The insider’s guide to leukocyte integrin signalling and function

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Abstract | The activation of leukocyte integrins through diverse receptors results in transformation of the integrin from a bent, resting form to an extended conformation, which has at least two states of ligand-binding activity. This highly regulated activation process is essential for T cell migration and the formation of an immunological synapse. The signalling events that drive integrin activation are complex. Some key players have been well-characterized, but other aspects of the signalling mechanisms involved are still unclear. This Review focuses on the integrin lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin), which is expressed by T cells, and explores how disparate signalling pathways synergize to regulate LFA1 activity.

Integrins are a large family of proteins that function as adhesion molecules and as receptors that are capable of transmitting information about both the chemical and mechanical properties of the external environment into the cell (a process termed outside-in signalling). They are transmembrane heterodimers, each consisting of one α-subunit and one β-subunit, of which a total of 18 α-subunits and 8 β-subunits have been identified. Integrins are unusual in that they exist on the cell surface mainly in an inactive form until they receive stimulating signals from other receptors (via inside-out signalling), and also because the end result of integrin activation is a dramatic shift in integrin conformation from a bent, compact shape to an extended, open one (FIG. 1). The binding of cytoplasmic proteins to the α- and β-subunit carboxy-terminal tails is an essential part of the activation process, as these interactions stabilize the extended integrin conformation and provide connections to the cytoskeleton.

As other aspects of integrin function have been recently reviewed elsewhere1–4, we focus here primarily on T cells and their predominant integrin, lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin), although we incorporate information gained from other integrins and leukocytes as appropriate. We first discuss, with some speculation, recent insights into the convergent inside-out signalling pathways that lead to LFA1 activation, and we then focus on the outside-in signalling that is controlled by LFA1 itself and directed back into the T cell.

Where is integrin function important?

There are two distinct T cell activities in which the β2 integrin LFA1 is implicated. The first is cell arrest and migration on surfaces expressing the LFA1 ligand, intercellular adhesion molecule 1 (ICAM1), and the second is in making contact with antigen-presenting cells (APCs), with subsequent formation of immunological synapses5. The failure to activate integrins can have life-threatening consequences, as exemplified by the leukocyte adhesion deficiency (LAD) disorders (BOX 1). T cell arrest occurs primarily on postcapillary venules at sites of infection or injury and on high endothelial venules in the lymph nodes. Signalling through other cell membrane receptors activates LFA1, enabling leukocytes to withstand the shear force of blood flow. The process involves the formation of transient attachments to the vasculature that cause the leukocytes to slow down (through fast and slow rolling) and ultimately leads to their firm adhesion and transmigration into the lymph node or injury site6,7. There is mounting evidence that integrins have no significant role in the migration of non-stimulated recirculating lymphocytes once these cells are within the shear-free environment of the lymph node8,9.

By contrast, when T cells interact in vitro with ICAM1-expressing antigen-laden APCs to form an immunological synapse, LFA1 forms a circular adhesive ring around the T cell receptor (TCR) cluster, and this is termed the peripheral supramolecular activation cluster (pSMAC)5. The strength of this contact reduces the amount of antigen necessary for T cell stimulation. To date, there are only a few studies suggesting that this same role is essential in vivo in mice undergoing immune challenge. T cell priming defects have been noted in LFA1-deficient CD4+ T cells, which produce reduced levels of interferon-γ (IFNγ) and interleukin-2 (IL-2), suggesting a faulty interaction with APCs8. Icam1−/− mice...
have reduced memory CD8+ T cell activity, and the functions of both effector and memory CD8+ T cells are reduced in LFA1-deficient mice. These transgenic models point to a positive role for LFA1 in immune responses in vivo, but more investigation is needed to pinpoint where it is important.

**Structural features of integrin activation**

The signalling events leading to integrin activation that have been most intensively investigated in leukocytes are those induced by chemokine receptors, the TCR or selectin ligands. This agonist signalling is ultimately sensed by the cytoplasmic tails of integrin α- and β-subunits and, as a direct consequence, the bent heterodimer flips open into an extended conformation (in a manner analogous to the opening of a jack knife). This conformational change exposes the globular headpiece, which, in integrins like LFA1, contains a ligand-binding α-subunit I domain (αI domain) (FIG. 1a). In fully activated integrins, the hybrid domain of the β-subunit leg has swung away from the α-subunit and the headpiece is in the ‘open’ conformation. The opening of the headpiece represents the final stage of integrin activation and occurs as a result of the βI domain pulling down on the α7 helix in the αI domain. This high-affinity form exists only transiently unless stabilized by ligand binding.

Recent structural studies have shed some light on how these dramatic conformational changes can occur. The three distinct integrin forms exist in equilibrium on the cell membrane and, in the absence of any restraint on conformation, shifts in this equilibrium can be caused by relatively small intracellular changes. Early stages of activation can give rise to various extended conformations of LFA1, in which the legs can be open or closed and the β-subunit hybrid domain may or may not be swung out (FIG. 1b). On-rates for ICAM1 binding vary considerably and are greater when there is headpiece ‘opening’. This is consistent with the observation that lateral forces from shear flow or buffeting effects, which have been predicted from modelling to result in leg separation, dramatically increase the rate of integrin activation.

However, in the absence of applied force perpendicular to the membrane, the final pairing between ICAM1 and the various activation states of LFA1 is unstable. It is only when external force is applied via binding of immobilized ICAM1 that full activation takes place, through the opposing forces supplied by ICAM1 and the cytoskeleton. The high stability of this complex is a result of catch bond formation between ICAM1 and the αI domain, and also possibly between the αI and βI domains; these bonds stiffen the β-subunit leg domain and result in stable, high-affinity LFA1 (REF. 13).

**A reductionist view of integrin activation**

Using accumulated information and a model of integrin activation in Chinese hamster ovary (CHO) cells, Ginsberg and colleagues reconstructed a core inside-out signalling pathway, in which activation of platelet integrin αIIbβ3 to its high-affinity form was
Box 1 | LAD-III and the importance of kindlin 3

The essential requirement for integrins in immune function is highlighted by the features of several severe immunodeficiency diseases. The leukocyte adhesion deficiency (LAD) disorders types I–III have the common phenotype of a lack of immune responsiveness, leading to life-threatening infections, but the causes differ for each disorder48. The leukocytes of patients with LAD-I lack expression of β2 integrins owing to β2 subunit mutations; in LAD-II, they lack fucosylated selectin ligands as a result of a dysfunctional GDP–fucose transporter; and in LAD-III, leukocytes express integrins normally but have a blockade in the signalling pathway that leads to integrin activation. Glanzmann’s thrombasthenia is another haematopoietic disorder, in which mutation in the genes encoding the αIIb or β3 integrin subunits causes loss of expression of this integrin on platelets, resulting in severe bleeding.

The cause of LAD-III has been a mystery for the last decade since these patients were first identified. LAD-III is an autosomal recessive disorder, and patients have been identified from Turkish, Arabic, Maltese and African-American backgrounds47–50. The disease, which has clinical features of both LAD-I and Glanzmann’s thrombasthenia, is marked by recurrent life-threatening infections, increased bleeding times and sometimes also osteoporosis. To date, the only effective treatment is bone marrow transplantation, which restores immune function and cures the osteoporosis48,51. The LAD-III mutant gene was identified by us and other groups to be KINDLIN3 (also known as FERM73–50). This identification was inspired by the characteristics of Kindlin3−/− mice, which have adhesion-deficient platelets and leukocytes39. Kindlin 3, which is specifically expressed in cells of haematopoietic origin, belongs to a small family (comprising kindlins 1, 2 and 3) of scaffold-type molecules. These proteins essentially comprise a FERM domain intersected with a plekstrin homology (PH) domain. Different classes of KINDLIN3 mutations have been identified, but all result in decreased mRNA expression and loss of protein. The final proof that kindlin 3 loss is responsible for the adhesion defect came from the re-introduction of wild-type KINDLIN3 cDNA into cells from a patient with LAD-III, as this led to reversal of the disease phenotype in vitro49,50. Interestingly, this disease is not restricted to humans, but also occurs in dogs (in which it is termed canine LAD)51.

The signalling pathways leading to LFA1 activation have been investigated using three different modes of stimulation: first, chemokine signalling (through G protein-coupled receptors (GPCRs) that are expressed by all leukocytes); second, TCR triggering (usually through monoclonal antibody-induced crosslinking of TCR-associated CD3 chains); and third, selectin binding to the ligands P-selectin glycoprotein ligand 1 (PSGL1), CD44 and E-selectin ligand 1 (ESL1) on neutrophils41. Here, we discuss chemokine-mediated signalling to LFA1 on T cells, as this has been most thoroughly investigated, and we compare it with TCR signalling when possible. With regard to the TCR, it has been difficult to untangle the signals involved exclusively in integrin activation from other TCR-mediated pathways, but there are parallels with chemokine-mediated signalling. Selectin-mediated signalling models have largely focused on the behaviour of LFA1 on neutrophils. We also indicate where we incorporate mechanisms that have been investigated for other integrins, but not so far for LFA1.

Inside-out signalling events in leukocytes

Recently, a clearer picture has emerged of the molecular events that cooperate in T cells to bring about the dramatic conversion of integrins from the bent conformation to the extended high-affinity form. There has been a particular focus on LFA1 because of its essential role in leukocyte function and, in part, because of the availability of monoclonal antibodies that detect the different conformations of human LFA1 (although unfortunately such antibodies are not available for the mouse receptor). KIM127 is a widely used monoclonal antibody that recognizes a β2 subunit epitope (on epidermal growth factor (EGF)-like domain 2) that is cryptic on bent LFA1, but exposed when the integrin extends38 (Fig. 1a). High-affinity LFA1 is detected using monoclonal antibodies specific for either the 327C or the 24 epitope. The location of the 24 epitope on the specificity-determining loop of the β2 subunit I-like domain has recently been structurally confirmed19,20 (Fig. 1a).

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Inside-out signalling: diverse events lead to LFA1 activation

Chemokines, such as CC-chemokine ligand 19 (CCL19) and CCL21, are expressed by the vasculature following an inflammatory stimulus and signal through GPCRs on leukocytes. The result of this signalling is the extension of LFA1, and this enables it to bind to its ligand, ICAM1, with intermediate affinity22–24. In the presence of shear forces, conversion of LFA1 to its fully activated form occurs within milliseconds to enable firm arrest of T cells22.

Key players in this signalling pathway are RAP1 and its guanine nucleotide-exchange factors (GEFs), talin, kindlin 3 and phosphatidylinositol 4-phosphate 5-kinase type 1 (PIP5K1y87) (Fig. 2). Moreover, it is now recognized that recycling vesicles are of major
A cytoskeletal protein of 270 kDa. It consists of an N-terminal globular head domain (50 kDa) that contains four FERM subdomains and a longer C-terminal rod domain (220 kDa) that includes actin and vinculin binding sites. The FERM3 subdomain contains a phosphotyrosine-binding (PTB) domain that binds the β-subunit of integrins at the membrane-proximal NPXY site. The talin FERM domain has unique properties compared with other PTB domain-containing proteins, as it has a second binding site on the β-subunit tail in closer proximity to the membrane.

Figure 2 | Essential stages of inside-out signalling. The pathway is subdivided into four steps for the sake of presentation, but may not be representative of a temporal sequence. a | A crucial step is RAP1 activation via its guanine nucleotide-exchange factors (GEFs) CALDAG-GEF1 (Ca2+ and diacyglycerol-regulated guanine nucleotide exchange factor 1; also known as RASGRP2) and C3G (also known as RAPGEF1). T cell receptor (TCR) signalling activates phospholipase Cγ1 (PLCγ1), which generates the Ca2+ and diacylglycerol (DAG) necessary for CALDAG-GEF1 activation. Chemokines stimulate other PLCs. TCR triggering also activates a WAVE2–ARP2/3–ABL complex that associates with and phosphorylates C3G–CRKL (which is inhibited by the E3 ubiquitin ligase CBLB). b | Another important step is the recruitment of recycling vesicles that transport lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin) and RAP1, together with other LFA1-activating complexes, to the plasma membrane. The conformation of vesicular LFA1 is unknown. c | The activation of phospholipase D1 (PLD1) via chemokine stimulation leads to the generation of phosphatidic acid. Phosphatidylinositol-4-phosphate 5-kinase type 1γ87 (PIP5K1γ87) is subsequently activated, resulting in the generation of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). Talin binds to PIP5K1γ87 and to PtdIns(4,5)P2. d | Prior to LFA1 extension, talin and possibly kindlin 3 (also known as FERM3) are at the plasma membrane, in position for binding to LFA1. Talin is reported to be already weakly bound to LFA1 at this stage. Kindlin 3 binding may be necessary for the extension of LFA1. ADAP, adhesion- and degranulation-promoting adaptor protein (also known as FYB); ARP2/3, actin-related protein 2/3; CRKL, CRK-like protein; MST1, mammalian STE20-like protein kinase 1 (also known as STK4); RAPL, regulator of adhesion and cell polarization enriched in lymphoid tissues (also known as RASSF5); RIAM, RAP1–GTP-interacting adaptor molecule (also known as APBB1IP); SKAP55, SRC kinase-associated phosphoprotein of 55 kDa (also known as SKAP1); WAVE2, WASP-family verprolin homologous protein 2.

The RAP1 GEFs. The switching of inactive GDP-bound RAP1 to active GTP-bound RAP1 occurs through interaction with appropriate GEFs. Thus, a crucial step on the path to integrin activation is the delivery of RAP1 GEFs to the membrane and their activation. The candidate GEFs that have been identified so far in T cells are C3G (also known as RAPGEF1) and CALDAG-GEF1 (Ca2+ and diacylglycerol-regulated guanine nucleotide exchange factor 1; also known as RASGRP2).
There is evidence for involvement of CALDAG-GEF1 in RAP1 activation following signalling in response to TCR, chemokine receptor and platelet-activating agonists25–27 (FIG. 2a). Silencing of CALDAG-GEF1 in primary human T cells using small interfering RNA (siRNA) reduces RAP1 activity and disrupts LFA1-mediated adhesion to ICAM1 (REF. 26). The important role of CALDAG-GEF1 is further emphasized in Cald1−/− mice, in which there is also a failure of RAP1 activation in response to many agonists, as well as substantial defects in the function of β1, β2 and β3 integrins in neutrophils and platelets28. However, mouse lymphocytes do not express CALDAG-GEF1, indicating a role for an alternative RAP1 GEF.

The second RAP1 GEF, C3G, forms a complex with CRK-like protein (CRKL), and siRNA silencing of either C3G or CRKL causes a reduction in T cell adhesion to ICAM1 and to fibronectin following TCR crosslinking, implicating C3G in adhesion mediated by both β2 and β1 integrins, respectively29 (FIG. 2a). A converse experiment demonstrated that overexpression of C3G enhances LFA1-mediated adhesion following TCR crosslinking in Jurkat T cells29. Transport of the CRKL–C3G complex is dependent on a mechanism involving the tyrosine kinase ABL in association with the cytoskeletal regulatory proteins WSP-family verprolin homologous protein 2 (WAVE2; also known as WASF2) and actin-related protein 2/3 (ARP2/3)30.

Phospholipase C (PLC) proteins are involved in the delivery of these GEFs to the membrane. Following either TCR or chemokine receptor stimulation, PLC generates Ca2+ and diacylglycerol (DAG), which are required for the activation of CALDAG-GEF1 and, subsequently, of RAP1 (REFS 25,26). PLCγ1 is activated following TCR stimulation, but chemokine receptor signalling appears to trigger other PLCs25. More information is needed to understand how the GEFs reach the plasma membrane. CRKL–C3G may be present in the cytosol rather than in RAP1-containing vesicles (see next section), indicating a separate mode of arrival at the membrane29.

Delivery of RAP1- and LFA1-containing vesicles to the leading-edge plasma membrane. The presence of the GTPase RAP1 at the plasma membrane is a key element in LFA1-activating signalling originating from both chemokine receptors and the TCR. Expression of constitutively active RAP1 enhances the affinity and clustering of LFA1 and promotes T cell migration and interaction with antigen-loaded APCs30,31. These same LFA1 activities can be blocked by inhibiting RAP1 activity, through overexpression of dominant-negative RAP1 or of the GTPase activating proteins (GAPs) RAP1GAP or signal-induced proliferation-associated protein 1 (SPA1), or by silencing RAP1 itself26,31.

RAP1 contains a C-terminal CAAL (cysteine-aliphatic-aliphatic-leucine) motif that is irreversibly modified by a geranylgeranyl lipid group, which dictates membrane localization and makes vesicle transport feasible. Vesicles containing RAP1 are delivered to the T cell membrane29,32,33 (FIG. 2b), although whether this occurs in response to GPCR and TCR signalling or is part of constitutive trafficking still needs to be determined. The vesicles contain both early endosomal markers (such as early endosome antigen 1 (EEA1) and Rab5A) and late endosomal markers (such as Rab11), which are typical of endocytic and exocytic recycling vesicles, respectively29,32,33. Although RAP1 is detected in intracellular vesicles29,32,33, a recent report suggests that it is constitutively bound to the plasma membrane34. However, supporting the recycling option is the evidence that expression of a dominant-negative form of Rab11 blocks RAP1 delivery to the membrane, linking RAP1 transport and localization with vesicle exocytosis35.

A further issue concerns the identity of the compartment where RAP1 is activated — is RAP1 already in its active GTP-bound form in the vesicles or does the conversion from inactive GDP-bound to active GTP-bound RAP1 occur at the cell membrane? A green fluorescent protein (GFP)-conjugated reporter for RAP1 activity was observed only in leading-membrane ruffles of TCR-activated Jurkat T cells, suggesting that RAP1 activation occurs at the cell membrane36,37. By contrast, data from chemokine-activated primary T cells show that the function of the vesicular RAP1–MST1 complex that contributes to LFA1 transport to the leading-edge membrane (see next section) is dependent on active GTP-bound RAP1 (REF. 33). A possible explanation might be that the crucial assembly of the GTP-bound RAP1–RPL–MST1 module occurs at the cell membrane as part of the vesicle-docking process.

What other cargo do the vesicles contain? Kinashi and colleagues showed that the RAP1 downstream effectors RAPL and MST1 are also components of the recycling vesicles that contain RAP1 (REF. 1). RAPL acts as an LFA1 transporter, delivering this integrin to the leading edge of migrating T cells and to the pSMAC at T cell–APC contact zones33,35. The serine/threonine kinase MST1 is a downstream effector of RAP1–RAPL and interacts with RAPL via its coiled-coil domain33. A module that contains the adaptor proteins RIAM, SKAP55 and ADAP (adhesion- and degranulation-promoting adaptor protein; also known as FYB) has also been demonstrated to convey active RAP1 to the membrane28–30 (FIG. 2b). Recently, Rudd and colleagues have provided a link between this module and the RAP1–RAPL–MST1 complex by demonstrating that vesicular SKAP55 controls the localization of RAP1–RAPL at the membrane41. Like MST1, SKAP55 binds the RAPL coiled-coil domain and thus it can compete with MST1, suggesting the existence of two types of RAP1–RAPL complex. The binding of ADAP to SKAP55 prevents SKAP55 proteolysis, so it is reasonable to suggest that ADAP is also a component of the SKAP55-containing complexes. Thus, RAP1 may be associated with RAPL–MST1, RIAM–SKAP55–(ADAP) or RAPL–SKAP55–(ADAP)34,36–38. There are probably additional versions of these core modules that may also be carried in RAP1-containing vesicles.
Its product, phosphatidic acid, GTPases and is activated. Phospholipase D1 functions

Box 2 | Negative regulators of LFA1 activity

Lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin) must switch between active and inactive conformations to function effectively. Mutation of LFA1 to create a constitutively active high-affinity conformation causes T cells to adhere without releasing, giving a lack-of-motility phenotype in vivo, resembling the functional failure of LFA1-deficient T cellsα38,39. We know too little about the processes involved in integrin inactivation, but there is an increasing catalogue of negative regulators of LFA1 activity in T cells. A provisional list includes the GTPase CDC42, which inhibits the activation of high-affinity LFA1 (REF. 40); the GTPase RHOH, which is speculated to interfere with RAP1 activationα26; the E3 ubiquitin ligase CBLB, which inhibits the active RAP1 guanine nucleotide-exchange factor activity of the C3G–CRKL (CRK-like protein) complexα27; and the cytosolic protease calpain, which promotes de-adhesion, possibly by cleaving the talin head from its rod domainα28. Phosphatidylinositol-4-phosphate 5-kinase type 1γ90 (PIP5K1γ90) is a second PIP5K1γ isoform that differs from PIP5K1γ87 by a 28-amino-acid C-terminal talin-binding extension. It is located in the uropod and is speculated to sequester talin as part of a de-adhesion mechanismα31. Removal of a so-far-unidentified RAP1-dependent restraint causes instant firm adhesion to ICAM1 (REF. 66). This step does not cause LFA1 extension, implying that the bent form can have substantial ligand-binding properties when correctly primed. Finally, phosphorylation at key sites on the integrin β-subunit can have an inhibitory effect. Tyrosine phosphorylation of the conserved NPXY site on β3, β1A and β7 subunits favours the binding of docking protein 1 (DOK1), thereby interfering with talin bindingα32. T cells express DOK proteins, including DOK1, so it is possible that talin binding to β2 integrins is similarly regulatedα33. Although individual mechanistic details are known, how these disparate inhibitory factors affect the process of turning integrin activity on and off needs much further investigation. There is at present no unifying scheme that incorporates even a few of them, but it is also possible that these regulators act individually as local ‘checks and balances’.

Talin. In resting T cells, talin is largely cytosolic, and its delivery to the membrane is another major feature of inside-out signalling. In the CHO cell model for αlβ3 integrin activation, the scaffold protein RIAM recruits talin to the membrane, but whether a similar process occurs during LFA1 activation in T cells has still to be investigatedα34. Talin binds directly to PtdIns(4,5)P2 via four positively charged lysine residues in PIP5K1γ87 and α4β1 integrin. This binding interferes with the talin head-to-rods auto-inhibited conformation, causing some unfoldingα34. In resting T cells, a proportion of talin is bound to the GEF VAV1 in a manner that appears to limit the full binding potential of talinα35,36 (FIG. 2d). However, this talin–VAV1 complex appears to be already weakly bound to inactive α4β1 integrinα36. The membrane-proximal NPXY site in the cytoplasmic tail of the integrin β-subunit has been identified as the major binding target of the talin FERM domainα34, but whether this site is accessible under these circumstances is uncertain.

A speculation is that this membrane positioning of talin, potentially in complex with VAV1 and aligned for optimal interaction with the integrin β-subunit tail, is set up by the events of inside-out signalling, but is held in reserve until the next stage of high-affinity integrin generation.

Kindlin 3. Kindlin 3 is the protein recently discovered to be mutated in patients with LAD type IIIα37–39 (BOX 1). Kindlin 3 binds to the integrin membrane-distal NXXY site, which has the sequence NPLF in the LFA1 β2 subunit tail, and this substantially aids the binding of talin to LFA1 (REF. 3). One idea is that occupation by kindlin 3 of the more accessible C-terminal binding site remodels the β2 tail to optimize the binding potential of the talin siteα36,37 (FIG. 2d). Another option is that kindlins indirectly enhance the binding of talin to integrins as follows. An event distinct from those discussed so far is that an inactive integrin may be maintained in this state by negative regulation (BOX 2). An important restraint for several integrins is filamin, a cytoskeletal protein that occupies the same site as talinα34. A possibility is that a complex of kindlin 3 and the filament-binding protein migfilin (also known as FBLP1) uncouples filamin from this binding site, leaving it free for talinα34,36. T cells do express migfilin (I.P. and N.H., unpublished observations) and the β2 integrin subunit binds filamin, although with low affinityα34, so this mechanism of action for kindlin 3 could contribute to the enhancement of LFA1 activity in T cells.

Following chemokine or phorbol ester stimulation, LFA1 in T cells from patients with LAD-III fails to extend, suggesting an important role for kindlin 3 at this stageα34. Thus, cooperative binding of kindlin 3 and talin, or potentially kindlin 3 binding alone, may be sufficient

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to disturb the αβ tail connections and shift integrin conformation from the bent form to the extended form that represents intermediate-affinity LFA1. This extension of LFA1 represents the end stage of inside-out signalling.

**Outside-in signalling events in leukocytes**

Integrin outside-in signalling refers to integrin-mediated signalling that leads to adhesion strengthening and the final stage of integrin activation. Extended LFA1 with an exposed but ‘closed’ headpiece is able to engage its ligand with intermediate affinity. As we describe, LFA1–ICAM1 binding initiates an integrin-proximal tyrosine kinase signalling step. We speculate that the products of these signalling events cooperate with talin and the RAP1 effector RAPL that are already located at the plasma membrane as a result of inside-out signalling (Fig. 3a). All are in position to bring about the final stage of integrin activation, in which a further shape change in the headpiece of LFA1 allows high-affinity binding to ICAM1 while the T cell is engaged with an appropriate target cell or migrating on the vasculature.

**SRC and SYK family tyrosine kinases.** SRC family tyrosine kinases are constitutively associated with the integrin β-subunits in platelets and myeloid cells. Haematopoietic cell kinase (HCK), FGR and LYN tyrosine kinases have similar but partially redundant roles56. Following integrin binding to ligand, the SRC kinases autophosphorylate and then phosphorylate the nearby immunoreceptor tyrosine-based activation motif (ITAM)-containing proteins DAP12 and/or Fc receptor γ-chain (FcRγ). The phosphorylated ITAMs act as docking sites for the tandem SH2 domains of spleen tyrosine kinase (SYK)57,58. SRC kinases then phosphorylate SYK, thereby initiating a cascade of downstream signalling.

In T cells, the SRC and SYK family tyrosine kinases LCK and ζ-chain-associated protein kinase of 70 kDa (ZAP70), respectively, are involved in the outside-in signalling step24 (Fig. 3b). Blocking ZAP70 function using siRNA or pharmacological inhibitors prevents the conversion to high-affinity LFA1, indicating that active phosphorylated ZAP70 is required for the conformational shift that changes the ligand-binding affinity of LFA1 from intermediate to high. Unlike SRC–SYK signalling in platelets and myeloid cells, the kinases LCK and ZAP70 are already associated with LFA1 in T cells and LCK is 'phosphorylation primed', possibly as part of a T cell pool of phosphorylated LCK24,59. As these kinases are further phosphorylated following LFA1 binding to ICAM1, they are early — if not the first — mediators of outside-in signalling.

**The importance of talin.** Talin is of fundamental importance for the formation of high-affinity integrins56,60. Experiments using isolated talin and monomeric αIIbβ3 integrin on nanodiscs have shown that talin binds...
directly to the β-subunit, causing integrin extension and activation. Moreover, the α- and β-subunit tails of LFA1 undergo separation when exposed to the overexpressed talin FERM domain. Expression of talin is essential for the integrity of the LFA1 high-affinity focal zone in T cells migrating on ICAM1 (REF 63), and Talin 1−/− mice are unable to make LFA1-mediated contacts between T cells and APCs.

There are several stages of talin binding that lead to its full interaction with integrins. Already mentioned is the binding of talin to PtdIns(4,5)P2 at the membrane, a stage that occurs during inside-out signalling (FIG. 5a). In the subsequent binding steps, which lead to the generation of fully stabilized high-affinity integrins, talin occupies both the NPXY site and, uniquely among phosphotyrosine-binding (PTB) domain-containing proteins, another site on the β-subunit tail in closer proximity to the membrane. The molecular detail of how this stage is brought about is suggested by the findings of Garcia-Bernal and colleagues, who used the activation of α4β1 integrin in T cells as their model, as discussed below.

A role for phosphorylated ZAP70 in delivering talin to integrins. Similarly to the signalling cascade downstream of LFA1, the sequence of events that follow α4β1 integrin ligand binding require the activation of ZAP70. As mentioned, the talin–VAV1 complex assembles at the T cell membrane in response to local PtdIns(4,5)P2 generation, which is stimulated by chemokines. This alignment of talin at the membrane favours an initial weak binding to the β1 subunit tail. Phosphorylation of VAV1 by active ZAP70 leads to dissociation of the talin–VAV1 complex, releasing talin to occupy its sites on the β1 tail and thus completing the conversion of α4β1 integrin to its high-affinity form (FIG. 5b). It is predicted that similar events occur when LFA1-associated ZAP70 is activated, as VAV1 is also a phosphorylation target when T cells are migrating on ICAM1 (REF 24).

Thus, synchrony of action between the products of inside-out signalling (FIG. 5a) and ZAP70 activation, which is directly controlled by LFA1–ICAM1 binding (FIG. 5b), has the consequence of bringing about the final stage of talin binding that yields a fully active high-affinity integrin (FIG. 5c).

RAP1 and RAPL: it isn’t all about talin. Recently, the Kinashi group demonstrated that RAP1-activated RAPL is required for the second phase of LFA1 adhesion strengthening. As mentioned, the RAPL binding site on the LFA1 α-subunit tail features two lysine residues (K1097 and K1099) that are close to the membrane, as well as the conserved GFFKR motif. Therefore, for LFA1, it seems reasonable to suggest that RAPL might contribute to the subsequent allosteric changes that separate the α- and β-subunit cytoplasmic tails and lead to the high-affinity conformation. LFA1 may therefore be stabilized not only by talin binding to the β2 subunit, but also by RAPL binding to the opposing LFA1 α-subunit (FIG. 5c).

Kindlin 3 shows up again. There is strong evidence that kindlin 3 has a vital role in the inside-out phase of the signalling that leads to integrin activation. However, there are substantial clues that it is also involved in outside-in signalling (FIG. 3c). Kindlin3−/− platelets use αIIbβ3 integrin to adhere when their inside-out signalling defect is reversed by treatment with Mn2+, but they do not spread, suggesting that the turnover of αIIbβ3 integrin is prevented, probably owing to faulty cytoskeletal attachments. Similarly, lymphocytes that were transfected with KINDLIN3 cDNA containing an unusual LAD-III mutation (that was identified in an African-American patient with the disorder) were adhesion-competent but migration-incompetent, again implying defects in downstream connections.

As kindlins are essentially scaffold proteins that lack any intrinsic activities, they need to recruit other proteins in order to function. The family members kindlin 1 and kindlin 2 are expressed in non-haematopoietic cells and bind integrin-linked protein kinase (ILK) and migfilin, which can both lead to cytoskeletal attachments. Which partners kindlin 3 might co-opt in T cells and other haematopoietic cells has yet to be determined.

Effect of outside-in signalling on LFA1 function. What are the effects of high-affinity LFA1 on T cell function? First, during migration, high-affinity LFA1 is localized to the mid-cell lamella focal zone or focal dots and functions to anchor the cell, while intermediate-affinity LFA1 operates at the more dynamic leading edge sampling surface contacts. Second, T cell arrest on the vasculature under flow conditions occurs in milliseconds. Control of the high-affinity conformation by LCK–ZAP70 that is already in complex with LFA1 helps to explain how arrest can happen so quickly. The LFA1–LCK–ZAP70 complex, which possibly also contains other proteins such as talin and kindlin 3, is localized to the lamella of the polarized T cell. With the complex ‘ready to fire’, this region of the cell is therefore equipped to initiate rapid, stable contact with ICAM1 and the subsequent activation of signalling events that promote cell spreading and motility. Such fast action would be most beneficial during an ongoing immune response, when it is essential for T cells to respond rapidly to stimulated vasculature at sites of infection or injury.

Several unique features of TCR signalling. TCR-induced activation of LFA1 would be expected to differ from chemokine- and selectin-mediated signalling, as it takes place within the protected environment of a lymph node or tissue and therefore in the absence of shear force, which is influential in generating high-affinity LFA1 following chemokine signalling. It has been difficult to detect LFA1 affinity changes following crosslinking with CD3-specific mAbs, the assay most frequently performed to mimic TCR activation. In addition, a recent report shows that signals from the TCR appear to ‘prime’, but not extend, the bent form of LFA1, and it is only when LFA1 binds to immobilized ICAM1 on an APC that headpiece opening, which is characteristic of high-affinity LFA1, occurs. These findings imply that the bent form of LFA1 has ligand-binding capability. Another explanation is that a pool of already extended LFA1 binds ICAM1 and
Box 3 | LFA1 microclusters and the cytoskeleton

Integrin avidity, lateral mobility or ‘clustering’ greatly increase the ability of lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin) to attach strongly and withstand the shear stress inflicted by the flow of circulating blood. An accepted view is that microclustering and association with the cytoskeleton are a consequence of LFA1-mediated outside-in signalling. Primary T cells migrating on endothelium display small dispersed ‘focal dots’ of high-affinity LFA1 scattered at the interface with intercellular adhesion molecule 1 (ICAM1). These clusters coalesce into a focal zone of high-affinity LFA1 when T lymphoblasts migrate on ICAM1 (REFS 63, 69). In the situation of T cell receptor (TCR) stimulation, in which contact with ICAM1 in vitro serves as a surrogate for an antigen-presenting cell (APC), LFA1 microclusters arise at the T cell periphery and relocate towards the ‘bulls-eye’ central supramolecular activation cluster (cSMAC) contact area, similarly to TCR clusters. Although both are attached to cortical actin and carried inwards by its retrograde flow, LFA1 clusters last longer and do not travel as far, possibly owing to a diffusion barrier created by other proteins. Interestingly, the behaviour of α4β1 integrin is quite different in this context. When bound to vascular cell adhesion molecule 1 (VCAM1), α4β1 integrin stalls the centripetal movement of the TCR microclusters by tethering cortical actin.

How are integrin clusters linked into the cytoskeleton? There are several possible pathways, but this is not a fully explored topic. The LFA1 present in the T cell focal zone is tethered by talin and talin is present in the focal dots. The talin rod domain binds to the actin cytoskeleton both directly and indirectly through vinculin. An LFA1-stimulated actin cloud forms in the presence of ADAP–SLP76, but not in the absence of this complex, indicating further downstream connections with the cytoskeleton.

Figure 4 | Selectin inside-out signalling leads to intermediate-affinity LFA1 and slow rolling. Signalling from E-selectin to its ligands P-selectin glycoprotein ligand 1 (PSGL1) and CD44 on neutrophils gives rise to a defined pathway consisting of a SRC family tyrosine kinase (FGR, or HCK and Lyn), an immunoreceptor tyrosine-based activation motif (ITAM)-containing protein (Fc receptor γ-chain (FcγR) or DAP12) and spleen tyrosine kinase (SYK). Downstream of SYK lies the TEC family kinase Bruton’s tyrosine kinase (BTK), which activates phospholipase Cy2 (PLCy2). Also illustrated are recent findings showing a role for CALDAG-GEFI (Ca2+ and diacylglycerol-regulated guanine nucleotide exchange factor 1; also known as RASGRF2) and RAP1 downstream of SYK. To date, there is little information about other mediators that are important in chemokine receptor- and T cell receptor-mediated inside-out signalling in T cells, making comparisons difficult. ICAM1, intercellular adhesion molecule 1; LFA1, lymphocyte function-associated antigen 1 (also known as αLβ2 integrin). Figure is modified, with permission, from REF. 81 © (2010) The American Society of Hematology.

TCR signalling exhibits several exclusive features that distinguish it from other signalling pathways that lead to active LFA1. First, apart from its central role in the T cell ‘motor machinery’, actin reorganization contributes to the generation of active LFA1, and it appears to make a larger contribution to this process following TCR stimulation than in response to other signalling pathways, potentially reflecting a role in LFA1 microclustering. The adaptor protein SH2 domain-containing leukocyte protein of 76 kDa (SLP76; also known as LCP2) is a key component of TCR signalling to β1 and β2 integrins. Following TCR signalling, outside-in signalling recruits the SLP76–ADAP complex to LFA1 microclusters and is necessary for stabilizing both T cell LFA1 (REF. 76) and platelet αIIbβ3 integrin clusters under shear-flow conditions. ADAP– T cells and platelets have diminished microclusters and spreading capacity, lack SLP76 localization with VAV1 and have diminished filamentous actin assembly.

Second, the TEC family kinase IL-2-inducible T cell kinase (ITK) is associated with linker for activation of T cells (LAT) and is an essential mediator of TCR signalling leading to β1 and β2 integrin activation. ITK is upstream of PLCγ1 and protein kinase C (PKC) in the signalling pathway that leads to RAP1 activation. Thus, a role of ITK may be to activate CALDAG-GEFI and/or C3G (both of which are possible RAP1 GEFs) following CD3 crosslinking.

Selectin signalling lacks an outside-in step

Binding of E-selectin and P-selectin to their ligands PSGL1, CD44 and ESL1 on neutrophils induces rolling behaviour on the endothelial surface. The selectin-mediated rolling becomes slower when ICAM1 is incorporated in the assay, suggesting that LFA1 activating signals are being generated. The pathway includes the SRC family kinases FGR or HCK and Lyn, the ITAM-containing proteins DAP12 and FcγR, and SYK. This sequence of signalling events leads to the activation of the TEC family kinase Bruton’s tyrosine kinase (BTK) and PLCγ2, although there is a lack of agreement about phosphoinositide 3-kinase.
involved. A key demonstration linking this signaling pathway to integrin behaviour was provided by the demonstration that LFA1-mediated slow rolling was absent in Syk- and Pdgfl- neutrophils.

The end result of stimulation of the selectin-mediated signalling pathway in neutrophils is the generation of intermediate-affinity LFA1 and slow rolling on ICAM1 (REF 82). Crucially, this signalling does not lead to high-affinity LFA1 and adhesion strengthening. This suggests that, although the cell is exposed to the same shear-flow conditions, some aspect of the chemokine-mediated signalling pathway is missing in the neutrophil signals stimulated by selectin binding. It would be of great interest to identify where the signalling pathways differ in this respect. A recent publication shows that the SYK-directed component of slow rolling is dependent on CADLAG-GEFI and RAP1 (REF 83). Information is needed about the SRC and SYK family members more directly associated with neutrophil LFA1, as well as about any involvement of talin and kindlin 3, which are both part of the chemokine-mediated signalling pathway.

Closing comments

The signalling events that lead to increased LFA1 activity take place at the T cell membrane and require the co-assembly of pathway components that arrive by different routes. An understanding of the basic details of this signalling has only been achieved recently and, as might be expected, the findings have generated speculation and new questions, as we have attempted to highlight in this Review. A key question is whether the recycling vesicles that contain LFA1 and RAP1 are influenced by signalling. The identity of other vesicle passengers that are possibly already assembled into ‘working module’ form is also an issue of interest. RAP1 is crucial to both inside-out and outside-in signalling, but its roles have still to be understood with precision. Currently, RAP1 function as an active GTPase seems to be essential for the conveyance of RAPI, LFA1 and the serine/threonine kinase MST1 to the membrane, but the MST1 targets are not yet identified. In the CHO cell model, a complex of RAPI, RIAM and talin drives αIIβ3 integrin activation at cell membrane level. How is talin delivered to the plasma membrane in leukocytes and what are its associates, other than potentially VAV1? How many GEFs are involved in activating RAP1, are they stimuli specific and how are they transported to the membrane? We don’t fully understand the differences between the signalling events induced by the different agonists — chemokines, TCIR ligands and selectins — that lead to an increase in LFA1 activity. It seems that the outcome, in terms of changes to LFA1 conformation, is not identical for each. Finally, how similar to LFA1 activation is the activation of α4β1 integrin and other leukocyte integrins? Despite the fact that many challenging questions remain, it is motivating that there is now a framework for asking targeted questions about integrin signalling and a number of well-developed models for testing such ideas.

16. This study reconstructed the activation of platelet integrin αIIbβ3 in a CHO cell model using the minimum number of signalling elements, and this has provided a framework for integrin signalling in other cell types, including T cells.
24. This was the first report to demonstrate the need for tethered chemokine in the rapid extension of LFA1, priming the integrin for high-affinity signalling and firm adhesion under flow conditions.
34.References 30 and 31 were early studies highlighting the importance of RAP1 for LFA1 activation in vivo.
REVIEWS


35. This study demonstrated SKAP1 binding to RAPL: this interaction integrates the two signaling modules RAP1–RAPL and SLPE6–ADAP/SKAP1 in inside-out signaling, leading to LFA1 activation in T cells.

36. Katagiri, K. et al. LFA1 expression is a major effector of Rap1 responsible for delivering both RAP1 and LFA1 to the lymphocyte membrane of migrating cells and to the triad-like structures in the extracellular matrix.


49. The importance of PLD1 and PIP5K1 as upstream mediators in LFA1 affinity modulation following TCR activation are highlighted.


53. The authors declare no competing financial interests.

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