The tyrosine phosphatase PTPN22 discriminates weak self peptides from strong agonist TCR signals

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T cells must be tolerant of self antigens to avoid autoimmunity but responsive to foreign antigens to provide protection against infection. We found that in both naive T cells and effector T cells, the tyrosine phosphatase PTPN22 limited signaling via the T cell antigen receptor (TCR) by weak agonists and self antigens while not impeding responses to strong agonist antigens. T cells lacking PTPN22 showed enhanced formation of conjugates with antigen-presenting cells pulsed with weak peptides, which led to activation of the T cells and their production of inflammatory cytokines. This effect was exacerbated under conditions of lymphopenia, with the formation of potent memory T cells in the absence of PTPN22. Our data address how loss-of-function PTPN22 alleles can lead to the population expansion of effector and/or memory T cells and a predisposition to human autoimmunity.

The maintenance of tolerance in naive T cells requires that the T cell antigen receptor (TCR) signaling machinery discriminate between low-affinity interactions of self peptide and major histocompatibility complex (MHC), which provide survival signals but not activation signals in the periphery1, and signals from pathogen-derived peptides that stimulate effector T cell responses and the development of memory. Transient lymphopenia exacerbates this situation, as stimulation of naive T cells by weak interactions with self peptide–MHC and interleukin 7 (IL-7) combine to drive slow homeostatic proliferation and the conversion of naive T cells to a memory phenotype2-4. Homeostatic population expansion following lymphopenia has been linked to the development of autoimmunity in humans after infection4 or immunosuppressive therapy5,6 and in autoimmunity-prone mice of the nonobese diabetic strain7. That last study showed that transient lymphopenia combined with genetic predisposition in those mice precipitated autoimmune disease7.

Among the genes identified in genome-wide association studies that have been linked to greater susceptibility to autoimmunity are those encoding hematopoietic phosphatases8. It has long been recognized that inhibitory tyrosine phosphatases dampen T cell responses and the development of memory. The cytoplasmic tyrosine phosphatase PTPN22 has attracted much attention, as it is encoded by an allele associated with major histocompatibility complex (MHC), which provide survival signals but not activation signals in the periphery1, and signals from pathogen-derived peptides that stimulate effector T cell responses and the development of memory. Transient lymphopenia exacerbates this situation, as stimulation of naive T cells by weak interactions with self peptide–MHC and interleukin 7 (IL-7) combine to drive slow homeostatic proliferation and the conversion of naive T cells to a memory phenotype2-4. Homeostatic population expansion following lymphopenia has been linked to the development of autoimmunity in humans after infection4 or immunosuppressive therapy5,6 and in autoimmunity-prone mice of the nonobese diabetic strain7. That last study showed that transient lymphopenia combined with genetic predisposition in those mice precipitated autoimmune disease7.

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The tyrosine phosphatase PTPN22 has attracted much attention, as it is encoded by an allele associated with major risk for the development of various autoimmune diseases, including rheumatoid arthritis and type 1 diabetes10. Ptpn22-deficient naive mice accumulate T cells of the effector and/or memory phenotype to a greater extent than do wild-type naive mice11,12. Two groups have independently reported the generation of mice with a knock-in mutation of Ptpn22 that encodes mutant PTPN22 with substitution of tryptophan for the arginine at position 619, a model for a disease-associated PTPN22 single-nucleotide polymorphism13,14. Both reported an effect of the knock-in mutation on T cell homeostasis that was similar to, albeit milder than, that reported for Ptpn22-deficient mice; this suggests the single-nucleotide polymorphism acts, in mice at least, as a loss-of-function allele. On a mixed genetic background, the knock-in mice develop many features of autoimmunity13. These papers suggest that loss of expression or function of PTPN22 affects mainly the activation of effector T cells, as the activation of naive T cells was unaffected. In humans and mice with either PTPN22 variants or Ptpn22−/− alleles, there is population expansion of effector and/or memory T cells. Two key remaining issues are what drives the population expansion of effector and/or memory T cells in the presence of loss-of-function PTPN22 alleles and whether this population expansion contributes to loss of self-tolerance.

We show here that naive T cell responses were influenced by loss of PTPN22. In T cells from mice of the OT-1 strain (which have transgenic expression of an MHC class I–restricted (ovalbumin (OVA)-specific) TCR), PTPN22 was critical in limiting the response to weak agonist peptides but not the response to strong agonist peptides. Ptpn22−/− naïve T cells proliferated more than wild-type naïve cells did, and in contrast to wild-type naïve cells, they acquired full effector function under conditions of lymphopenia. Ptpn22−/− CD8+ cytotoxic T lymphocytes (CTLs) became potentially self-reactive and produced inflammatory cytokines in response to self peptide. Our data identify a vital role for PTPN22 in the ability of the TCR to discriminate among its ligands and provide insight into the mechanism by which this phosphatase acts in human disease.
RESULTS
Enhanced homeostatic proliferation of Ptpn22−/− T cells

Studies of mice with a polyclonal T cell repertoire have shown that in the absence of PTPN22, T cells of the effector and/or memory phenotype accumulate in secondary lymphoid tissues\textsuperscript{11,12}. Whether such population expansion of effector and/or memory cells results from the persistence of cells driven to respond by foreign antigens or high-affinity antigens or whether it stems from a loss of the homeostatic regulation of naive T cells by self antigens or low-affinity antigens has not been determined. The fact that enhanced signaling is apparent only in Ptpn22−/− effector T cells and not in Ptpn22−/− naive T cells would suggest the former\textsuperscript{11,12}. To determine the main cause of T cell dysregulation in this model, we back-crossed mice with global deletion of Ptpn22 (generated by crossing mice with loxP-flanked alleles of Ptpn22 exon 1 and mice with transgenic expression of Cre recombinase driven by the gene encoding prostate (Prm-Cre)) with mice of the H-2K\textsuperscript{b}-restricted OT-I strain with congenital deficiency of alleles of RAG1 (Rag1\textsuperscript{−/−}) progeny (called ‘Ptpn22−/− OT-I’ mice) and assessed their progeny (called ‘Ptpn22−/− OT-I’ mice) here.

Comparison of thymuses from 7-week-old mice showed that Ptpn22−/− OT-I mice had approximately 40% fewer CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive thymocytes than Ptpn22+/+ OT-I mice had (Supplementary Fig. 1a–d). This may have been linked to premature expression of the OT-I TCR, as polyclonal Ptpn22−/− CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive thymocytes are unaffected\textsuperscript{11,12}; however, the differentiation of CD8\textsuperscript{+} single-positive thymocytes appeared to be normal. Thus, Ptpn22−/− and Ptpn22+/+ OT-I single-positive thymocyte subpopulations were equivalent in terms of both cell number (Supplementary Fig. 1d) and phenotype (data not shown). These data were consistent with findings suggesting that disease-associated PTPN22 variants do not affect negative selection\textsuperscript{15}.

The number of CD8\textsuperscript{+} T cells was similar in the lymph nodes of 7-week-old Ptpn22+/+ and Ptpn22−/− OT-I mice (Fig. 1a). Expression of the TCR, the negative regulator CD5, the coreceptor CD8, the integrin CD11a (LFA-1) and the immunoregulatory molecule CD98 by Ptpn22+/+ and Ptpn22−/− OT-I mice was similar, as assessed by flow cytometry (Supplementary Fig. 1e). Overall, both Ptpn22+/+ OT-I cells and Ptpn22−/− OT-I cells maintained a naive phenotype, with uniformly high expression of the adhesion molecule CD62L and the cytokine receptor CD127 (IL-7R\textalpha) and no expression of the activation marker CD69 (Supplementary Fig. 1e). However, there were indications of elevated basal activation in the Ptpn22−/− OT-I T cells. For example, a significantly higher proportion of Ptpn22−/− OT-I T cells than Ptpn22+/+ OT-I T cells expressed Ki67, a marker of proliferation (Fig. 1b,c). The inflammatory chemokine receptor CXCR3 was expressed on 10–30% of Ptpn22−/− OT-I T cells but was expressed on only 3–7% of Ptpn22+/+ OT-I T cells, whereas expression of the activation and memory marker CD44 was very slightly, but consistently, higher on Ptpn22−/− cells than on Ptpn22+/+ cells (Fig. 1d,e). Flow cytometry showed consistent, albeit modest, enhanced staining of the key transcription factors T-bet and eomesoderm (Eomes) in Ptpn22−/− naive OT-I T cells relative to that in their Ptpn22+/+ counterparts (Fig. 1f,g).

The OT-I Rag1−/− background is mildly lymphopenic due to the absence of B cells and CD4\textsuperscript{+} T cells, and we hypothesized that this might account for the signs of activation in the naive Ptpn22−/− OT-I T cells. We reasoned that in a more profoundly lymphopenic setting this effect would be enhanced. We transferred 5 × 10\textsuperscript{6} Ptpn22+/+ (CD45.1\textsuperscript{+}) OT-I T cells and Ptpn22−/− (CD45.2\textsuperscript{+}) OT-I T cells together into Rag1−/− hosts at a ratio of ∼1:1 (Fig. 2a). After 7 d data not shown) and 14 d (Fig. 2b), the number of donor cells recovered was low and the ratio of Ptpn22+/+ cells to Ptpn22−/− cells was equivalent, consistent with the slow homeostatic proliferation that is driven by contact with self ligands in a lymphopenic environment. At 4 weeks after transfer, both Ptpn22+/+ and Ptpn22−/− cells had increased in number (Fig. 2c,d). At this time point, we recovered Ptpn22−/− cells at a significantly greater frequency (Fig. 2c) and number (Fig. 2d).

Slow homeostatic proliferation under conditions of lymphopenia is driven by a combination of self peptide–MHC and IL-7. Typically, the increased availability of IL-7 in a lymphopenic environment amplifies...
the weak TCR signal. To determine whether responsiveness to TCR stimulation or IL-7 was affected by loss of PTPN22, we repeated the cell transfers described above and treated recipients with either PBS or monoclonal antibody (mAb) to the receptor for IL-7 (IL-7R) every 3 d. At 14 d after transfer in the presence of mAb to IL-7R, a significantly higher proportion of Ptpn22−/− cells than Ptpn22+/+ cells had proliferated (Fig. 2b). Although the total number of cells recovered of each genotype was five- to tenfold lower after treatment with mAb to IL-7R (data not shown), which indicated that both cell types were similarly responsive to IL-7 survival signals, blockade of IL-7R clearly revealed that weak TCR signaling drove the enhanced responsiveness of Ptpn22−/− T cells.

To determine if the effect on T cell homeostatic proliferation noted above was a general feature of Ptpn22 deficiency, we used polyclonal CD4+ T cells from two distinct Ptpn22-deficient mouse strains. In mice with germline Ptpn22 deficiency, Ptpn22 is deleted in all cell types, whereas in dLck-Cre mice, deletion of loxP-flanked alleles by Cre recombinase expressed from the distal promoter of the gene encoding the kinase Lck (dLck) occurs in post-positive selection thymocytes. These experiments also addressed whether the activity of polyclonal Ptpn22−/− T cells was due to alterations in thymic selection rather than to an inherent alteration in the responsiveness of naive T cells. We used sublethally irradiated wild-type recipients to avoid the responses to gut antigens that can occur in Rag1−/− recipients and so that we could measure proliferation of transferred polyclonal T cells 'preferentially' in response to homeostatic cytokines and low-affinity self antigen. We obtained naive CD44lo CD4+ T cells from wild-type (CD45.1+) mice, Ptpn22−/− (CD45.2+) mice and dLck Ptpn22−/− (GFP+) mice, labeled the cells with CellTrace Violet dye (to assess proliferation), mixed them and cells at a ratio of 1:1:1 and transferred the mixture into irradiated recipients. After 14 d, we recovered donor cells from the hosts. T cells from both Ptpn22-deficient strains were present in equal proportions and in significantly greater numbers than those of wild-type cells (Fig. 2e). Furthermore, flow cytometry showed that proportionally fewer wild-type cells had completely diluted the dye (Fig. 2f), indicative of slower rates of proliferation.

The data reported above showed that lymphopenia drove the proliferation of naive polyclonal and OT-1 Ptpn22−/− T cells to a greater extent than their Ptpn22+/+ counterparts. The absence of cognate antigen and the slow rate of proliferation in the OT-1 model, together with the increased proliferation of Ptpn22−/− cells in the presence of blockade of IL-7R, suggested that this population expansion was driven by low-affinity self peptide–MHC.

PTPN22 restrains TCR signaling induced by weak agonists

We set out to test directly the hypothesis that PTPN22 expression is essential for the regulation of TCR responses to low-affinity peptide–MHC. Many altered peptide ligands that vary in their potency to stimulate OT-I thymocytes and T cells have been characterized. We assessed the effect of Ptpn22 deficiency in the response of naive T cells to three peptides: the agonist peptide SIINFEKL (N4), the partial agonist peptide SIITFEKL (T4), and the very weak agonist SIIIFGEL peptide (G4). Of note, the T4 ligand is a 'threshold agonist' for the negative selection of OT-I thymocytes; such ligands are thought to be particularly relevant for autoimmunity.

To investigate early signaling, we mixed Ptpn22−/− (CD45.1+) OT-I cells and Ptpn22−/− OT-I (CD45.2+) cells at a ratio of 1:1, stimulated the cells in vitro with N4, T4 or G4 peptide and measured phosphorylation of the mitogen-activated protein kinase Erk by flow cytometry. The proportion of OT-I cells containing phosphorylated Erk was maximal by 15 min of stimulation with N4 and had reached a plateau at that time, and the kinetics and magnitude of this response were equivalent for Ptpn22+/+ and Ptpn22−/− OT-I cells (Fig. 3a). At these early time points, stimulation with either T4 peptide or G4 peptide induced only a very low proportion of cells with phosphorylated Erk (Fig. 3a), consistent with reports that weaker agonists induce the activation of mitogen-activated protein kinase Erk with delayed kinetics. At later time points, between 30 and 120 min after stimulation with T4 or G4 peptide, significantly more Ptpn22−/− OT-I cells than Ptpn22+/+ OT-I cells were positive for phosphorylated Erk (Fig. 3b). Furthermore, weak agonist–induced activation of the kinase p90 ribosomal S6 kinase (RSK), which is downstream of Erk, was also significantly enhanced in the absence of PTPN22 at these time points (Fig. 3d).

TCR-induced activation of the GTPase Ras–mitogen-activated protein kinase pathway is important for upregulation of expression of the early activation marker CD69 (ref. 23). As expected, 4 h of stimulation with the strong agonist peptide N4 induced high expression of CD69 (Fig. 3e), with Ptpn22−/− and Ptpn22−/− OT-I cells responding similarly over a range of peptide concentrations (Fig. 3f). Consistent with their enhanced phosphorylation of Erk, naive Ptpn22−/− OT-I cells

Figure 2  Lymphopenia-induced proliferation is inhibited by PTPN22. (a) Flow cytometry of cells from Ptpn22+/+ (CD45.1+) and Ptpn22−/− (CD45.2+) OT-I donor mice (used in b–d). Numbers adjacent to outlined areas indicate percent CD45.1− (Ptpn22+/+) cells (left) or CD45.1+ (Ptpn22−/−) cells (right). (b) Frequency of Ptpn22+/+ and Ptpn22−/− donor cells recovered from the lymph nodes and spleens of Rag1−/− recipient mice given intravenous transfer of cells from donor mice as in a (mixed at a ratio of 1:1:1) and then treated with PBS (left) or mAb to IL-7R (right), assessed 14 d after cell transfer. (c,d) Frequency (c) and absolute number (d) of Ptpn22+/+ and Ptpn22−/− donor cells recovered from lymph nodes and spleens of recipient mice at 30 d after cell transfer as in b. Each symbol (b–d) represents an individual host mouse (n = 5 per group); small horizontal lines indicate the mean. (e) Frequency of donor cells recovered from lymph nodes and spleens of irradiated CD45.1+CD45.2+ F1 recipient mice 14 d after transfer of CellTrace Violet–labeled polyclonal naive CD4+ T cells from wild-type (CD45.1+), Ptpn22−/− (CD45.2+) and dLck Ptpn22−/− (GFP+) donor mice (mixed at a ratio of 1:1:1) (Day 14), and frequency of donor cells in the injection mixture before transfer (Inj mix). Each symbol represents an individual host mouse (n = 4 per group); small horizontal lines indicate the mean. (f) Dilution of CellTrace Violet (CTV) in cells recovered from recipient mice as in e at day 14 after transfer. Numbers in plots indicate percent cells in which CellTrace Violet dye had been completely diluted (bracketed lines). *P < 0.05, **P < 0.01 and ***P < 0.001 (Student’s t-test). Data are from one experiment of at least two experiments.
were more responsive than were Ptpn22+/+ OT-I cells to stimulation with T4 or G4 peptide, as indicated by a substantially greater proportion of CD69+ cells in the population of CD69+ cells in the Ptpn22−/− population (Fig. 3e,f). These data showed that PTPN22 suppressed early biochemical signals in response to weak ligands without decreasing the response to strong agonist ligand.

TCR-induced expression of transcription factors regulates the fate of CD8+ T cells. Expression of the transcription factors T-bet and c-Myc is required for the differentiation of protective effector T cells, whereas expression of Eomes is associated with the formation of memory cells. The induced expression of T-bet and c-Myc in both Ptpn22+/+ and Ptpn22−/− OT-I cells was directly correlated with the strength of signaling received via the TCR (N4 > T4 > G4) (Fig. 4a and Supplementary Fig. 2a,b). T-bet and c-Myc had higher expression in Ptpn22−/− T cells than in Ptpn22+/+ cells in response to all three peptide ligands, although the difference in the upregulation of c-Myc expression in response to the weakest stimulus, G4, did not reach statistical significance (Fig. 4a and Supplementary Fig. 2a,b). In contrast, Eomes expression was inversely correlated with strength of signal (N4 < T4 < G4), and stimulation with N4 or T4 induced higher expression of Eomes in Ptpn22+/− OT-I cells than in Ptpn22+/+ OT-I cells, whereas stimulation with the weakest G4 peptide resulted in equivalent upregulation Eomes expression in cells of the two genotypes (Fig. 4a and Supplementary Fig. 2c). Therefore, the greatest difference between Ptpn22−/− cells and Ptpn22+/+ cells in their upregulation of expression of the memory-associated Eomes protein was evident after stimulation with the strong agonist N4, whereas for the other transcription factors, it was particularly apparent after stimulation with a weak agonist.

The transcription factor IRF4 has been linked to the 'preferential' population expansion and effector function of high-affinity CD8+ T cell clones. IRF4 expression is upregulated proportionally to the strength of TCR signaling, while forced IRF4 expression facilitates the population expansion of CD8+ T cells in response to ligands of lower affinity. In response to the weak ligands T4 and G4, there was significantly higher expression of IRF4 in Ptpn22+/− OT-I cells than in Ptpn22+/+ OT-I cells, whereas equivalent upregulation of IRF4

Figure 3 PTPN22 limits triggering of the TCR induced by weak agonists in naive T cells. (a) Frequency of CD8+ T cells containing Erk phosphorylated at Thr202 and Tyr204 (p-Erk(T202,Y204)) among Ptpn22+/+ and Ptpn22−/− OT-I cells stimulated (Stim) for 0–30 min (horizontal axis) with 1 µM N4, T4 or G4 peptide (key). (b) Intracellular staining of phosphorylated Erk (as in a) and of RSK phosphorylated at Thr359 and Ser363 (p-RSK(T359,S363)) in Ptpn22+/+ and Ptpn22−/− OT-I cells left unstimulated (US) or stimulated (Stim) for 2 h with 1 µM T4 or G4 peptide (key). P < 0.05 (unpaired Student’s t-test). (c, d) Time course for the activation of Erk and RSK in Ptpn22−/− and Ptpn22+/+ OT-I cells stimulated for 0–120 min (horizontal axis) with T4 or G4 peptide (key), assessed as phosphorylation as in b. (e) Surface expression of CD69 on Ptpn22+/+ and Ptpn22−/− OT-I cells left unstimulated (basal expression) or stimulated for 4 h with 1 µM N4, T4 or G4 peptide (key). (f) Frequency of CD69+ cells among Ptpn22−/− and Ptpn22−/− OT-I cells stimulated with various concentrations (horizontal axis) of N4, T4 or G4 peptide (key). Data are from one experiment representative of at least three experiments (mean and s.d. of three replicates in c–d).

Figure 4 PTPN22 inhibits TCR-induced metabolic changes, transcription factor expression and proliferation. (a, b) Expression of T-bet, c-Myc and Eomes (a) and IRF4 (b) in Ptpn22+/+ and Ptpn22−/− Rag1−/− OT-I cells stimulated for 24 h with 1 µM N4, T4 or G4 peptide, assessed by flow cytometry. (c) Size (forward scatter (FSC)) and glucose uptake of Ptpn22+/+ and Ptpn22−/− Rag1−/− OT-I cells stimulated as in a,b, assessed by flow cytometry, AU, arbitrary units. (d) Dilution of CellTrace Violet dye in Ptpn22+/+ and Ptpn22−/− Rag1−/− OT-I cells left unstimulated or stimulated for 48 h with N4, T4 or G4 peptide. *P < 0.01 and **P < 0.001 (two-tailed unpaired Student’s t-test). Data are from one experiment of four (a–c) or three (d) experiments (mean and s.d. of three replicates in a–c).
expression occurred in response to the strong ligand N4 (Fig. 4b and Supplementary Fig. 2d).

IRF4 has been shown to regulate the majority of the TCR affinity-driven transcriptional changes in CD8+ T cells, including those of genes encoding molecules required for key metabolic functions as the cells shift from oxidative phosphorylation to aerobic glycolysis to cope with the bioenergetics demands involved in proliferation and the development of effector function 28. In keeping with this, we found that when stimulated with the weak agonist peptides T4 and G4, Ptpn22−/− OT-I cells were larger than their Ptpn22+/+ counterparts, as indicated by increased intensity of forward scatter, and had elevated uptake of glucose (Fig. 4c and Supplementary Fig. 2e,f). In contrast, stimulation of Ptpn22+/+ and Ptpn22−/− OT-I T cells with N4 resulted in equivalent increases in both forward scatter and glucose uptake (Fig. 4c and Supplementary Fig. 2e,f). Furthermore, stimulation for 24 h with weak agonists also induced higher expression of the transferrin receptor CD71 (Supplementary Fig. 2g), but not of the amino acid transporter CD98 (Supplementary Fig. 2h), by Ptpn22−/− OT-I cells. These data were consistent with the proposal that the greater upregulation of IRF4 expression in Ptpn22−/− T cells contributed to their ‘preferential’ activation in response to weak ligands. We assessed the effect of Ptpn22 deficiency on TCR-induced proliferation as dilution of CellTrace Violet dye. As has been reported for polyclonal T cells activated with mAb to the invariant signaling protein CD3 and mAb to the coreceptor CD28 (ref. 12), after 48 h of stimulation with peptide, Ptpn22−/− OT-I cells proliferated to a greater extent than did Ptpn22+/+ OT-I cells (Fig. 4d). Together these data indicated that in naive Ptpn22+/+ T cells, PTPN22 acted to restrain TCR-induced cell growth, metabolism, the expression of transcription factors and proliferation. For many parameters, these effects were most evident after stimulation with a weak agonist rather than after stimulation with a strong agonist and provided a basis for the observed population expansion of effector and/or memory cells in the absence of PTPN22.

**PTPN22 limits cytokines induced by weak agonists**

Thus far, our data linked PTPN22 to regulation of the response of naïve T cells to low-affinity antigens, with few differences between Ptpn22−/− cells and Ptpn22+/+ cells in their activation in response to high-affinity antigens. Published data have suggested that PTPN22 is particularly important in regulating effector T cell responses 11,12; therefore, we generated effector Ptpn22+/+ and Ptpn22−/− OT-I CTLs and assessed their recall responses to N4 and to the variant peptides, T4 and G4. To generate CTLs in vivo, we mixed naïve Ptpn22+/+ (CD45.1+) OT-I cells with Ptpn22+/+ (CD45.2+) OT-I cells at a ratio of 1:1 and transferred the mixture into Rag1−/− recipient mice given transfer of Ptpn22+/+ (CD45.1+) Rag1−/− OT-I cells and Ptpn22−/− (CD45.2+) Rag1−/− OT-I cells (mixed at a ratio of ~1:1), followed by infection of host mice with LM-OVA and analysis at day 7 after infection (Day 7), and frequency of donor cells in the injection mixture before transfer (Inj mix). Each population is represented by an individual host mouse (n = 6 per group); small horizontal lines indicate the mean. (b–d) Frequency of IFN-γ− cells among CTLs obtained from host mice as in a and restimulated for 4 h ex vivo with 1 μM N4 (b), T4 (c) or G4 (d). Symbols connected by lines indicate paired Ptpn22+/+ and Ptpn22−/− CTL samples from the same host mouse. (e–g) Frequency of IFN-γ− cells among Ptpn22+/+ and Ptpn22−/− CTLs generated in vitro by stimulation with N4 peptide followed by population expansion and differentiation with IL-2, then restimulated for 4 h with N4 (e), T4 (f) or G4 (g). Each symbol represents an individual mouse (n = 3 per group); small horizontal lines indicate the mean. (h) Expression of IFN-γ and TNF in Ptpn22+/+ and Ptpn22−/− CTLs generated as in e–g and then restimulated with 10 μM RTYTYEKL. Numbers in quadrants indicate percent cells in each. (i,j) Frequency of IFN-γ− cells among Ptpn22+/+ and Ptpn22−/− CTLs generated in vitro by stimulation with N4 peptide, followed by population expansion and differentiation with IL-2 (i) or IL-15 (j) and restimulation with various concentrations (horizontal axis) of RTYTYEKL. ND, not detectable. *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed paired Student’s t-test). Data are from two experiments (a–d) or at least four experiments (e–j).
genotypes (Supplementary Fig. 3c), which suggested that the differentiation of CTLs under these optimal in vitro conditions after stimulation with strong agonist was not overtly altered in the absence of PTPN22. Nonetheless, restimulation revealed the same bias as before; the weak agonists T4 and G4 stimulated substantially more Ptpn22<sup>−/−</sup> CTLs than Ptpn22<sup>+/+</sup> CTLs to produce IFN-γ (Fig. 5e–g), the inflammatory cytokines TNF and GM-CSF and the inflammatory chemokine CCL3 (Supplementary Fig. 4), but the strong agonist N4 did not.

Given the ability of weak antigens to stimulate responses by naive Ptpn22<sup>−/−</sup>-T cells, we sought to determine whether Ptpn22<sup>−/−</sup> effector CTLs had the ability to break tolerance in vitro. A peptide derived from β-catenin, RTTYEKL, has been demonstrated to act as an endogenous positively selecting ligand for OT-I thymocytes, whereas mature naive OT-I T cells are completely unresponsive to this peptide<sup>2</sup>. We generated OT-I CTLs as described above and restimulated them with RTTYEKL. The response of Ptpn22<sup>−/−</sup>-CTLs to the self peptide was much greater than that of Ptpn22<sup>+/+</sup> CTLs, with a fivefold and threefold greater frequency of Ptpn22<sup>−/−</sup> cells than Ptpn22<sup>+/+</sup> cells producing IFN-γ and TNF, respectively (Fig. 5i,ii). Finally, we assessed the recall responses of CTLs grown in vitro under less-inflammatory conditions; that is, CTLs differentiated in the presence of IL-15. Under these conditions, Ptpn22<sup>−/−</sup> CTLs were unresponsive to restimulation with the self peptide RTTYEKL, whereas a small but detectable proportion of Ptpn22<sup>−/−</sup> cells responded by producing IFN-γ (Fig. 5j). Together these data indicated that in effector CTLs, PTPN22 was critical for regulating TCR sensitivity, and deficiency in PTPN22 became permissive for the production of an inflammatory response to self antigen.

### Homeostatic proliferation–induced Ptpn22<sup>−/−</sup> memory T cells

Having determined a notable role for PTPN22 in the ability of the TCR to discriminate among its ligands in both naive CD8<sup>+</sup> T cells and effector CD8<sup>+</sup> T cells, we addressed the role of this phosphatase in memory cells. We mixed naive CD45-congenic Ptpn<sup>22+/+</sup> OT-I T cells and Ptpn<sup>22−/−</sup> OT-I T cells at a ratio of 1:1 and transferred the mixture into Rag1<sup>−/−</sup> recipients, which we either infected with LM-OVA or left uninfected. After 30 d, both Ptpn<sup>22+</sup> effector cells and Ptpn<sup>22−/−</sup> cells were predominantly of a CD44<sup>hi</sup> memory phenotype, with similar expression of CD62L, KLRG1 and CD127 regardless of whether the host was infected with LM-OVA or not (data not shown). In uninfected control hosts in which the cells had expanded their populations in response to lymphopenia only, more Ptpn<sup>22−/−</sup> OT-I T cells than Ptpn<sup>22+/+</sup> OT-I cells produced IFN-γ upon restimulation with each peptide (N4, T4 or G4) in vitro, and this was particularly notable upon restimulation with the weaker ligands T4 and G4 (Fig. 6a–c). Ptpn<sup>22−/−</sup> memory cells induced by infection with the strong agonist LM-OVA had IFN-γ recall responses to T4 that were significantly greater than those of Ptpn<sup>22+/+</sup> memory cells, although this difference was smaller than the difference between Ptpn<sup>22+/+</sup> and Ptpn<sup>22−/−</sup> naive and effector CD8<sup>+</sup> T cells (Fig. 6d–f). Furthermore, the recall responses of LM-OVA-induced Ptpn<sup>22+/+</sup> and Ptpn<sup>22−/−</sup> memory cells to N4 or G4 peptide were equivalent (Fig. 6d–f). Of note, in the Rag1<sup>−/−</sup> hosts, lack of competition from endogenous cells diminishes contraction of the antigen-specific effector cell populations after pathogen elimination in the lymphopenic host. Therefore, the proinflammatory capacity of the LM-OVA-induced Ptpn<sup>22+/+</sup> and Ptpn<sup>22−/−</sup> memory cells may have been influenced by the lymphopenic environment in which they were generated. Overall, these memory cells were more similar in their response to altered peptide ligands than were Ptpn<sup>22+/+</sup> or Ptpn<sup>22−/−</sup>-naive and effector T cells.

The data reported above suggested that for Ptpn<sup>22+/+</sup> cells, PTPN22 limited the generation of fully functional memory CD8<sup>+</sup> T cells in the context of lymphopenia but not in the context of strong antigen or inflammation. Consistent with this, fewer Ptpn<sup>22−/−</sup>/memory CD8<sup>+</sup> T cells were able to produce IFN-γ when they were induced by lymphopenia than when they were induced by LM-OVA (Fig. 6g). In contrast, Ptpn<sup>22−/−</sup> memory cells induced by antigen or homeostatic proliferation were equally effective at producing IFN-γ in response to either weak peptide or strong peptide (Fig. 6h). Thus, in the absence of PTPN22 expression, homeostatic proliferation was sufficient to induce the development of highly inflammatory memory CD8<sup>+</sup> T cells, whereas in PTPN22-sufficient cells, this process needed initial stimulation with cognate antigen.

### PTPN22 regulates LFA-1-dependent adhesion

The stimulation of Ptpn<sup>22−/−</sup>-effector T cells via the TCR results in increased activation of the guanine nucleotide–exchange factor Rap1, which links ‘inside-out’ TCR stimulation to the function of the integrin CD11a (LFA-1)<sup>11</sup>. We hypothesized that the greater sensitivity of naive and effector Ptpn<sup>22−/−</sup>-OT-I T cells to weak ligands may have been associated with an enhanced ability to form stable

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**Figure 6** PTPN22 limits the proinflammatory capacity of memory T cells induced by homeostatic proliferation. (a–f) Frequency of IFN-γ<sup>+</sup> cells among Rag1<sup>−/−</sup>-OT-I memory T cells recovered from Rag1<sup>−/−</sup>-recipient mice 30 d after transfer of CD45-congenic donor Ptpn<sup>22+/+</sup> or Ptpn<sup>22−/−</sup>-OT-I T cells and Ptpn<sup>22+/+</sup> or Ptpn<sup>22−/−</sup>-effector T cells (mixed at a ratio of 1:1) followed by no infection (and thus homeostatic (lymphopenia-induced) proliferation; a–c) or infection of the host with LM-OVA (d–f), with restimulation of the recovered cells in vitro with various concentrations (horizontal axes) of N4 (a,d), T4 (b,e) or G4 (c,f). (g,h) Frequency of IFN-γ<sup>+</sup> cells among Ptpn<sup>22+/+</sup> memory cells (g) or Ptpn<sup>22−/−</sup> memory cells (h) induced by LM-OVA (Ag) or homeostatic proliferation (HP) after transfer into Rag1<sup>−/−</sup> recipients as in a–f, followed by restimulation with N4, T4 or G4 (horizontal axes). Each symbol represents an individual mouse (n = 5 per group); small horizontal lines indicate the mean. *P < 0.05, **P < 0.01 and ***P < 0.001 (paired a–f) or unpaired (g,h) Student’s t-test. Data are from one experiment representative of two experiments.
LFA-1-dependent cell contacts. Using a flow cytometry–based assay, we monitored the formation of conjugates of naive OT-I T cells and peptide-loaded antigen-presenting cells (APCs). Neither Ptpn22−/− OT-I cells nor Ptpn22−/− OT-I cells formed large numbers of conjugates with APCs loaded with the irrelevant control peptide SIY, whereas at all time points assessed, Ptpn22+/+ and Ptpn22−/− OT-I T cells were equally efficient at forming conjugates with N4-loaded APCs (Fig. 7a). At later time points (2–4.5 h), Ptpn22−/− OT-I cells formed significantly more conjugates with T4- or G4-loaded APCs than did Ptpn22+/+ OT-I cells (Fig. 7a). The formation of conjugates with peptide-loaded APCs was blocked by mAb to LFA-1 (Fig. 7a), which showed that this integrin was central to this interaction. To confirm that the greater stability of conjugates was functionally relevant, at various times after stimulation we disrupted conjugates by adding blocking antibody to LFA-1. Blockade of LFA-1 after 30–60 min of stimulation with G4 peptide had a more profound effect on the responsiveness of Ptpn22−/− OT-I cells than on that of Ptpn22+/+ OT-I cells, while such blockade did not affect the response of cells of either genotype to stimulation with the strong agonist N4 (Fig. 7b).

We assessed whether PTPN22 influenced the activity of LFA-1 in CTLs as well as in naive T cells by monitoring the adhesion of Ptpn22+/+ or Ptpn22−/− OT-I CTLs to the LFA-1 ligand ICAM-1 under conditions of flow. We obtained approximately 30% more adherent Ptpn22−/− T cells than adherent Ptpn22+/+ cells under these conditions (Fig. 7c). These data indicated that PTPN22 regulated LFA-1-dependent adhesion in a manner that was important for limiting the formation of conjugates of both naive T cells and effector T cells with APCs presenting weak and/or self antigens; the function of PTPN22 in wild-type mice is therefore to limit these responses while not impairing robust responses to strong agonist antigens.

**DISCUSSION**

The ability of T cells to discriminate low-affinity interactions versus high-affinity interactions is critical for the induction of protective immunity and the maintenance of self-tolerance. We found here that PTPN22 acted to limit the activation of naive and effector T cells and the development of memory T cells in response to very weak antigens and self antigens. The early responses of naive OT-I T cells to weak agonist peptides were enhanced in the absence of PTPN22, whereas the responses of PTPN22-deficient cells to strong agonist peptides were indistinguishable from those of PTPN22-sufficient cells, as shown before following stimulation with anti-CD3 and anti-28 (refs. 10–12). Thus, PTPN22 is a critical regulator of the ability of the TCR to discriminate among its ligands: it permits the activation of T cells by cognate antigens while restraining potentially damaging inflammatory responses to weaker ligands, which may include autoantigens.

In contrast to early responses, such as the activation of Ras and Erk, cell growth and glucose uptake, the later proliferative responses of Ptpn22−/− T cells to stimulation with peptides of all affinities were enhanced. Therefore, in addition to having a role in initial TCR signaling pathways, in which it acts as a brake on responses to weak agonists, PTPN22 has a more general role in tempering population expansion following activation. The latter role is coincident with the upregulation of PTPN22 expression during effector cell differentiation.

We have provided evidence that regulation of the ‘inside-out’ signaling pathway by PTPN22 is important for the discrimination of ligands, as the ability of Ptpn22−/− T cells to form LFA-1-dependent conjugates with APCs pulsed with low-affinity peptide was enhanced. PTPN22 negatively regulates the activation of T cells, at least in part, by dephosphorylating critical tyrosine residues in TCR-proximal kinases such as Lck and Zap70 (ref. 33); that, in turn, limits TCR-induced activation of Rap1 and subsequent ‘inside-out’ signaling to LFA-1 (ref. 11). Elevated Rap1 activity has been shown to enhance the activation and proliferation of T cells (ref. 34). We found that greater formation of conjugates by naive PTPN22-deficient T cells was directly correlated with enhanced responses to weak ligands, which influenced downstream expression of the transcription factors c-Myc, T-bet and IRF4.

IRF4 has been shown to be an important ‘rheostat’ of TCR affinity that limits the response of OT-I T cells to weak agonists (ref. 28). Consistent with that, upregulation of IRF4 expression and downstream metabolic and effector cell function was enhanced in response to weak agonists in Ptpn22−/− mice. Conversely, Ptpn22−/− mice had higher Eomes expression in response to the stronger ligands that might favor the production of long-lived memory cells. Our data fit with a model in which wild-type cells, PTPN22 limits T cell responses to weak agonists by restraining TCR and ‘inside-out’ signaling pathways and thereby diminishes upregulation of the expression of critical transcription factors such as IRF4 and restrains effector cell differentiation.

The activation of T cells in response to weak antigens needs to be restrained, particularly in conditions of lymphopenia, as homeostatic proliferation is associated with the development of autoimmunity (ref. 21).
The secondary autoimmunity that develops in patients with relapsing-remitting multiple sclerosis after leukocyte-depletion therapy with alemtuzumab has been shown to be a consequence of the homeostatic proliferation–induced population expansion of T cells. Many genes linked to susceptibility to autoimmunity that have been identified in genome-wide association studies encode molecules that are involved in T cell regulation and can increase the risk of lymphopenia-induced autoimmunity. The PTPN22 variant that encodes a product with substitution of tryptophan for the arginine at position 620 is a risk allele for the development of several autoimmune diseases, although the mechanism of this predisposition is unknown. Mouse studies have shown that homozygous expression of the variant Ptpn2 allele results in a phenotype similar to that of PTPN22 deficiency, albeit with slightly milder characteristics, and is sufficient for autoimmunity to develop spontaneously in an autoimmune-prone genetic background. Our data have shown that PTPN22 both limited the population expansion of T cells and restrained the gain of proinflammatory function under homeostatic proliferation conditions. It is possible that the expression of disease-associated PTPN22 alleles in the context of transient or chronic lymphopenia in human patients might increase the probability that autoimmune processes will develop.

PTPn22+/− and Ptpn22−/− memory T cells induced by high-affinity antigen during infection were similar in their ability to produce proinflammatory cytokines. Thus, PTPN22 function was important in restraining the development of inflammatory memory cells in response to homeostatic proliferation without markedly impeding their development during infection. PTPN22 was previously thought to have a role in maintaining tolerance exclusively in effector T cells. The importance of this role is emphasized by the finding that a self peptide derived from β-catenin was sufficient to induce abundant production of inflammatory cytokines in Ptpn22−/− CTLs but not in Ptpn22+/− CTLs. Therefore, low expression of PTPN22 in naive T cells is sufficient and necessary to limit TCR activation in response to partial and weak agonists, whereas high PTPN22 expression in effector cells is needed to prevent responses of highly sensitive effector T cells to true self antigens.

A related phosphatase, PTPN2, has been reported to restrain the homeostatic proliferation of mouse T cells and regulate responses to low affinity peptides. Given that both PTPN2 and PTPN22 target proximal signaling molecules, it is perhaps not unexpected that their absence in mice would produce similar phenomena. Moreover, variants of the genes encoding both PTPN2 and PTPN22 have been identified in genome-wide association study screens as being linked with the same autoimmune diseases. Further study of the physiological roles of these regulatory proteins and their disease-associated variants are thus of fundamental importance for improved understanding of the mechanisms of activation of the immune system, tolerance and autoimmunity.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.J.S. designed and did most in vitro experiments; R.I.B. designed and did most in vivo experiments and in vitro analyses of the formation of T cell conjugates; V.L.M. designed and did analyses of adhesion under shear flow; R.Z. designed experiments and led the overall project; and R.J.S. and R.Z. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice, cell transfer and infection with *L. monocytogenes*. The progeny of mice with loxP-flanked alleles of *Ptpn22* exon 1 and *Prm-Cre* mice (with transgenic expression of Cre recombinase driven by regulatory elements of the gene encoding protamine), a cross that results in mice with global *Ptpn22* deficiency, backcrossed ten times to the C57BL/6J background have been described; those mice were crossed to a Rag1−/− OT-I background.19 Mice with loxP-flanked alleles of *Ptpn22* exon 1 were crossed with the dLck-Cre strain.20 CD3ε-deficient (Gd3ε−/−), Rag1−/−, C57BL/6J, CD45.1+ and CD45.2+ mice were bred in-house at the University of Edinburgh. In some experiments, recipient mice were sublethally irradiated before cell transfer. For cell transfer, 2.5 × 105 to 5 × 105 CD45-congenic *Ptpn22*+/+ T cells and *Ptpn22*−/− OT-I T cells or sorted naive CD4+ T cells were mixed at a ratio of 1:1 and were injected intravenously into recipient mice. In some experiments, mice were infected intravenously with 1 × 106 colony-forming units of an attenuated strain of OVA-expressing *L. monocytogenes* with deletion of the actin assembly–inducing protein ActA21 (a gift from H. Shen). Age-matched (7–12 weeks) and sex-matched mice were used in all experiments. Where stated, groups of mice given intraperitoneal administration of 300 μg mAb to IL-7R (A7R34; a hybridoma prepared in-house) every second day over 4 weeks were used: phycoerythrin–anti-CD4 (RM4.5), allophycocyanin–anti-CD4 (eFluor 450–anti-CD62L (MEL-14), phycoerythrin–anti-CD25 (PC61.5), phycoerythrin–indotricarbocyanine–β CD127 (A7R34), Alexa Fluor 488–anti-IFN-γ (H129.19), allophycocyanin–anti-CD62L (MEL-14), phycoerythrin–anti-CD69 (H1.2F3), fluorescein isothiocyanate–anti-CD62L (MEL-14), phycoerythrin–anti-CD4 (RM4.5), Alexa Fluor 488–anti-CD25 (PC61.5), phycoerythrin–anti-CD132 (554457) (all BD Biosciences), Pacific Blue–anti-CD45.2 (104), Brilliant Violet 421–anti-CD45.2 (104), Pacific Blue–anti-CD45.2 (104), allophycocyanin–anti-CD69 (H1.2F3), allophycocyanin–anti-CD73 (TY/11.8), Brilliant Violet 421–anti-CD127 (A7R34), Alexa Fluor 488–anti-IFN-γ (XM1G1.2) and peridinin chlorophyll protein–cyanine 5.5–anti-CXCR3 (H57–597), allophycocyanin–anti-KLRG1 (2F1), peridinin chlorophyll protein–cyanine 5.5–anti-ICAM-1. Effector CD8+ T cells at a density of 1 × 106 cells per ml in adhesion medium (RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, l-glutamine, antibiotics and 50 μM 2-mercaptoethanol) were expanded and/or they were differentiated for a further 4 d in 20 ng/ml recombinant human IL-2 or mouse IL-15 (Peprotech). For cytokine recall responses, cells were restimulated for 4 h in the presence of 2.5 μg/ml brefeldin A (Sigma) before fixation and intracellular staining (antibodies identified above). For analysis of glucose uptake, following 24 h of stimulation with peptide, cells were incubated for 45 min with 10 μM 2-NBDG, then were washed twice in PBS before fixation in 2% paraformaldehyde and analysis by flow cytometry. Relative glucose uptake was calculated as the mean fluorescence intensity of 2-NBDG staining after stimulation, with subtraction of background fluorescence.

Flow cytometry and antibodies. The following conjugated antibodies were used: phycoerythrin–anti-CD4 (RM4.5), allophycocyanin–anti-CD4 (RM4.5), phycoerythrin–indotricarbocyanine–anti-CD8 (eBioscience); fluorescein isothiocyanate–anti-CD27 (LG.7F9), allophycocyanin–eFluor 780–anti-CD44 (IM7), eFluor 450–anti-CD62L (MEI-14), phycoerythrin–anti-CD62L (MEI-14), phycoerythrin–anti-CD69 (H1.2F3), fluorescein isothiocyanate–anti-CD71 (R71217), phycoerythrin–anti-CD98 (RL388), phycoerythrin–anti-CD127 (A7R34), fluorescein isothiocyanate–anti-TCRβ (H57–597), allophycocyanin–anti-CD11a (2F1), peridinin chlorophyll protein–cyanine 5.5–anti-ICAM-1, fluorescein isothiocyanate–anti-CD69 (H1.2F3), phycoerythrin–anti-T-bet (4B10), Alexa Fluor 647–anti-Eomes (Dan1Imag), eFluor 450–anti-IRF-4 (3E4), fluorescein isothiocyanate–anti-Ki-67 (SolA15), phycoerythrin–anti-granzyme B (NG2B), allophycocyanin–anti-TNF (MP6-XT22), phycoerythrin–anti-GM-CSF (MP1-22E9) and fluorescein isothiocyanate–anti-perforin (eBioOMAK-D; all from eBioscience); fluorescein isothiocyanate–anti-CD4 (RM4.5), allophycocyanin–anti-CD5 (53-7.3), fluorescein isothiocyanate–anti-CD11a (2D7), phycoerythrin–anti-CD11b (554457) (all BD Biosciences), Pacific Blue–anti-CD4 (RM4.5), Alexa Fluor 488–anti-CD25 (PC61.5), phycoerythrin–anti-CD45.2 (104), Pacific Blue–anti-CD45.2 (104), allophycocyanin–anti-CD69 (H1.2F3), allophycocyanin–anti-CD73 (TY/11.8), Brilliant Violet 421–anti-CD127 (A7R34), Alexa Fluor 488–anti-IFN-γ (XM1G1.2) and peridinin chlorophyll protein–cyanine 5.5–anti-TNF (MP6-XT22; all from BioLegend); and fluorescein isothiocyanate–anti-CD45.1 (A20; Abcam). Unconjugated antibody to Erk phosphorylated at Thr359 and Ser363 (9344) and rabbit anti-c-Myc (D844C12) were from Cell Signaling Technologies and were counterstained with goat anti-rabbit (A21244; Molecular Probes). Live/Dead Aqua and Cell Tracker Violet dyes and the fluorescent glucose analog 2-NBDG were from Life Technologies. For intracellular staining, cells were fixed in Phosflow Lyse/Fix Buffer 1 (BD) or Foxp3 Fix/Permeabilization Buffer (eBioscience) before staining with antibody in Permeabilization/Wash buffers (eBioscience). For conjugate-formation assays, a published method was adapted.42 Splenocytes from CD3ε-deficient (Gd3ε−/−) mice were labeled with 1 μM CFSE (carboxyfluorescein succinimidyl ester; Molecular Probes), then were loaded for 2 h at 37°C with 1 μM N4, T4, G4 or SIY T-cell–peptide (Peptide Synthetics) were added to culture medium at the appropriate concentrations. For growth of CTLs, OT-I cells were stimulated for 2 d with 0.01 μM N4, washed, then their populations were expanded and/or they were differentiated for a further 4 d in 20 ng/ml recombinant human IL-2 or mouse IL-15 (Peprotech). For cytokine recall responses, cells were restimulated for 4 h in the presence of 2.5 μg/ml brefeldin A (Sigma) before fixation and intracellular staining (antibodies identified above). For analysis of glucose uptake, following 24 h of stimulation with peptide, cells were incubated for 45 min with 10 μM 2-NBDG, then were washed twice in PBS before fixation in 2% paraformaldehyde and analysis by flow cytometry. Relative glucose uptake was calculated as the mean fluorescence intensity of 2-NBDG staining after stimulation, with subtraction of background fluorescence.

Adhesion under shear flow. The adhesion of effector CTLs was assessed as described.37, 40 μl μ-slip (ibidi) were coated overnight at 4°C with 6 mg/ml ICAM-1. Effector CD8+ T cells at a density of 1 × 106 cells per ml in adhesion medium (RPMI medium with 0.1% BSA, 40 mM HEPES and 2 mM MgCl2) were injected into a flow system that used a silicone tubing loop connected to a Multi-phaser NE-1000 syringe pump (New Era Pump Systems). Cells were allowed to flow over the ICAM-1-coated plates for 10 min at a continuous shear flow rate of 0.5 dynes per cm². Cells were monitored by microscopy, and adherent cells in the field of view were counted manually at intervals of 1 min.

Statistical analysis. Prism software was used for Student’s *t*-test (paired or unpaired; two tails) and analysis of variance.

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