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Title: Super-resolution imaging of the cytoplasmic phosphatase PTPN22 links integrin-mediated adhesion with autoimmunity

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Abstract: Integrins are transmembrane heterodimers that play a fundamental role in the migration of leukocytes to sites of infection or injury. Here, we provide evidence that the protein tyrosine phosphatase PTPN22 is a novel regulator of LFA-1 signaling in effector T-cells. PTPN22 co-localizes with its substrates at the leading edge of cells migrating on ICAM-1. Gene targeting, or expression of the autoimmune disease-associated PTPN22-R620W variant, results in hyper-phosphorylation of integrin signaling intermediates. Super-resolution imaging reveals that in the steady state PTPN22-R620 exists in large clusters that disaggregate upon LFA-1 stimulation, permitting increased association with its binding partners at the membrane. Failure to retain PTPN22-R620W molecules at the membrane leads to increased LFA-1 clustering and integrin-mediated cell adhesion. Our data define a novel mechanism for fine-tuning integrin signaling in T-cells, and a new paradigm of autoimmunity in man in which disease susceptibility is underpinned by inherited perturbations of integrin function.

One Sentence Summary: PTPN22 is a negative regulator of integrin signaling and loss-of-function mutants increase cell adhesion.

Introduction

Integrins function as adhesion receptors that control cell-cell and cell-matrix interactions, thereby regulating migration of cells into tissues. Lymphocyte Function-associated Antigen 1 (LFA-1; CD11a/CD18; αLβ2) is the major integrin used by T-cells which, besides adhesion and migration, transduces environmental cues that impact on a wide range of cellular functions including cell differentiation, proliferation, cytokine production, cytotoxicity and cell survival (*1-6*). Optimal function requires changes in conformation and clustering of LFA-1 in ways that promote cell adherence, achieved through two distinct yet overlapping signaling pathways (*7*). Inside-out signaling is initiated by antigen or chemokine receptors, resulting in conformational changes in LFA-1 that increase the affinity of interactions with ligand, such as intercellular adhesion molecule (ICAM)-1, and binding of cytoplasmic signaling modules to integrin tails (*8, 9*). Outside-in signaling is initiated following LFA-1 engagement with ICAM-1, and leads to Src and Syk-mediated protein tyrosine phosphorylation, membrane translocation of Rap1 and binding of talin and kindlin-3 to the cytoplasmic tail of the β2 subunit (*10*). While counter-regulation of LFA-1 dependent protein tyrosine phosphorylation is required for repeated cycles of adhesion, de-adhesion, protrusion and contraction (*11, 12*), the protein tyrosine phosphatases that support this function are not well understood.

PTPN22, known as Lyp in man and PEP in mouse, is a protein tyrosine phosphatase that dephosphorylates Src and Syk family kinases (*13*). Interest in PTPN22 has grown considerably since initial reports of strong associations between a missense single nucleotide polymorphism in the *PTPN22* gene (1858C>T, encoding R620W) and a growing number of autoimmune diseases including type I diabetes, rheumatoid arthritis and systemic lupus erythematosus (*14*).

Functional studies have focused on how the phosphatase regulates antigen receptor signaling in lymphocytes, but precisely how the R620W variant confers susceptibility to autoimmune disease is unknown. Some studies have suggested that it is a loss-of-function mutation, while others point to gain-of-function (*15, 16*). Here, we investigated whether PTPN22 regulates LFA-1-mediated signaling because its substrates, Lck and ZAP-70, are also phosphorylated following LFA-1 engagement (*17, 18*). Our experiments were also motivated by the possibility that perturbations in integrin signal transduction might play a role in provoking immune-mediated inflammatory diseases in individuals carrying PTPN22 genetic polymorphism.

Results

PTPN22 co-localizes with its substrates at the leading edge of migrating T-cells

To examine whether PTPN22 regulates outside-in integrin signaling, activated primary human T-cells were plated onto ICAM-1 coated glass, prior to fixing, staining and imaging by confocal microscopy. PTPN22 mainly polarized to the lamellipodium at the leading edge of migrating T-cells (ICAM-1) with some staining in the uropod, while PTPN22 was not polarized in non-migrating T-cells on glass coated with poly-L-lysine (PLL) (Fig. 1A). Specificity of staining was confirmed by gene knockdown, patterns of staining obtained with monoclonal and polyclonal antibodies to PTPN22, and by localization in PTPN22-GFP expressing T-cells (fig. S1A-C). Confocal microscopy and fluorescence intensity plots revealed phosphorylation of activatory tyrosine residues on Lck (pY394), ZAP-70 (pY493) and Vav (pY174) to be co-localized with PTPN22 at the leading edge of migrating cells (Fig. 1A and fig. S1D), but not non-migrating cells (fig. S1E). Analysis and quantification of Total Internal Reflection Fluorescence (TIRF) microscopy images confirmed membrane proximal associations between PTPN22 and

phosphorylated substrates at the leading edge of migrating T-cells, when compared to non-migrating PLL controls (fig. S2A and B).

Integrin signaling induces association between PTPN22 and its phosphorylated substrates

Biochemical analysis of whole cell lysates confirmed inducible phosphorylation of integrin signaling intermediates in migrating T-cells in response to different integrin ligands (fig. S3A and B); specificity of the response was confirmed using soluble anti-ICAM-1 antibodies (fig. S3C). PTPN22 immunoprecipitates from non-migrating and migrating cells were then probed with an anti-phosphotyrosine antibody. While a single phosphoprotein band was detected in non-migrating T-cells, many more phosphoproteins were detected in migrating T-cells, suggesting inducible association of PTPN22 with phosphotyrosine substrates (fig. S3D). Three of these bands resolved to molecular weights of phosphorylated Lck (56kD), ZAP-70 (70kD) and Vav (118kD). Their specificity and association with PTPN22 over time were confirmed by immunoblotting of anti-PTPN22 immunoprecipitates using anti-phosphospecific antibodies (Fig. 1B). Importantly, PTPN22 associated only with phosphorylated protein substrates, as demonstrated by blotting for total proteins, indicating that phosphorylation was required for protein PTPN22-substrate interaction in migrating cells.

PTPN22 is a negative regulator of integrin signaling; R620W is a loss-of-function mutant

Targeting of *PTPN22* with siRNA depleted protein expression in migrating T-cells by ~50-60%, without affecting LFA-1 expression (fig. S3E), but increased ICAM-1 induced phosphorylation of Lck(pY394), ZAP-70(pY493) and Vav(pY174) when compared to control siRNA in migrating cells (Fig. 1C). Unlike pY493-ZAP-70, increased phosphorylation of the regulatory

Y319-ZAP-70, which is not a target of PTPN22 (*13*), was less pronounced. *PTPN22* knockdown was also associated with increased cell motility (Fig. 1D), while overexpression of wild-type PTPN22-R620 in PTPN22 sufficient T-cells, but not a catalytically inactive C227A mutant or control vector, reduced cell motility (Fig. 1E). The phosphatase activity of PTPN22 was therefore required for regulation of integrin-mediated cell motility. Integrin dependent phosphorylation of the classical mitogen activated protein kinase ERK1/2 (extracellular-signal-regulated kinase) was also observed following LFA-1 engagement (Fig. 1C), consistent with its localization in nascent adhesion complexes and its contribution to the "motor phase" of lamellopodium protrusion (*19-21*). This was blocked by the Lck inhibitor PP2 (fig. S3F), demonstrating that integrin-induced Lck activation is upstream of ERK phosphorylation, as previously reported (*18*). In keeping with these data, ERK was hyper-phosphorylated in migrating *Ptpn22*$^{-/-}$ deficient murine T-cells (Fig. 1F), and in human T-cells carrying either one or two copies of the *PTPN22* genetic variant encoding PTPN22-W620 (Fig. 1G). These data indicated that PTPN22 is a negative regulator of LFA-1 signaling, and suggested that the R620W variant is a loss-of-function mutant.

PTPN22 and Csk localize in membrane proximal clusters that decluster during LFA-1 induced migration.

Diffraction limited microscopy showed significant localization of PTPN22 at the leading edge of migrating T-cells following LFA-1 engagement (Fig. 1A). To obtain quantitative imaging data of this phenomenon at the membrane, TIRF-dSTORM super-resolution microscopy and quantitative cluster analysis was employed. These techniques quantify protein (localization) and cluster number, as well as cluster size and density of PTPN22 protein localization at the membrane of

stationary cells or at leading edge of migrating T-cells. Visualization of single molecules can be represented by point maps and cluster heat maps. Using this approach, PTPN22 was found to be highly clustered at the plasma membrane of non-migrating T-cells (PLL), while analysis of migrating T-cells revealed smaller, less dense clusters (Fig. 2A), confirmed by quantitative cluster analysis (Fig. 2B and C). Quantification by Ripley's K-function, a measure of clustered-ness, also revealed a substantial reduction in clustering (peak of L(r)-r curve) and the presence of smaller clusters (position of the peak on the x-axis) at the leading edge (Fig. 2D), further confirming that PTPN22 was substantially less clustered in migrating T-cells. Csk also exhibited this declustering phenomenon upon LFA-1 stimulation (15% of localizations in clusters versus 30% in non-migrating cells), although the heat maps revealed more cluster heterogeneity when compared to PTPN22 (fig. S4A-C). Concurrent with LFA-1 induced declustering of PTPN22 and Csk, we detected increased association between the phosphatase and kinase in both PTPN22 and Csk immunoprecipitates (fig. S4D and E), while the adaptor protein associated with glycolipid enriched microdomains (PAG, also known as Csk-binding protein), a binding partner of Csk (*22*), had notably less Csk and PTPN22 associated with it after ICAM-1 stimulation (fig. S4F and G). Consistent with these findings, tyrosine phosphorylation of PAG, known to regulate PAG and Csk binding (*23*), was also reduced upon LFA-1 engagement (fig. S4H). These data indicated that de-clustering of both PTPN22 and Csk coincides temporally with increased PTPN22-Csk complexing.

Nanoscale organization of PTPN22-R620 and W620 clusters in migrating T-cells

Closer examination of PTPN22-R620 localizations at the nanoscale level revealed that the transition from the non-migrating to the migrating state was associated with three cluster

characteristics. Firstly, there was a modest reduction in the total number of clusters (Fig. 3A, closed symbols). Secondly, PTPN22-R620 clusters become smaller. This is based on a marked reduction in the % of PTPN22 localizations within clusters (Fig. 3B, closed symbols), a finding in keeping with the cluster heat maps, and the changes in diameter and density of clusters upon ICAM-1 stimulation (Fig. 2A-C). Thirdly, there was a marked increase in the number of PTPN22-R620 localizations at the leading edge (Fig. 3C, closed symbols). Thus, following LFA-1 stimulation PTPN22 molecules disperse from clusters, accumulating at the membrane in the vicinity of receptor proximal signaling intermediates.

The cluster characteristics of the disease associated W620 mutant phosphatase were similar to the common R620 variant in non-migrating cells (Fig. 3D upper panel; compare open versus closed symbols for PLL in Fig. 3A-C). In contrast, migrating T-cells expressing the PTPN22-W620 variant had increased numbers and density of clusters (Fig. 3A and B, open symbols), evident in region point maps and heat maps (Fig. 3D lower panel). Indeed, the amplitude of Ripley's K-function also suggested that PTPN22-W620 was more clustered (Fig. 3E). Unexpectedly, the total number of PTPN22-W620 molecules at the membrane was significantly reduced following LFA-1 stimulation, when compared to PTPN22-R620 (Fig. 3C, open versus closed symbols for ICAM-1). This finding was in keeping with the point maps, showing reduced numbers of localizations outside of clusters (Fig. 3D lower panels, and fig. S5A), which could not be explained by differences in protein expression at the whole cell level (fig. S5B and C).

By using simulated data to model the consequence of varying the density of the non-clustered background of molecules surrounding clusters we found that both linearity and the gradient of

Ripley's K-function depend on the number of non-clustered localizations (fig. S5D and E). These simulations, together with the observed Ripley's K-function data in Figure 3E, indicated that differences between PTPN22-R620 and W620 clustering were not due to changes in clustering behaviour of W620 *per se*, but a consequence of the lack of localizations outside of clusters, a finding in keeping with the pointillist maps (fig. S5A). We surmised that declustered PTPN22-W620 molecules are not retained at the membrane in migrating T-cells to the same extent as R620. To explore the mechanism behind this difference in membrane localization we tested whether the P1 domain mutation in PTPN22-W620 compromised binding to SH3-domain protein partners, such as Csk, following integrin engagement. Csk immunoprecipitates were generated from homozygous donor-derived PTPN22-R620 or W620 expressing T-cells migrating on ICAM-1 and probed for PTPN22. The results clearly demonstrated a reduced level of Csk and PTPN22-W620 association in migrating T-cells compared to R620 (Fig. 3F). This suggests that upon LFA-1 stimulation, retention of PTPN22 at the membrane is dependent on the formation of a complex with Csk, or other SH3 domain containing proteins.

PTPN22 associates with the LFA-1 signaling complex and regulates LFA-1 clustering

LFA-1 mediated adhesion and migration of T-cells is regulated by conformational changes in the extracellular domain of the α and β chains, and physical clustering of heterodimers at the membrane (*24-26*). Furthermore, Lck and ZAP-70 associate with the cytoplasmic tail of engaged LFA-1 (*17, 27*). Initial evidence for an association between LFA-1 and its regulator, PTPN22, derived from confocal images showing LFA-1 and PTPN22 co-localized at the leading edge (Fig. 4A). By TIRF microscopy, co-localization of LFA-1 and PTPN22 was also observed at the interface with ICAM-1 at the leading edge (Fig. 4B), returning a Manders' co-

localization coefficient (MCC) of 0.43 ± 0.14. This association was also supported by co-immunoprecipitation experiments, in which LFA-1-PTPN22 interactions were observed to increase as a function of LFA-1 engagement (Fig. 4C). To address the mechanism for this association, we examined immunoprecipitates from the human leukemic Jurkat T-cell line and its Lck-deficient derivative JCaM1.6 (which expresses comparable levels of ZAP-70 and PTPN22 to the parental cell line), and found that PTPN22 does not appear to complex with LFA-1 in the absence of Lck (Fig. 4D and fig. S6). Thus, PTPN22 associates with the LFA-1 signaling complex in an Lck dependent manner, where it interacts with substrates to regulate integrin signaling.

To examine the relationship between regulation of LFA-1 signaling by PTPN22 and LFA-1 function, we first investigated how loss of PTPN22 function might impact LFA-1 clustering at the cell surface. Super-resolution TIRF-dSTORM maps showed increased clustering of LFA-1 at the leading edge of migrating T-cells expressing the W620 variant (Fig. 5A), corroborated by Ripley's K-function (Fig. 5B). While there were no differences in the total number of LFA-1 localizations, nor number of clusters (Fig. 5C and D), the percentage of LFA-1 molecules participating in clusters was increased in T-cells expressing the W620 variant (Fig. 5E). Thus, loss of PTPN22 function is associated with increased LFA-1 clustering at the leading edge of migrating T-cells.

Integrin-dependent adhesion is increased in T-cells expressing the loss-of-function PTPN22-W620 variant

In light of these findings we evaluated the functional consequences of enhanced LFA-1 clustering by directly comparing the adhesion of R620 and W620 expressing T- cells under conditions of shear flow. T- cells expressing the disease-associated W620 variant were more adherent to ICAM-1 (Fig. 6A). *Ptpn22* deficient murine T-cells phenocopied the disease-associated variant, being more adherent under shear-flow (Fig. 6B), and more adherent in de-attachment assays (Fig. 6C). Thus, loss-of-function PTPN22 mutants that enhance LFA-1 signaling increase LFA-1 clustering and cell adhesion. Taken together, our results indicate that in migrating T-cells, PTPN22 disperses from large membrane proximal clusters into smaller clusters capable of interacting with the LFA-1 adhesome where fine-tuning of signals can regulate integrin clustering and adhesion. If PTPN22 localization at the membrane is compromised, regulation of integrin signals is uncoupled, and both integrin clustering and cell adhesion are enhanced.

Discussion

Protein tyrosine phosphatases are now established as key regulators of integrin signaling (*11, 12*). Indeed, the PTP-PEST family of phosphatases, which comprises PTPN12 as well as PTPN22, can be added to a growing list of negative regulators of integrin function that includes RhoH, DOK1, calpain and the ubiquitin ligase SHARPIN (*28-31*). Single molecule localization microscopy has allowed us to image PTPN22 at the nanoscale level, and to document that membrane proximal declustering is linked both temporally and spatially to its inhibitory function. This is in contradistinction to the opposing clustering behaviour reported previously for

kinase associated signaling modules (*32*, *33*), which has been shown to digitize signaling and increase signal transduction fidelity (*34*). Whether clustering in the steady state is unique to PTPN22, or a common mechanism for sequestering phosphatases from their substrates will require further study.

The negative regulatory functions of native PTPN22 were confirmed by gene targeting in mouse and human T-cells. We also found that a catalytically active phosphatase was required to regulate integrin dependent cell motility. Experiments using PTPN22-W620 expressing T-cells from homozygous donors indicated that the disease-associated variant was a loss-of-function mutant, at least in the context of LFA-1 signaling. The basis for this functional difference is underpinned in part by impaired binding of the mutated P1 polyproline domain of PTPN22 with SH3 domain containing proteins, notably Csk. The spatiotemporal dynamics of PTPN22-Csk interactions and the impact of disrupting these associations on PTPN22 function are complex, and may be signal specific. For example, Vang and colleagues demonstrated that dissociation of PTPN22 from Csk is a prerequisite for targeting of the phosphatase to plasma membrane lipid raft domains where it attenuates T-cell receptor (TCR) signaling (*35*). PTPN22-W620 partitioned into rafts more efficiently, while forced dissociation of PTPN22 from Csk using a Csk-SH3 domain also reduced TCR signals. These data support the gain-of-function hypothesis with respect to the W620 variant and TCR signaling, and suggest that the impact of the disease associated mutant is context dependent. While uncoupling of the PTPN22-W620-Csk association seems a consistent feature relevant to TCR and integrin dependent signaling (*36*, *37*), we cannot rule out the possibility that disrupting interactions between PTPN22 and other SH3 domain containing proteins could contribute to the signaling phenotypes reported. A comparative

biochemical analysis of the PTPN22-R620 and W620 interactomes, using technologies recently reported for PAG would provide a systematic and unbiased approach to address this issue (*38*).

We present a model depicting how PTPN22 regulates LFA-1 signaling and how expression of the PTPN22-W620 mutant enhances LFA-1 signaling and integrin-dependent adhesion (Fig. 7). According to this model, PTPN22 exists in large clusters in the steady state, sequestered from its substrates. De-clustering is triggered by active signals – an event we are now studying in the context of TCR as well as LFA-1 stimulation – and one that targets clustered pools of both PTPN22 and Csk. Precisely how the clusters disaggregate is not known, but the process serves to deliver monomers of PTPN22 to the membrane zone permitting interactions with its binding partners. Lck is constitutively associated with the β chain of LFA-1 (*17*), and is required for recruitment of PTPN22 to the LFA-1 adhesome. In phosphatase deficient cells, LFA-1 induced phosphorylation of these intermediates goes unchecked, manifesting as hyper-phosphorylation of signaling intermediates and augmented integrin dependent signaling, cell motility and adhesion. The outcome of expressing PTPN22-W620 is the same, except that in this case total cellular levels of phosphatase are equivalent to those in R620 expressing T-cells, but the W620 variant fails to bind Csk (or other SH3 domain containing proteins). Instead, PTPN22-W620 is distributed throughout the rest of the cell rather than being retained in the vicinity of the adhesome. Failure to attenuate LFA-1 signals is associated with much larger, denser clusters of LFA-1 at the cell surface, equipping the cell with domains of increased adhesiveness.

How might increased cell adhesiveness translate to altered cell function in vivo? We suspect that the consequences of increased adhesion under shear flow conditions could perturb multiple

phases of lymph node and tissue homing, including adhesion of cells on vascular endothelium coupled to transmigration into tissues, trafficking across high endothelial venules within lymphoid organs, and interactions between cells or with the surrounding extracellular matrix. Integrins have other functions besides adhesion and migration, since they promote interactions between T-cells and antigen presenting cells in ways that underpin the earliest steps in T-cell activation and differentiation (*9, 39*), as well as downstream effector responses, such as cytokine expression and cytotoxic functions (*4-6*). We propose that aberrations of integrin function be included in the repertoire of mechanisms underpinning predisposition to autoimmune disease in individuals carrying loss-of-function *PTPN22* mutations (*40*). Loss of immune tolerance, however, will depend on the balance of function between effector and regulatory T-cells, since our work has previously demonstrated that increased adhesiveness of *Ptpn22* deficient Tregs is in fact associated with greater potency (*41*).

MATERIALS AND METHODS

Antibodies and integrin ligands

Primary antibodies: Mouse monoclonal antibody (Ab) and affinity-purified goat polyclonal Ab**,** both raised against human PTPN22, were purchased from R&D Systems. Antibodies to Vav1 (C-14), ZAP-70 (1E7.2), Lck (3A5), Csk (C20), were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies: p-Src (Tyr416), p-ZAP70 (Tyr319/SykTyr352), p-ZAP70 (Tyr493/Syk-Tyr526), p-Erk (Thr202/Tyr204) (197G2), α/β-tubulin, β-actin (Cell Signalling Technology), p-Vav1(Tyr174) (EP5107) (Abcam), and anti-PAG (PAG-C1) and anti-CD11a (LFA-1). mAb38 was a kind gift of Dr Nancy Hogg. *Secondary antibodies for immunoblotting detection*: Sheep anti-mouse IgG-HRP (Amersham), goat anti-rabbit Ig-HRP (DAKO), mouse monoclonal light chain specific anti-goat IgG (Jackson Laboratory), goat anti-mouse IgG, goat anti-rabbit IgG, donkey anti-mouse IgG, donkey anti-rabbit IgG, donkey anti-goat IgG, and IgG1, IgG2b Zenon® antibody labelling kits were all from Life Technologies. *Integrin ligands:* Recombinant human ICAM-1/CD54 Fc chimera, recombinant murine ICAM-1/CD54 Fc chimera, recombinant VCAM-1 and fibronectin (R&D Systems).

Mice

Ptpn22 deficient mice were generated and genotyped for use in experiments as described (*41*). The line was re-derived into the Biological Services Unit at King's College London and bred on a C57BL/6 background for 10 generations in SPF conditions, in compliance with Home Office regulations and local ethically approved guidelines. Sex and age matched *Ptpn22*$^{+/+}$ and *Ptpn22*$^{-/-}$ littermates were used in experiments.

Media, cell culture and transfection

Human T-cell complete medium: IMDM, 10% FBS, penicillin-streptomycin. Murine T-cell complete medium: Glutamax-RPMI, 10% FBS, 50 μM βME, 100 μM sodium pyruvate, 20 mM HEPES, penicillin-streptomycin. Cell migration medium: Glutamax-RPMI, 50 μM βME, 100 μM sodium pyruvate, 25 mM HEPES, penicillin-streptomycin. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphoprep (Stemcell Technologies) and stimulated with 1 □g/ml phytohaemagglutinin (PHA) (ThermoScientific) in IMDM media supplemented with 10% FCS and penicillin-streptomycin (PAA) for the first 48 hours and supplemented with 20 ng/mL of IL-2 (Aldesleukin, Novartis) for up to 10 days. T-cells were purified using a pan T-cell isolation kit (cat no. 130-091-156, Miltenyi Biotec) to a purity of > 97%, determined by flow cytometry. Donors belonging to TwinsUK (www.twinsuk.ac.uk), and selected on the basis of their rs2476601 genotype (*PTPN22* C1858T, corresponding to PTPN22-R620W protein), also provided PBMC samples after informed consent. Transfection of primary human T-cell blasts was achieved using the Amaxa Nucleofector and human T-cell Nucleofection Kit, programme T-020 (Lonza). Plasmid DNA (2 μg) and siRNA pools (Invitrogen, Thermo Fisher Scientific) were used to transfect 10^7 T-cells. Murine T-cells were generated from splenic and lymph node cell suspensions, adjusted to 3 x 10^6 cells/ml, and cultured in complete medium. Cells were stimulated with 1 μg/ml concanavalin A (ConA, Sigma) for 48 hours, subjected to a Ficoll gradient and re-suspended in complete medium supplemented with 20 ng/ml of IL-2 at a density of 2 x 10^6 cells/ml. All adhesion and migration assays were conducted with murine T-cells after culture for 4-5 days in IL-2. The human leukemic T cell line, Jurkat, and its Lck-deficient derivative JCaM1.6, were also used in experiments (*42*).

Generation of PTPN22-GFP constructs

A pEF5HA plasmid encoding PTPN22 (a kind gift from Dr Nunzio Bottini, La Jolla Institute for Allergy and Immunology, California, USA) was sequenced and used as a plasmid backbone for site directed mutagenesis to generate a panel of PTPN22 mutants; a 3' GFP fragment was introduced by sub-cloning. All constructs were verified by sequencing. Targeted mutations were introduced using the following specific primers pairs:

R620W-Fwd (CCACTTCC TGTATGGACACCTGAATCATTTA) and

R620W-Rev (TAAATGATTCAGGTGTCCATACAGGAAGTGG)

C227A-Fwd (TGTTCCCATATGCATTCACGCCAGTGCTGGCTGTGGAAGGACTGG) and

C227A-Rev (CCAGTCCTTCCACAGCCAGCACTGGCGTGAATGCATATGGGAACA

T-cell stimulation and signaling with integrin ligands

32 mm glass coverslips (VWR International) were coated in 6-well sterile plates with integrin ligands ICAM-1-Fc (3 µg/ml, unless indicated otherwise), VCAM-1-Fc (3 µg/ml), fibronectin (10 µg/ml) or poly-L-lysine (PLL; Sigma) overnight at 4°C, washed 3X with PBS and blocked for 1 hour at room temperature with 2% BSA in PBS. Blasting T-cells were rested in medium for 30 min, then added to coverslips ($3x10^6$/coverslip) and incubated for 20 min at 37°C. After 20 min unbound cells were aspirated and 1 ml of lysis buffer added to coverslips (1x 6-well plate; $2x10^7$ cells) prior to lysis on ice for 20 min. Lysates were cleared by centrifugation and diluted in sample buffer for immunoblotting or immunoprecipitation experiments.

Immunoblotting and immunoprecipitation

Cells were lysed directly in 2X SDS-PAGE sample buffer or in lysis buffer containing 1% Triton X-100 from Sigma, containing phosphatase and protease inhibitors (Roche). Proteins were separated by SDS-PAGE and transferred to immobilon-P polyvinylidene difluoride membranes by standard immunoblotting techniques. After incubation with primary and secondary antibodies, blots were visualised by SuperSignal chemiluminescent reaction (Pierce Biotechnology) in a Chemidoc Station (BioRad). For immunoprecipitation, 1-2 µg of control or specific antibody was added to lysates overnight at 4°C followed by the addition of 20 µl magnetic beads (Millipore) for 1 hour at 4°C. Beads were washed 3X in lysis buffer prior to elution with 20 µl boiling 2X SDS-PAGE sample buffer. Lysates and immunoprecipitates were used immediately, or stored at -80°C until analysis.

Immunofluorescence staining and microscopy

Coverslips, glass bottom dishes (Mattek) or 8-well glass bottomed microscopy chambers (Ibidi) were coated overnight at 4°C with human recombinant ICAM-1-Fc (3 µg/ml) or 0.01% Poly-L-Lysine (PLL), washed 3X in HBSS and blocked in 5% BSA for 1 hour. T-cells were resuspended at $2.5x10^5$ cells/ml in migration medium (HBSS, 10mM HEPES equilibrated overnight in incubator conditions) and added to ICAM-1-Fc coated glass. After 20 min of migration cells were pH shift fixed (3% PFA-kPIPES 80 mM pH6.8 for 5 min supplemented with 2mM Mg^{2+} and 5mM EGTA followed by 3%-PFA Borax 100 mM pH11 for 10 min.) and permeabilised using 0.1% Triton at 4°C for 5 min. Autofluorescence was quenched using 1 mg/ml $NaBH_4$ for 15 min. Chambers were blocked with 10% goat serum for 1 hour followed by incubation with primary antibody overnight and appropriate secondary antibody for 20 min. at room temperature.

Confocal microscopy was performed with a Zeiss LSM700 Axioimager M2 system at 63X magnification (Zeiss, Plan-Apochromat 63x/1.40 Oil M27, zoom 4, 512x512 pixel scan). TIRF images were obtained with Zeiss observer Z1 (inverted) microscope equipped with TIRF slider (Zeiss, Plan-Apochromat, 100X/1.40 Oil DIC (UV) VIS-IR). Images were collected, processed and analysed using Slidebook 5.5 (3i) or ImageJ software. Colocalization of signaling proteins was determined according to the method of Dunn and colleagues (*43*). Briefly, TIRF images were processed with the automatic method of local background subtraction by median filtering and finally by small value subtraction using SlideBook6 software. Four regions (1.6 x 1.6 μm) were chosen in the front lamella of the cell. Subsequently, Manders' colocalization coefficient (MCC) was calculated with the inbuilt SlideBook6 function. Results from five regions were averaged per cell in four different experiments and analysed using Prism 6 Graphpad software.

Stochastic Optical Reconstruction microscopy (dSTORM)

dSTORM imaging was performed on a Nikon N-STORM microscope using a 100X 1.46N.A. oil immersion TIRF objective. Cells were imaged under TIRF illumination with a 647 nm laser with photo-activation at 405 nm in oxygen scavenging buffer (including 50 μg/ml glucose oxidase, 25 μg/ml horseradish peroxidase and 75 mM cysteamine in base buffer, pH8.0). Fluorescence was collected on an Andor iXon EM-CCD camera. Acquisition time was between 5 and 15 min with an integration time of 10 ms. Molecular coordinates were calculated by Nikon NIS N-STORM software using a photon threshold of 3000 per point spread function.

Cluster analysis

The output from the NIS software is in the form of pointillist x-y coordinates of the localized fluorophores. Data were divided into non-overlapping 2 x 2 μm square regions, avoiding cell boundaries. Ripley's K-function can be used to quantify the level and size scale of molecular clustering and has previously been demonstrated in T-cells *(44-46).* The K-function is calculated for each region with the Excel plugin SpPack (*45*), using the equation:

$$K(r) = \frac{A}{n^2}\sum_{i=1}^{n}\sum_{j=1}^{n}\delta_{ij}$$

Where δ_{ij}=1 if the distance between molecules *i* and *j* is less than *r*, and is otherwise 0. Therefore δ_{ij} represents drawing concentric circles of radius *r* around each point, *i*, and counting how many other points, *j*, are encircled. As defined, the *K(r)* value scales linearly with the circle area for increasing *r* and is therefore converted into the L-function using:

$$L(r) = \sqrt{K(r)/\pi}$$

This function is represented by a plot of L(*r*)-*r* versus *r*. In the case of a completely spatially random (CSR) distribution of molecules, L(*r*)-*r* = zero for all *r*. If L(*r*)-*r* is positive, this represents clustering on a particular spatial scale, *r*. Negative L(*r*)-*r* represents a more regular distribution than CSR (negative clustering). Edge effects are corrected by means of a toroidal wrap. 95% confidence intervals are calculated by simulating 100 CSR distributions with the same total molecular density as the experimental data. In order to test the effect of reducing the density of monomers (i.e. the CSR background) on the Ripley's K-function curves we analysed simulated data. A 3 x 3 μm region was simulated with a single Gaussian profile cluster at its centre (s.d. = 100 nm, 100 points) that was then overlaid with a CSR background. We then varied the density of the CSR overlay and analysed the resulting Ripley's K-function curves. In

the presence of zero background, the curve will decay linearly to negative infinity at increasing *r*, however in the presence of increasing background, the curve will asymptotically approach zero (as is the case for a pure CSR distribution). To generate the pseudo-coloured cluster maps, the degree of clustering of each molecule is calculated using Getis's variant of Ripley's K-function (*48*). This is simply the L(r) value, omitting averaging over all molecule in the region (j). For each molecule, i, therefore, this value is given by:

$$L(r)_i = \sqrt{\frac{A}{n}\sum_{j=1}^{n}\delta_{ij} / \pi}$$

Molecules at the edge of the region of interest have their L(r) corrected using a buffer region of width *r*. To generate the cluster maps, the L(r) values are interpolated onto a 5 nm resolution grid using Matlab and the L(r) colour surface pseudo-coloured. To extract cluster statistics, the map is thresholded at a value of L(r)=200, with areas above this value considered to be clusters. Clusters were separated using an 8-connectivity rule allowing us to extract the number of clusters, cluster sizes, number of molecules per cluster and other parameters.

Flow cytometry

T-cell blasts were stained in cold FACS buffer (0.5% BSA, 0.01% sodium azide) with antibodies and live/dead discrimination, washed, fixed with 2% PFA and analysed using a FACSCalibur. Data analysis was performed using FlowJo software (Treestar Incorporated).

Time-lapse microscopy; attachment under shear flow

Glass bottomed flow chambers (μ-slide $VI^{0.4}$, ibidi) were coated with ICAM-1-Fc (5 μg/ml) and T-cell blasts (1 x 10^6 cells/ml) were flowed over glass at 0.5 dyn/cm² for 8-10 min in migration

media. Wide-field time-lapse movies were acquired in 10 areas per slide. Cell numbers were counted over time and data presented as mean ± SD values.

Time-lapse microscopy; detachment under shear flow

T-cells generated from *Ptpn22*$^{+/+}$ and *Ptpn22*$^{-/-}$ mice (5 x 10^6 cells/ml) in migration media, allowed to adhere to ICAM-1-Fc (5 μg/ml) coated glass bottomed flow chambers (μ-slideVI$^{0.1}$, ibidi) for 10 min and migration media (incubator equilibrated overnight) applied at shear flow rates from 5dyn/cm^2 to 30dyn/cm^2. Wide-field time-lapse images were acquired for 10 min and cell counts accrued every minute. Data were plotted as cell number (Y0)/time(X0) and One Phase decay Y=(Y0-plateau)*exp(-K*X)+ plateau applied to calculate the phase decay half-life, a value representing the rate at which T-cells detach from the glass over time, where low values indicate increased detachment.

Statistical analysis

All analyses were performed using Prism 6.0 software (GraphPad, La Jolla, CA). Distributions of data points and their variance were determined and parametric or non-parametric tests applied, as appropriate. Comparisons between two groups were evaluated using Mann Whitney U test; unpaired Student's *t* tests were used for normally distributed data. Comparison of 3 or more independent conditions was determined using the rank based non-parametric Kruskall-Wallis H test, or Tukey's Ordinary One-Way ANOVA multiple comparisons test for normally distributed data. For cell motility, cell adhesion and clustering data comparisons between groups were determined using unpaired two-tailed *t* tests. Student's *t* tests were also applied to compare

densitometric measurements between independent immunoblotting experiments. Differences were considered significant when $P<0.05$.

Supplementary Materials

Fig. S1. PTPN22 and its phosphosubstrates localize to the leading edge of migrating T-cells.

Fig. S2. TIRF microscopy confirms colocalization of PTPN22 with its phosphorylated substrates.

Fig. S3. Integrin engagement leads to inducible phosphorylation of PTPN22 substrates in migrating T-cells.

Fig. S4. Csk declusters on LFA-1 engagement and associates with PTPN22 in migrating T-cells.

Fig. S5. Cluster characteristics and expression of PTPN22-R620 and W620 in migrating T-cells.

Fig. S6. PTPN22 associates with LFA-1 in an Lck-dependent manner.

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FIGURES LEGENDS

Fig. 1. PTPN22 is a negative regulator of LFA-1 signaling, co-localizing with its phosphorylated substrates at the leading edge of migrating T-cells. (**A**) Primary human T-cell blasts were layered onto glass slides coated with poly-L-lysine (PLL) or ICAM-1 prior to staining with mouse anti-PTPN22 and the indicated anti-phosphospecific antibodies, and imaged by confocal microscopy. The direction of migration is indicated (large white arrows). Scale bars 10 μm. Data represent analysis of 30-40 cells from 4 independent experiments. (**B**) Mouse anti-PTPN22 or control IgG immunoprecipitates were generated from T-cells layered onto PLL or ICAM-1 coated plates for the indicated time, prior to immunoblotting with anti-phosphospecific antibodies; anti-PTPN22 (goat polyclonal), anti-Lck and anti-ZAP-70 blots were used to evaluate levels of total protein. (**C**) T-cells were transfected with scrambled (Scram) or specific (PTPN22) siRNA and cultured for a further 48 hours prior to plating onto PLL or ICAM-1. After 20 min adherent cells were harvested and lysates resolved by SDS-PAGE prior to immunoblotting with the indicated antibodies. Blots were probed with goat anti-PTPN22 to confirm PTPN22 depletion, and with anti-tubulin antibodies to control for protein loading. (**D**) T-cell blasts were transfected with scrambled siRNA (Scram) or PTPN22 specific siRNA and cultured for 24 hours prior to plating onto ICAM-1. Migration of single cells was tracked by time-lapse microscopy. Dot plots show mean speeds (± SD) of T-cells for each siRNA condition; **** $P<0.0001$. (**E**) T cells were mock transfected (no DNA), or transfected with the indicated GFP expression vectors and velocity of cells quantified as for (D); ns – not significant, **** $P<0.0001$. (**F**) Murine T-cells were generated from lymph nodes of *Ptpn22*$^{+/+}$ or *Ptpn22*$^{-/-}$ littermates and allowed to migrate for 20 min on PLL or ICAM-1 coated plates. Lysates from adherent cells were probed with anti-phospho-ERK antibodies, or anti-ERK to control for protein loading. Fold changes in

expression relative to phospho-ERK in non-migrating (PLL) cells are shown (n = 3); * *P*=0.042. (**G**) Human T-cell blasts were generated from genotyped donors expressing PTPN22-R620 (RR), PTPN22-R620W (RW) or PTPN22-W620 (WW) and allowed to migrate on PLL or ICAM-1 coated plates for 20 mins. Lysates were probed for phospho-ERK and total ERK expression. Fold changes relative to phospho-ERK in non-migrating (PLL) cells are shown (right panel; n = 8 per genotype). Pairwise comparisons (two-tailed *t* test): RR/RW **P*=0.0337; RW/WW **P*=0.0144; RR/WW ***P*=0.0093.

Fig. 2. PTPN22 exists in large clusters which disperse upon LFA-1 engagement. (**A**) Primary T-cell blasts were generated from peripheral blood of PTPN22-R620 homozygous donors and layered onto PLL or ICAM-1 coated plates for 20 min. prior to fixing, permeabilisation and staining with mouse anti-PTPN22 antibodies. Images were acquired using a Nikon N-STORM microscope and molecule distributions analysed using cluster analysis algorithms. For each condition N-STORM images are representative of PTPN22 molecule distributions at the whole cell level and 1 μm^2 region maps, selected from the migrating T-cell leading edge. Pointillist (Gaussian fitted) and pseudo-coloured heat maps are representative of cluster data acquired after processing of N-STORM image-regions using the cluster analysis algorithm. Scale bars, 5 μm for PLL and 10 μm for ICAM-1 images. Data are representative of multiple experiments. (**B**) Cluster analysis of images was used to define the diameter of PTPN22-R620 clusters, and (**C**) the number of localizations per cluster, for >900 clusters in non-migrating (PLL) and migrating cells (ICAM-1); **** P<0.0001. (**D**) Ripley's K-function curves (mean ± SEM) were generated to quantify the degree of clustering for non-migrating (PLL, solid line) and migrating T-cells (ICAM-1, dashed line) expressing PTPN22-R620.

Fig. 3. Retention of PTPN22-W620 at the membrane is impaired. Primary T-cell blasts were generated from peripheral blood of PTPN22-R620 and W620 homozygous donors and layered onto PLL or ICAM-1 coated plates for 20 min. prior to fixing, permeabilisation and staining with mouse anti-PTPN22 antibodies. N-STORM images were acquired as described for Fig. 2, and cluster analysis used to define (**A**) the number of PTPN22-R620 and W620 clusters per region (n=126 regions), (**B**) the percentage of PTPN22 localizations in clusters (n=126 clusters) and (**C**) the number of PTPN22 localizations per region (n=126 regions). Bars represent mean values ± SD; * P<0.02, **** P<0.0001, ns – not significant. (**D**) Pointillist (Gaussian fitted) and pseudo-coloured heat maps are representative of cluster data acquired after processing of N-STORM image-regions, as described for Fig. 2A. Scale bars, 5 μm for PLL and 10 μm for ICAM-1. (**E**) Ripley's K-function curves (mean ± SEM) were constructed to quantify the degree of clustering of PTPN22-R620 (dashed line) and PTPN22-W620 (solid line) in T-cells migrating on ICAM-1. Data are representative of three independent experiments. (**F**) Csk immunoprecipitates were generated from non-migrating (PLL) or migrating (ICAM-1) T-cells expressing PTPN22-R620 (RR) or W620 (WW) derived from homozygous donors, and probed with goat anti-PTPN22 antibodies, or anti-Csk to control for protein loading. Quantification of PTPN22/Csk association relative to PLL for 3 independent experiments is shown (right panel). Bars represent mean values; Tukey's Ordinary One-Way Anova multiple comparisons test *** P<0.0005, ** P<0.005, ns – not significant.

Fig. 4. PTPN22 colocalizes with LFA-1 at the leading edge of migrating T-cells. (**A**) T-cells migrating on ICAM-1 were stained with antibodies to PTPN22 (goat polyclonal) and LFA-1 (mab38) and imaged by (**A**) confocal microscopy, or (**B**) TIRF microscopy. PTPN22 (green) and

LFA-1 (red) co-localize at the leading edge. Direction of migration is shown (white arrows). In B, higher magnification shows co-localization of PTPN22 and LFA-1 (panel B merge, small white arrows). Bright field images with intensity scales for PTPN22 and LFA-1 stained cells are also shown (right panels). Data are representative of regions selected from the leading edge of 30-40 cells in 4 independent experiments. (**C**) Expression of LFA-1 and PTPN22 in anti-LFA-1 or IgG control immunoprecipitates derived from non-migrating (PLL) and migrating (ICAM-1) T-cells was determined by immunoblotting. (**D**) Jurkat T-cells and their JCam1.6 Lck-deficient derivative were plated onto PLL for 20 min. Cell lysates were prepared and probed with antibodies to Lck and ZAP-70 (upper panel). Mouse anti-PTPN22 immunoprecipitates were generated from both Jurkat lines and probed with antibodies to PTPN22 (goat) and LFA-1 (lower panel).

Fig. 5. Expression of the PTPN22-W620 variant enhances LFA-1 clustering at the leading edge of migrating T-cells. (**A**) T-cells from homozygous donors expressing PTPN22-R620 or PTPN22-W620 were layered onto PLL or ICAM-1 coated plates for 20 min. prior to fixing and staining with anti-LFA-1. Images were acquired and molecule distributions analysed as described in Figure 2. For each PTPN22 variant representative images are shown for cell surface LFA-1 molecule distributions. Single regions were used to generate pointillist and pseudo-coloured cluster heat maps. Scale bar 5 μm. (**B**) Ripley's K-function curves (mean ± SEM) were constructed to quantify the degree of clustering of surface LFA-1 in PTPN22-R620 (dashed line) and PTPN22-W620 (solid line) expressing T-cells migrating on ICAM-1. Data are representative of three independent experiments. (**C**) Cluster analysis of N-STORM images derived from PTPN22-R620 and PTPN22-W620 expressing T-cells migrating on ICAM-1 was used to

quantify the number of LFA-1 localizations per region (n = 83 regions), (**D**) number of LFA-1 clusters (n = 83 regions), and (**E**) the % of LFA-1 localizations in clusters (n = 83 clusters). Data represent mean values ± SD. ns – not significant, **** P<0.0001.

Fig. 6. T-cells expressing loss-of-function/expression PTPN22 mutants are more adherent under shear-flow. (**A**) PTPN22-R620 and PTPN22-W620 expressing T-cells derived from homozygous donors were flowed over glass slides coated with ICAM-1 (5 μg/ml) at a shear flow rate of 0.5dyn/cm^2 and imaged by time-lapse wide-field microscopy. Cells adhering to ICAM-1 coated glass were counted every minute for total of 8 min. Pooled data for each genotype are derived from 12 independent experiments; * P=0.049. (**B**) T-cells were generated from the lymph nodes of *Ptpn22*$^{+/+}$ and *Ptpn22*$^{-/-}$ littermates and adherence under flow quantified as described for (A). A representative experiment (left), and pooled data (right) show the mean number of adherent cells (± SD) after 10 min. for each genotype, based on 6 independent experiments; ** P=0.002. (**C**) Murine T-cells were prepared as described for (B) and allowed to adhere to ICAM-1 (5μg/ml) coated glass slides for 10 min. Shear force was applied at the indicated flow rates, and time-lapse wide-field movies acquired. Numbers of adherent T-cells were counted every minute for 10 min. Phase decay analysis generated half-life values for cell attachment over time. Data represent 11 independent experiments; ns – not significant, * P=0.0472, ** P=0.005. *** P=0.0008.

Fig. 7. Mechanistic model for regulation of integrin signaling by PTPN22. The transition from a low or intermediate LFA-1 affinity state (middle panel) to a high affinity state (left panel) is characterized by phosphorylation of Lck, ZAP-70 and Vav associated with the β2 chain

cytoplasmic tail of LFA-1. Subsequently, spatiotemporal regulation of LFA-1 signals is mediated by dispersal of PTPN22 and Csk from clusters, disassociation of PTPN22 and Csk from PAG at the membrane, and increased association of PTPN22 with Csk through the P1 domain of PTPN22 and SH3 domain of Csk. PTPN22-Csk complexes target phosphorylated substrates in the LFA-1 adhesome, leading to attenuation of LFA-1 signaling. While declustering of the loss-of-function PTPN22-W620 mutant is preserved, binding of PTPN22-W620 to Csk is impaired (right panel). The mutant phosphatase is not retained at the membrane, and in the absence of membrane proximal binding partners, such as Csk, PTPN22-W620 diffuses away from the membrane. As a consequence, LFA-1 signal intensity is augmented and sustained, further promoting LFA-1 clustering at the cell surface and increasing integrin-dependent adhesion (right panel). The spatiotemporal organization of PTPN22-R620 (PTP), PTPN22-W620 (PTP-W), Csk (Csk), talin (Tal), Lck, Vav1 (Vav), ZAP-70 (Zap) and Kindlin are illustrated. P – denotes phosphorylation on tyrosine residues.