematopoietic cells. The relocalization of Rap1 to the membrane is regulated by the adaptor protein RIAM. A next step is the recruitment of talin to the β-subunit of the integrin, bringing about the conversion of integrin conformation to one of higher affinity. Kindlin-3 also binds to the β-subunit and aids talin binding.

epithelial expression; kindlin-2 (FERM2), widespread expression; and kindlin-3, haematopoietic-cell-specific expression (Ussar et al., 2006). Both talin and kindlin-3 have similar FERM (protein 4.1, ezrin, radixin and moesin) domains that bind the β-subunit of integrins at the two NPxY sites (in which x denotes any amino acid) – talin at the membrane-proximal site and kindlin-3 at the membrane-distal site (Ma et al., 2008; Moser et al., 2008). Kindlin-3 knockout (*Fermt3*$^{-/-}$) mice have severe bleeding problems (Moser et al., 2008) similar to the *Rasgrp2*$^{-/-}$ mice (Bergmeier et al., 2007; Crittenden et al., 2004), suggesting that kindlin-3 is also a candidate gene involved in LAD-III.

A challenge for the future is to understand the settings in which all these molecular players that are associated with Rap1 lead to the active integrin state. However, once an increase in affinity or 'activation' has been achieved, integrins such as LFA-1 or α4β1 will bind firmly to ligand and signal back into the cell on which it is expressed. This is termed outside-in signalling and the effector functions of adhesion, cell spreading and migration depend on this signalling.

Consequences of outside-in integrin signalling

Altering affinity is not the only means of controlling the ability of integrins to bind their ligands. A further level of integrin-activity control is the regulation of avidity, and it is the outside-in signals from activated LFA-1 itself that appear to control the state of clustering. Macroclustering of LFA-1 on T cells follows on from the initial binding of LFA-1 to ICAM1 in multivalent form, thus strengthening adhesion (Kim et al., 2004). On macrophages, inactive LFA-1 is randomly distributed, but primed LFA-1 forms nanoclusters and then macroclusters when it binds to ICAM1 (Cambi et al., 2006). Whether the nature of the interaction with the cytoskeleton also controls clustering is unclear. We have shown that intermediate-affinity LFA-1 on T cells is attached to the

actin-binding protein α-actinin-1, whereas high-affinity LFA-1 is attached to the cytoskeletal protein talin (Smith et al., 2005; Stanley et al., 2008). On macrophages, the LFA-1 macroclusters colocalise with talin (Cambi et al., 2006). Considered together, the evidence suggests that the high-affinity LFA-1–talin connection favours the formation of stable clusters that further enhance adhesion.

It is well recognised that the successful functioning of T cells and other leukocytes depends on cytoskeletal remodelling (Billadeau et al., 2007; Vicente-Manzanares and Sanchez-Madrid, 2004). As outside-in signalling through leukocyte integrins causes cell spreading and migration, the implication is that cytoskeletal reorganisation is downstream of the integrin-specific signalling. For T cells, LFA-1-mediated adhesion to ICAM1 causes extensive F-actin remodelling (Porter et al., 2002), but little is understood of the integrin-proximal signalling that is involved. More detail is known about signalling in neutrophils and macrophages, which promotes β2-integrin-mediated adhesion, spreading, chemotaxis and other myeloid-specific functions, such as the production of reactive oxygen species and the release of cytokines and cytotoxic granules (Mocsai et al., 2002). The involvement of integrins in these activities is highlighted by the finding that neutrophils from β2-integrin-null mice are unable to carry out these functions.

The Src and Syk families of protein tyrosine kinases have been implicated in outside-in signalling in myeloid cells that express β2 integrins, in platelets that express integrin αIIbβ3, and also downstream of the TCR and BCR in lymphocytes (Fig. 3) (Abram and Lowell, 2007a). Syk is recruited through its tandem SH2 domains to an adaptor molecule(s) containing an immunoreceptor tyrosine-based activation motif (ITAM; see Box 1) that is di-phosphorylated by Src kinase (Abram and Lowell, 2007b). By this means, Syk is brought within range of Src, which then phosphorylates it.

Recent studies in platelets and myeloid cells suggest that the integrin-Src-Syk signalling cascade is more similar to immunoreceptor signalling than initially thought. Using retroviral reconstitution of myeloid cells expressing wild-type Syk versus Syk mutated in the SH2 domains, it has been demonstrated that these domains are needed for Syk activation (Abtahian et al., 2006; Mocsai et al., 2006). The identity of the ITAM-containing adaptor molecule(s) has been of interest because neither β3 nor β2 integrins contain ITAM motifs. For myeloid cells, a deficiency in the ITAM-containing γ-chain of the Fc receptor and DAP12 proteins leads to a failure to signal through their integrins (Mocsai et al., 2006). No direct association between integrins and ITAM-containing adaptors has so far been identified. An indirect association through an adaptor-associated linker molecule is possible or, perhaps the membrane lipid architecture ensures that the ITAM adaptor and integrin are in close proximity (Jakus et al., 2007).

T-cell model of LFA-1-mediated migration

Immune cells are rapid migrators. For example, they move at speeds of 5-40 μm/minute, which contrasts with 15 μm/hour for a fibroblast (Friedl and Weigelin, 2008; Mempel et al., 2004; Shimonaka et al., 2003; Smith et al., 2003). This difference in speed suggests that the regulation of integrins on a leukocyte differs from slower cells. Leukocytes lack the traditional focal adhesions and stress fibres that are associated with the migration of many slow cells but there is still much to learn about the molecular details of their integrin-containing adhesions.

Confocal microscopy images show that the expression of LFA-1 on a migrating T cell is not uniform, varying from a low level at the leading edge to a high level in the non-attached uropod at the rear
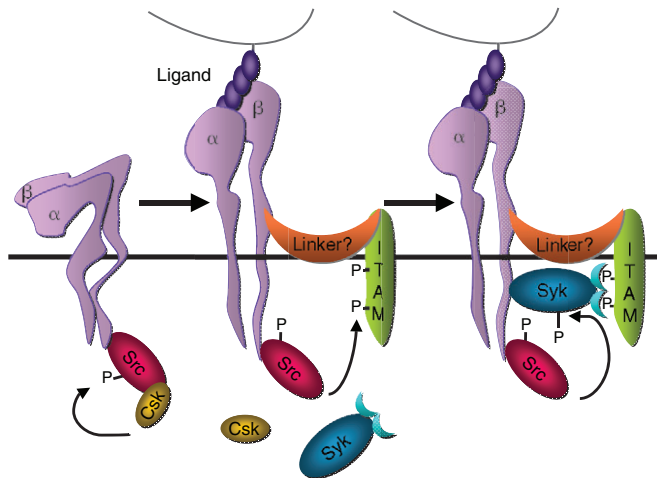
Fig. 3. Outside-in signalling associated with integrins on leukocytes. The inactive β2 and β3 integrins on myeloid cells and platelets, respectively, are constitutively associated with inactive Src kinase. Src is maintained in a C-terminal phosphorylated conformation by Csk kinase. The ligation of integrin to ligand prompts the dephosphorylation of the inhibitory Tyr by phosphatases, such as PTP1B and CD45, dissociation of Csk and autophosphorylation onto the activation loop of Src. Active Src can then dually phosphorylate an ITAM-containing adaptor that has been postulated to be associated with integrin through a linker. The phosphorylated ITAM recruits Syk through its tandem SH2 domains (pale blue). Syk then associates with the integrin β-tail and is in sufficient proximity to Src to be phosphorylated. Active Syk then phosphorylates downstream effectors, such as Vav1, Vav3 and SLP-76.

Fig. 4. The location of the LFA-1 conformation zones on a T cell migrating on ICAM1. The leading edge of the T cell expresses low levels of LFA-1 with intermediate affinity for ICAM1 that is recognised by monoclonal antibody (mAb) KIM127 (Stanley et al., 2008). The focal zone expresses higher levels of LFA-1, which is in the high-affinity conformation recognised by both mAb KIM127 and mAb 24 (Smith et al., 2005; Stanley et al., 2008). The uropod at the rear expresses the highest level of LFA-1, but little is known of its binding activity.

(Smith et al., 2005) (Fig. 4). Superimposed on these expression levels are different conformations of LFA-1 that are restricted in distribution. The projecting lamellipodium at the leading edge of the T cell expresses intermediate-affinity LFA-1 and the mid-cell 'focal zone' contains high-affinity LFA-1 (Smith et al., 2005; Stanley et al., 2008). The ligand-binding status of the high level of LFA-1 that is expressed by the uropod is uncertain. It is both the spatial organisation of LFA-1 and turn over of active LFA-1 that equips the cells to migrate efficiently. Surprisingly, the immune defects of mice that express a constitutively active form of LFA-1 mirror those that are observed in the *Lfa1*−/− mouse (Berlin-Rufenach et al., 1999; Semmrich et al., 2005). These mouse models clearly show the contribution of LFA-1 to immune function and the need to convert between LFA-1 conformations with different affinities for ICAM1. These different LFA-1 zones all have distinctive features, as outlined below.

The leading edge of the migrating cell
A T cell needs to rapidly scan its target, which might be a DC displaying bound antigen or a blood vessel. Such T cells express intermediate-affinity LFA-1 on the lamellipodial membranes that are rapidly protruding and retracting (Stanley et al., 2008). This conformation of LFA-1 allows the leading edge to participate in faster 'make-break' binding to ICAM1 than would high-affinity LFA-1. By contrast, the leading edge of the more slowly migrating endothelial cell expresses high-affinity αvβ3 integrin that would have less dynamic on-off kinetics (Kiosses et al., 2001). Alternative mechanisms of adhesion at the leading edge may have evolved to favour the biological activities of individual cell types.

Intermediate-affinity LFA-1 links to the cytoskeleton through α-actinin-1, an actin-bundling protein that is part of the major F-actin network propelling the cell forward (Stanley et al., 2008). Disruption
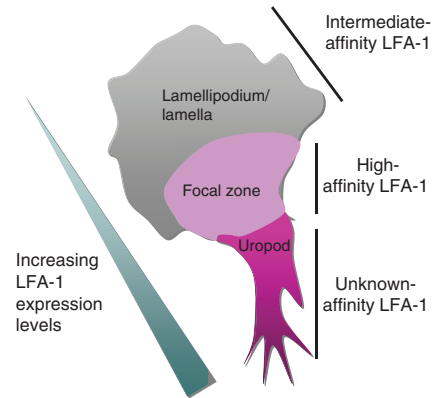
of this interaction prevents attachment of the leading edge to ICAM1, highlighting the importance of intermediate-affinity LFA-1 in this context in vitro. An in vivo role for α-actinin-1-associated intermediate-affinity LFA-1 in lymphocyte migration is supported by the following experiment that we performed. Naïve mouse lymphocytes were treated with a peptide competing for the α-actinin-1 binding site on LFA-1 and injected back into mice – this peptide prevented migration of these lymphocytes into peripheral LNs (Fig. 5A). The experiment shows that our in vitro findings are mirrored in vivo, and that the LFA-1–α-actinin-1 link is crucial for the important manoeuvre that T cells must make of crossing the endothelial barrier from the circulation into LNs.

The mid-cell 'focal zone'
There are other LFA-1-directed events that operate away from the leading edge of the migrating T cell. A distinctive zone of adhesion in the mid-cell region, probably corresponding to the lamellar region of the fibroblast, provides firm attachment to an ICAM1-expressing surface. The LFA-1 in the 'focal zone' binds ICAM1 with high affinity, as identified by monoclonal antibody 24 (see Fig. 1) (Smith et al., 2005) (Fig. 4). If the high-affinity conformation of LFA-1 is 'locked' in place by this antibody, turnover in the focal zone is prevented, but the leading edge remains motile, indicating that the generation of force at the leading edge is sufficient to move the T cell forward.

LFA-1 in the focal zone is associated with a second cytoskeletal protein, talin. If the talin link is disrupted, for example by talin knockdown, then the migrating T cell rounds up in shape and detaches (Smith et al., 2005). This type of evidence suggests that the focal zone provides support for the more dynamic attachments at the leading edge of the T cell. How talin becomes localised to the mid-cell region has been unclear. Nolz and colleagues have recently shown that signalling through the TCR leads to the formation of a complex between Wiskott-Aldrich syndrome protein (WASp)-family member 2 (WAVE2), actin-related proteins 2 and 3 (ARP2/3) and vinculin that is linked to both actin polymerisation and, via vinculin, to talin recruitment (Nolz et al., 2007). In fact, the WAVE2 complex is necessary to recruit talin to the IS where it stabilises LFA-1. It will
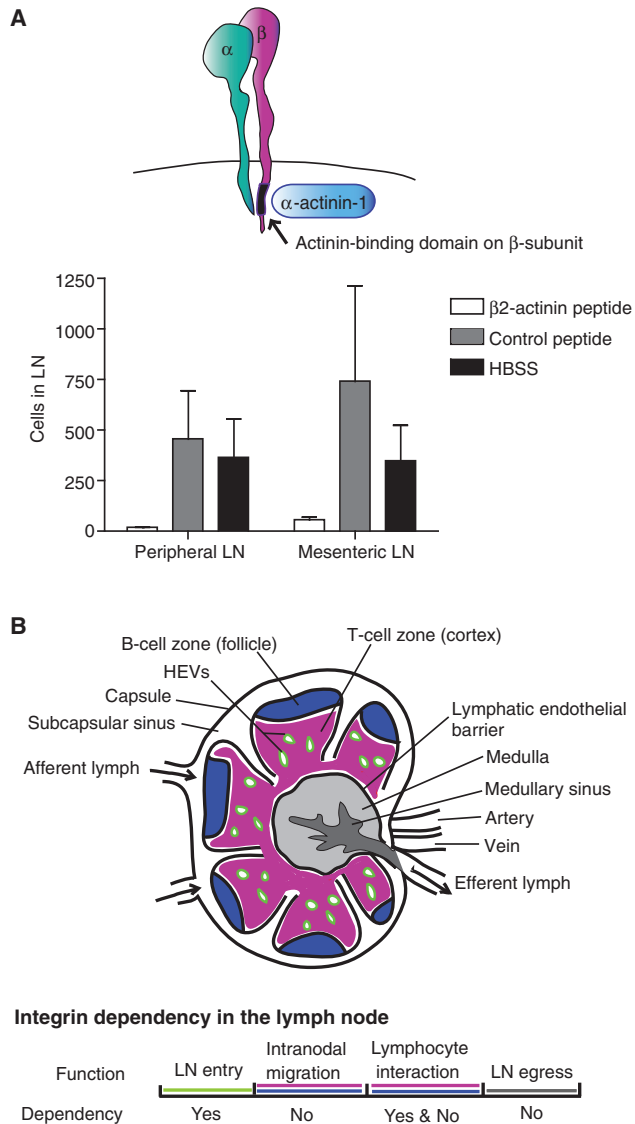
A

α β

α-actinin-1

Actinin-binding domain on β-subunit

Cells in LN

1250
1000
750
500
250
0

□ β2-actinin peptide
▨ Control peptide
■ HBSS

Peripheral LN   Mesenteric LN

B

B-cell zone (follicle)
HEVs
Capsule
Subcapsular sinus
Afferent lymph
T-cell zone (cortex)
Lymphatic endothelial barrier
Medulla
Medullary sinus
Artery
Vein
Efferent lymph

Integrin dependency in the lymph node

Function	LN entry	Intranodal migration	Lymphocyte interaction	LN egress
Dependency	Yes	No	Yes & No	No

Fig. 5. The role of LFA-1–α-actinin-1 in LN entry and schematic overview of a LN. (A) Effect of β2-actinin-blocking peptide on the migration of mouse T cells into peripheral and mesenteric LNs. The figure shows the effect on lymphocyte migration into LNs of T cells treated with a blocking peptide that consists of the α-actinin-1 binding site on the β2 cytoplasmic tail (top panel) linked to membrane-penetrating peptide penetratin-1 (β2-actinin peptide) (Stanley et al., 2008). Mouse LN T cells that had been labelled with the fluorescent dyes CFSE or SNARF-1 were incubated for 30 minutes with either the β2-actinin-blocking peptide or, alternatively, control peptide or Hanks buffered salt solution (HBSS), and injected intravenously into the host for 30 minutes. The numbers of fluorescently labelled T cells that successfully transmigrated into either peripheral or mesenteric LNs were quantified. The β2-actinin-blocking peptide, but not control treatments, severely retarded LN entry, implying a major role for the intermediate-affinity integrin bound to α-actinin-1 in this crucial step. (B). Schematic overview of a LN. Leukocytes enter the LN either through the afferent lymph into the subcapsular sinus, or the blood flow through HEVs. T cells migrate in the T-cell zone, thereby contacting resident DCs, which may activate the T cells if they are expressing appropriate antigen. B cells migrate to their follicles seeking an antigen stimulus. As a subsequent stage in their stimulation they encounter T cells at the T-cell–B-cell boundary zone. After migrating through the LN, lymphocytes exit through the medullary sinus into the efferent lymph. The lower panel shows a scheme to highlight integrin dependency (or the lack thereof) during the journey of the lymphocyte through the LN. Green, HEV (entry); purple, T-cell area (intranodal migration and lymphocyte contacts); blue, B-cell area (intranodal migration and lymphocyte contacts); grey, medulla (exit).

be interesting to examine whether a similar WAVE2 complex maintains talin in the focal zone of the migrating T cell.

The activity of high-affinity LFA-1 in the focal zone must be regulated for the T cell to detach and move forward (Semmrich et al., 2005; Smith et al., 2005). Speculation as to the molecular basis of this regulation is as follows. Inhibition of the GTPase RhoA causes prolonged LFA-1 attachment at the rear of the cell (Smith et al., 2003). This is a general feature of immune-cell migration as attachments of eosinophils and monocytes are also characterised by RhoA sensitivity (Alblas et al., 2001; Worthylake et al., 2001). RhoA-associated kinase (ROCK) is active at the rear of the cell and phosphorylates myosin light chain (MLC), which is necessary for myosin activity (Smith et al., 2003). Myosin is also localised to the rear of the polarised cell and, in T cells, this myosin is non-muscle myosin heavy chain IIA (MyH9) (Jacobelli et al., 2004; Morin et al., 2008; Smith et al., 2003). If myosin activity is blocked, the T cells remain attached to ICAM1, implying a role for myosin in LFA-1 turnover (Morin et al., 2008). This event mirrors those that occur when LFA-1 turnover at the focal zone is inhibited, so there may be a connection between high-affinity LFA-1, RhoA and myosin activity that needs to be formally tested. It is of interest that T-cell signalling leads to phosphorylation of MHC and myosin inactivity. This may constitute the T-cell 'stop' signal that overrides promigratory signals and occurs when a T cell encounters an antigen-loaded APC and active migration ceases (Dustin et al., 1997; Jacobelli et al., 2004).

The uropod – back of the cell and beyond

The uropod is a protruding structure found exclusively at the trailing edge of leukocytes, and has been somewhat neglected in models of T-cell function. We have been discussing migration that takes place on an ICAM1-expressing cell surface, which is essentially a two-dimensional environment; however, two observations suggest that the uropod may reveal its function in a three dimensional (3D) context, as found in tissues. First, the uropod was observed to act as an LFA-1-dependent tether for the T cell when confronted with a chemokine-expressing APC (Friedman et al., 2006). This 'backwards' attachment allowed the T cell to efficiently scan its immediate surroundings with its leading edge. How frequently T cells use this manoeuvre to interact with an APC in vivo needs further investigation. Second, when neutrophils transmigrate across a blood vessel in vivo, LFA-1 redistributes into a doughnut-shaped structure at the trailing edge that maintains a grip on the surface as the leading edge of the cell penetrates through the endothelial junction (Shaw et al., 2004).

Sixt and colleagues have now shown that the rear of the cell behaves independently of the front in a 3D context, and that its major function is the contraction of the nucleus of the cell and easing of its passage through small spaces (Lammermann et al., 2008). This rear contraction is myosin-ROCK dependent. It needs to be explored whether nuclear compression is also advantageous for squeezing through endothelial junctions and whether LFA-1 has a role here. The steps taken by leukocytes when they leave the blood and migrate into tissue will be discussed in the next section.

Extravasation – crossing the vasculature into lymph nodes and other tissues

Three overlapping adhesion events mediate the migration of leukocytes out of the blood and into tissues (Butcher, 1991). These sequential steps are required to enable the leukocytes to overcome the shear forces of blood flow. During the first step, circulating leukocytes are captured, become loosely attached to the vascular

endothelium and begin to roll along using their selectin molecules. This rolling is mediated by L-selectin on the leukocyte and E- and P-selectins on the endothelium. The selectins bind to sialyl-Lewis^X moieties that decorate a number of glycosylated counter-receptors (Uchimura and Rosen, 2006).

The next step is mediated by chemokines that are constitutively produced in LNs or induced in injured tissues and become tethered to proteoglycans on the vasculature (Forster et al., 2008; Rot, 1992). For example, naive T cells abundantly express the chemokine receptor CCR7, which binds both CC-chemokine ligand 19 (CCL19) and CCL21, both of which are presented by the high endothelial venules (HEVs) (Box 1) of LNs. Chemokines activate integrins α4β1 and LFA-1 by engaging the specific G-protein-coupled receptors (inside-out signalling) on leukocytes, which leads to the third step of the cascade – integrin-mediated leukocyte arrest on the vessel wall. The integrin α4β1 is important at this stage and can have a role in both leukocyte rolling and arrest (Tsuzuki et al., 1996). A new role for integrin α4β1 in lymphocyte homing to LNs has been recently investigated. In this scheme, the lipase autotaxin, which is expressed by HEVs, binds active integrin α4β1, generating lysophosphatic acid that, in turn, acts on G-protein-coupled receptors that then further amplifies the process of homing (Kanda et al., 2008).

Chemokines also activate LFA-1, enabling leukocytes to firmly attach to the endothelial surface and to subsequently migrate across it. *Lfa1*$^{-/-}$ mice have small LNs and make poor immune responses because their leukocytes cannot make this transition (Berlin-Rufenach et al., 1999). The crawling of the leukocytes through the vasculature is known as diapedesis, and this can occur by both paracellular (through endothelial-cell junctions) (Ley et al., 2007; Vestweber, 2007) and transcellular (through the endothelial cell itself) routes (Carman and Springer, 2008). The factors that impact on route selection are unclear but one in vivo study suggests that transmigration occurs at sites of least resistance (Wang et al., 2006).

The initial stages of the transcellular route are now being dissected. During migration, cells such as macrophages and DCs display multiple podosomes, which are dynamic integrin-containing structures, each surrounded by a ring of actin. Lymphocytes also form integrin-expressing podosome-like protrusions that appear to test the endothelial surface as the cell moves along (Carman et al., 2007). The endothelium responds by forming a cup-like docking structure around the leukocyte, which is rich in ICAM1 and VCAM1 (Barreiro et al., 2002; Carman and Springer, 2004). To achieve the step of penetrating the endothelial cell, the protrusions trigger recruitment of endothelial vesicles that are rich in the fusogenic proteins vesicle-associated membrane protein 2 (VAMP2) and VAMP3. These members of the soluble *N*-ethylmaleimide-sensitive fusion-protein-attachment-protein receptor (SNARE) family participate in the creation of a pore in the endothelium through which the lymphocyte migrates (Carman et al., 2007).

Lymph nodes and trafficking of lymphocytes
The LN receives continual input of circulating naive T cells and B cells from the blood, which enter the LN through the HEVs. Once in the LN, T cells migrate within the T-cell area and B cells move through this area to the B-cell follicles (Fig. 5B). The use of the two-photon laser-scanning microscope has considerably advanced understanding of both T- and B-cell trafficking within an intact LN (Cahalan and Parker, 2008; Miller et al., 2002). Both types of lymphocytes migrate along highly organised reticular-fibre networks that comprise two stromal-cell types: fibroblastic reticular cells

(FRCs; found in the T-cell cortex) and follicular DCs (FDCs; found in the B-cell follicles) (Bajenoff et al., 2006). These networks are based on fibrillar collagen matrix, extracellular-matrix molecules and a basement membrane that is surrounded by stromal fibroblastic cells (Gretz et al., 1997; Sixt et al., 2005). The FRC network influences lymphocyte recruitment in several ways. A subset of DCs in the T-cell zone is attached to the FRC network and positioned next to HEVs in a prime position for contact with newly recruited T cells (Bajenoff et al., 2003; Miller et al., 2002). The FRC network also serves as a conduit for signalling molecules such as lymph-borne chemokines (Gretz et al., 2000). Within the LN, the chemokine CCL21 is expressed at bioactive levels (Luther et al., 2002), and contributes to T-cell motility within the T-cell zone (Huang et al., 2007; Okada and Cyster, 2007; Worbs et al., 2007).

It has been an issue whether integrins are used by lymphocytes to travel within the LN, and an important recent study now shows that the behaviour of DCs within the LNs is unaffected by ablation of β1, β2, β7 and αv classes of integrins, making them essentially 'integrin nude' (Lammermann et al., 2008). Closer analysis of these DCs migrating in a 3D collagen gel in vitro reveals that the cells migrate by means of leading-edge protrusions that are created by expansion of the actin network. Chemotaxing neutrophils and B cells are also not compromised by the absence of integrins, suggesting a general mechanism for leukocyte migration within the tissues. In a two-photon microscopy study, *Itgb2*$^{-/-}$ T cells were observed to migrate in a similar manner to wild-type T cells (Woolf et al., 2007). After migrating through the LN, lymphocytes exit through the cortical sinus vessels in the medulla region into the efferent lymph, making use of the sphingosine-1-phosphate receptor (Cyster, 2005) and the mannose receptor CD31 that binds L-selectin (Irjala et al., 2001). Lymphocyte exit from the LN is LFA-1- and α4β1-independent (Arnold et al., 2004; Lo et al., 2005). The surprising conclusion from these studies is that leukocytes do not need integrins to migrate in the LNs. Perhaps they do not need the adhering capacity of a high-affinity integrin to attach to a target when shear force is absent, such as in a LN.

An important function of the LN is to bring T cells and APCs together. In response to tissue damage through infection or injury, the LN receives input from the afferent lymph that drains the surrounding peripheral tissue and delivers stimulated APCs, such as DCs and soluble antigen, to the LN. Circulating lymphocytes then arrive in the LN from the blood and scan the newly arrived antigen-laden DCs. There is not yet much information about the use of integrins under these circumstances when the beginnings of an immune response is underway in vivo. However, the frequencies of contacts between DCs and different subsets of T cells are reported to be unaffected by the lack of LFA-1 (Westermann et al., 2005). Despite this, expression of ICAM1 on mature DCs is necessary for long-lasting contacts and, consequently, for the full activation of the CD8$^+$ cytotoxic T-cell subset (Scholer et al., 2008). In the absence of ICAM1, the T cells express low amounts of interferon-γ (IFN-γ), which leads to lack of activation and, subsequently, to their deletion. Mice that lack ICAM1 are unable to respond to an antigenic re-challenge. Thus, the interaction of ICAM1 on the DC surface, presumably with LFA-1 on the T cell, is required for setting up the complete programme of T-cell survival and full responsiveness.

In brief, integrins such as LFA-1 are essential for the crucial step of lymphocyte entry into the LN, but are not needed for intranodal migration. Once interacting lymphocytes find each other in the LN, integrins are dispensable for establishing their contacts, but the

evidence – which is so far limited – suggests that they are needed for maintaining the stable interactions that result in efficient signalling.

Interactions between leukocytes

Naive T cells undergo a priming process in the LN where they are exposed to foreign antigen that is displayed on DCs. In recent years, much information has come from imaging this process in vivo using two-photon microscopy (Cahalan and Parker, 2008; Halin et al., 2005; Lammermann and Sixt, 2008), and three distinct phases have been defined (Mempel et al., 2004). T cells initially enter the LN and undergo a series of short, sequential encounters with DCs for about 8 hours. More stable T-cell–DC contacts then form over a 12-hour window, followed by T-cell detachment and a third phase of high motility and proliferation.

The IS is the term used to represent the zone of contact between a lymphocyte and an APC (Fig. 6). The classical IS has a symmetrical bull's-eye appearance that forms as a result of the clustering of antigen by the TCR on the surface of the APC (Dustin, 2008b; Grakoui et al., 1999; Monks et al., 1998). A central region of concentrated TCRs is termed the 'central supramolecular activating cluster' (cSMAC). LFA-1 forms a peripheral adhesive ring (pSMAC) that stabilises the IS. This LFA-1 ring also prevents access of the phosphatase CD45 to the central synapse region by confining it to a third distal ring (dSMAC) (Graf et al., 2007). This is necessary to prevent dephosphorylation of key kinases such as ZAP-70, thus maintaining signalling. Other leukocytes such as B cells (Carrasco et al., 2004) and natural killer (NK) cells (Osman et al., 2007) also form the classical bull's-eye IS when encountering their targets. For B cells, adhesion to ICAM1 recruits LFA-1 into a peripheral ring as it does with T cells, and this interaction lowers the affinity of binding between the BCR and antigen that is needed to trigger B-cell activation (Carrasco et al., 2004).

The spatial segregation of the cSMAC and pSMAC molecules precedes IS formation. Both LFA-1 and the TCR are associated with the actin cytokeleton as separate microclusters at the periphery of the spread T cell, and are carried by retrograde flow towards the IS (Kaizuka et al., 2007). The absence of LFA-1 from the cSMAC might be due to the lack of actin in the centre or to an exclusion effect in this protein-dense region. Separate TCR microclusters contain the TCR, ζ-chain-associated protein of 70 kDa (ZAP-70) and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) (Varma et al., 2006; Yokosuka et al., 2005). They represent an early event in the T-cell response to antigen and move continually from the periphery of the spread cell towards the cSMAC. At the cSMAC, ZAP-70 and SLP-76 dissociate from the TCR clusters, leaving them to enter the cSMAC where signalling is terminated. The persistence of microclusters correlates with T-cell activation levels. If the microclusters are immobilised through the binding of integrin $\alpha4\beta1$ to ligand, then the interaction with ZAP-70 is extended, their migration towards the cSMAC is impeded and TCR signalling is prolonged (Nguyen et al., 2008).

B cells undergo a similar process in generating BCR microclusters (Depoil et al., 2008). They undergo a distinctive spreading and contraction response that enhances the formation of antigen clusters that are destined for the cSMAC (Fleire et al., 2006). Clustered antigen in the cSMAC can then be presented to T cells. There are similarities as well as differences as to how B cells make use of LFA-1 and integrin $\alpha4\beta1$ in the process of antigen gathering and associated signalling (reviewed by Arana et al., 2008).

Another model is the 'multifocal synapse', in which multiple cSMACs form across a single cell (Brossard et al., 2005) (Fig. 6).
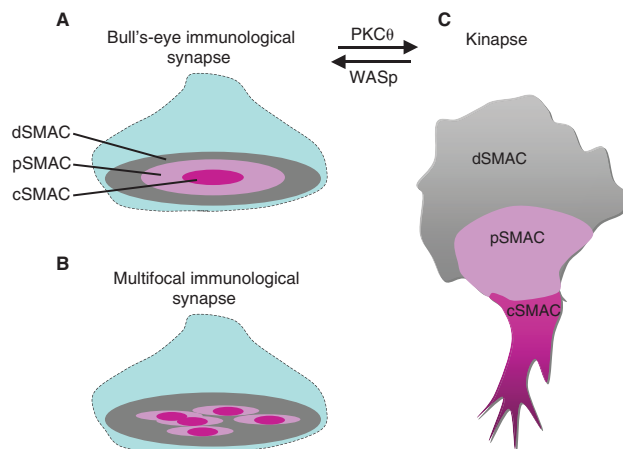

Fig. 6. Models of immunological synapses and a kinapse. (A)The bull's-eye model of an IS comprises three concentric zones. The cSMAC contains concentrated TCRs and PKCθ, and is where signalling is terminated. The surrounding pSMAC contains LFA-1 and talin. The dSMAC is enriched in the phosphatase CD45, which is excluded from the synapse to maintain optimal signalling. (B) The zones of the multifocal synapse are less-well defined. The synaptic interface contains many cSMACs, and the LFA-1-rich pSMAC is diffuse. (C) The kinapse represents a migrating cell that forms transient synapses. PKCθ mediates the transition from a stationary IS to a kinapse, whereas WASp mediates the reverse action. As with both the bull's-eye and multifocal models, signalling microclusters form in the distal area (dSMAC) and move through the pSMAC to the cSMAC, where signalling is terminated. In the polarised cell, the dSMAC corresponds to the lamellipodium, the pSMAC to the mid-region and the cSMAC to the uropod.

Although LFA-1 is still localised separately from the TCR, its distribution is more diffuse than the adhesive ring of the monocentric model. The nature of the IS can differ between closely related T cells (Thauland et al., 2008). One type of T cell, termed the T helper 1 (Th1) cell, forms a monocentric synapse, whereas cytotoxic Th2 cells form multifocal synapses. For Th1 cells, the targeted delivery of effector molecules is guaranteed by a ring of LFA-1–ICAM1 that provides a sealed barrier. By contrast, for Th2 cells that do not have such defined monocentric synapses, TCR signalling appears to last longer – that is, it is not so rapidly extinguished in the cSMAC. Granule-releasing NK cells also have monocentric synapses where LFA-1 forms a peripheral ring around a central cluster of receptors (Osman et al., 2007).

The bull's-eye model of the IS represents a highly stable synapse that lasts >30 minutes (Dustin, 2008b). However, T cells are often observed to adopt highly motile behaviour, scanning multiple DCs (Dustin, 2008b; Gunzer et al., 2000; Mempel et al., 2004). As they migrate across the surface of an APC, T cells form migratory synapses, also termed kinapses (Fig. 6) (Dustin, 2008a). The transition from stationary to migratory synapse is mediated by PKCθ, which promotes myosin II contraction at one side of the pSMAC, causing cell movement in that direction (Sims et al., 2007). Conversely, a concentric IS can be re-formed by WASp through the inhibition of myosin II contraction. Dustin has proposed the kinapse to contain the equivalent of the cSMAC at the leading edge of the cell, the pSMAC at the mid-region and the dSMAC at the uropod of the polarised T cell (Dustin, 2008b) (Fig. 6). It is of interest to compare this distribution to the zones of LFA-1 activity that we detect on a migrating T cell (Fig. 4). In particular, the pSMAC of the kinapse hypothetically corresponds to the region that we define as the focal

zone in our migration model. High-affinity LFA-1 is expressed in the focal zone, which is in keeping with the formation of a strongly adhesive LFA-1 bond in the pSMAC.

These models focus on an adhesive function for LFA-1; however, its role might be more active. Signalling through LFA-1 can involve the remodelling of F-actin (Porter et al., 2002), and an LFA-1-induced 'actin cloud' forms at the IS, which is associated with increased protein phosphorylation and a subsequent increased sensitivity to antigen (Suzuki et al., 2007). Any contribution of integrin signalling at the IS needs to be further investigated.

Myeloid cells (monocytes and neutrophils)

The myeloid cells known as neutrophils and monocytes are the first leukocytes to arrive at the site of an infection or injury (Ley et al., 2007; Petri et al., 2008). One of their roles is to prepare the site for the later arrival of the lymphocytes, but they are primarily involved, at least initially, in the process of engulfment and destruction of the invading pathogens. Myeloid cells use LFA-1 and α4 integrins in a manner that is similar to other leukocytes to negotiate the vasculature. However, another β2 integrin, Mac-1, comes into play. Neutrophils use LFA-1 to attach to the vasculature and Mac-1 to crawl to an endothelial junction, leading to emigration (Henderson et al., 2001; Phillipson et al., 2006). Proof for this role for Mac-1 comes most convincingly from the use of *Itgam*$^{-/-}$ neutrophils that attach very well to the vasculature but do not crawl. Although neutrophils display this behaviour after an inflammatory stimulus, a subset of monocytes patrol the vessels continuously in an LFA-1-dependent fashion (Auffray et al., 2007). Why neutrophils use Mac-1 whereas monocytes and lymphocytes use LFA-1 for their promigratory behaviour remains to be determined.

For their phagocytic activities, myeloid cells use Mac-1 and a third β2 integrin, p150,95 (Mayadas and Cullere, 2005). After binding to its ligands ICAM1 or fibrinogen, Mac-1 can deliver both survival signals (Whitlock et al., 2000) and pro-apoptotic signals (Zhang et al., 2003) to neutrophils. This has lead to the idea that Mac-1 controls the life span of the neutrophil. Survival is promoted by Mac-1 in conjunction with signalling that is activated by the pro-inflammatory chemokines that are encountered by the neutrophil as it travels towards the region of infection. However, after engulfment of pathogen by the neutrophil, Mac-1 switches on its apoptotic signal, leading to the activation of the cysteine proteases called caspases 1, 3 and 8 (Mayadas and Cullere, 2005). Survival and apoptosis signals are both controlled by the activation of the Ser-Thr kinase Akt. Understanding the role of Mac-1 in these processes in vivo has been extended through the use of *Itgam*$^{-/-}$ mice. These mice have an increased number of neutrophils at inflammatory sites, which is associated with reduced neutrophil phagocytosis and apoptosis (Coxon et al., 1996). These results confirm an essential role for Mac-1 in the programmed elimination of neutrophils that have already phagocytosed their target pathogens, in a process called phagocytosis-induced cell death.

The crucial role for myeloid cells in controlling infections is evident in patients who are affected by LAD-III (see above) and also LAD-I syndromes. LAD-I is caused by a germline point mis-sense or non-sense mutation in the gene that encodes the integrin-β2 subunit, causing faulty α-β subunit association (Hogg and Bates, 2000). For this reason, LAD-I patients fail to express any of the four members of the β2-integrin family. As in LAD-III, this leads to defects in the ability of leukocytes to leave the circulation, as well as in pathogen phagocytosis and neutrophil apoptosis. Both LAD-1 and LAD-III patients experience life-threatening infections unless they receive bone-marrow transplants.

Concluding comments

Studies that used LFA-1-, β2- and ICAM1-null mice have demonstrated the necessity for β2 integrins to achieve a successful immune response. It has therefore been surprising to learn that leukocytes migrate without using any integrins once they have gained entry into a LN. It has also been unexpected that mechanical stress, such as that provided by blood flow, is potentially a major factor in governing conformational change in integrins. Thus, integrins might perform as adhesion receptors only when a tight grip is needed, such as when leaving the blood stream to enter tissues. It is not yet clear where else they are needed. For example, there has been no analysis yet of whether they have a role in the leukocyte interactions in vivo that are the essence of an ongoing immune response. We also do not know whether the concept of the IS with its ring of LFA-1 is valid in vivo. Investigating these issues will be certain to throw up more surprises involving these fascinating receptors.

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