Immunology and Cell Biology (2016), 1–9

www.nature.com/icb

ORIGINAL ARTICLE

Plasmin and regulators of plasmin activity control the migratory capacity and adhesion of human T cells and dendritic cells by regulating cleavage of the chemokine CCL21

Natalie Lorenz[1,2,7], Evert Jan Loef[1,2,7], Inken D Kelch[1,2], Daniel J Verdon[1,2], Moyra M Black[1], Martin J Middleditch[1,3], David R Greenwood[1,2], E Scott Graham[4,5], Anna ES Brooks[1,2], P Rod Dunbar[1,2,8] and Nigel P Birch[1,4,6,8]

The homeostatic chemokine CCL21 has a pivotal role in lymphocyte homing and compartment localisation within the lymph node, and also affects adhesion between immune cells. The effects of CCL21 are modulated by its mode of presentation, with different cellular responses seen for surface-bound and soluble forms. Here we show that plasmin cleaves surface-bound CCL21 to release the C-terminal peptide responsible for CCL21 binding to glycosaminoglycans on the extracellular matrix and cell surfaces, thereby generating the soluble form. Loss of this anchoring peptide enabled the chemotactic activity of CCL21 and reduced cell tethering. Tissue plasminogen activator did not cleave CCL21 directly but enhanced CCL21 processing through generation of plasmin from plasminogen. The tissue plasminogen activator inhibitor neuroserpin prevented processing of CCL21 and blocked the effects of soluble CCL21 on cell migration. Similarly, the plasmin-specific inhibitor α_2-antiplasmin inhibited CCL21-mediated migration of human T cells and dendritic cells and tethering of T cells to APCs. We conclude that the plasmin system proteins plasmin, tissue plasminogen activator and neuroserpin regulate CCL21 function in the immune system by controlling the balance of matrix- and cell-bound CCL21.
Immunology and Cell Biology advance online publication, 2 August 2016; doi:10.1038/icb.2016.56

Guided migration of lymphocytes is essential to mount an effective immune response and to maintain efficient immune surveillance. Localised expression of chemokines provides important directional cues for the trafficking of immune cells to the secondary lymphoid tissues and regulates cell movement within the lymph node. Two chemokines, CCL19 and CCL21, have key roles in the homing of dendritic cells (DCs) and T cells. They are constitutively expressed by fibroblastic reticular cells within the T-cell zones of lymph nodes to guide leukocyte homing.[1,2] Both chemokines bind the G protein-coupled receptor CCR7[3] expressed on lymphocytes to regulate cell motility in the lymph node. Despite comparable binding affinities, CCL19 and CCL21 induce different phosphorylation patterns of the G protein-coupled CCR7 receptor[4] translating into distinctive functional effects, with CCL19, but not CCL21 stimulating receptor desensitization and internalisation.[5–7]

Whereas both CCL19 and CCL21 can drive chemotactic movement through polarisation of the cytoskeleton, only CCL21 can stimulate haptokinetic movement of immune cells through activation of integrins and cell adhesion. This functionality also contributes to transendothelial migration of naive T cells from the bloodstream to lymph nodes[8] and has a role in the tethering of T cells to antigen-presenting cells (APCs) such as DCs.[9] Haptokinetic movement requires immobilisation of CCL21 onto the extracellular matrix and cell surfaces.[10] Binding is mediated by a highly charged, 32-amino-acid C-terminal extension[11–13] found in CCL21 but not CCL19, which mediates high-affinity binding to glycosaminoglycans (GAGs). Growing evidence suggests that the mode of presentation of CCL21, either surface-bound or soluble, determines the cellular response to CCL21. Surface-bound but not soluble CCL21 arrests lymphocyte rolling on high endothelial venules by stimulating a conformational shift in the integrin lymphocyte function-associated antigen 1, inducing transendothelial migration of naive T cells from the bloodstream to lymph nodes.[8] Similarly, immobilized CCL21 gradients provide a strong

[1]School of Biological Sciences, University of Auckland, Auckland, New Zealand; [2]Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand; [3]Auckland Science Analytical Services, University of Auckland, Auckland, New Zealand; [4]Centre for Brain Research, Rangahau Roro, Aotearoa, New Zealand; [5]School of Medical Sciences, University of Auckland, Auckland, New Zealand and [6]Brain Research New Zealand, Rangahau Roro, Aotearoa, New Zealand
[7]These authors contributed equally to this work.
[8]Joint senior authors.
Correspondence: Professor PR Dunbar or Associate Professor NP Birch, School of Biological Sciences, University of Auckland, Thomas Building, 3a Symonds Street, Auckland 1010, New Zealand.
E-mail: r.dunbar@auckland.ac.nz or n.birch@auckland.ac.nz
Received 28 January 2016; revised 18 May 2016; accepted 3 June 2016; accepted article preview online 15 June 2016

haptotactic stimulus to guide murine DC migration from peripheral tissue towards lymphatic vessels *in vitro*.[14] Moreover, CCL21 bound to GAGs on the cell surface tethers T cells to APCs and promotes T-cell activation.[9] Surface-bound CCL21 also activates β_2 integrin on mature DCs promoting ICAM-1-dependent adhesion and motility, whereas the soluble form, generated by proteolytic truncation, influences the direction of migration.[15] Taken together, these findings support the emerging paradigm that matrix- or cell-bound CCL21 promotes cell adhesion as well as haptotaxis, whereas soluble CCL21 drives adhesion-independent chemokinesis or chemotaxis.[15] The conversion of surface-bound to soluble CCL21 is prompted through direct cell contact, as demonstrated most graphically by the Sixt group, who showed that murine DCs in contact with surface-bound CCL21 induce the release of a truncated, soluble CCL21, causing chemotaxis of nearby DCs.[15]

The proteolytic enzyme(s) that regulate CCL21 presentation and function have not been identified. Cleavage of CCL21 by DCs can be inhibited with the broad-spectrum protease inhibitor aprotinin,[15] suggesting the involvement of a serine protease either through direct cleavage of CCL21 or indirectly through activation of other proteases, such as members of the matrix metalloprotease family.[16] We hypothesised that plasmin cleaves basic amino acids found in the C terminus of CCL21[11,17] to release the C-terminal anchoring peptide and convert it from a surface-associated to soluble form. Plasmin hydrolyses peptide bonds following an arginine or lysine residue[18] and is most well known for its role in fibrinolysis through cleavage of fibrin. However, it also cleaves coagulation factors, hormones, metalloproteinases, growth factors, complement zymogens and matrix proteins. Many studies support a role for plasmin(ogen) and the plasminogen activators tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) in mediating cell migration in physiological and pathological conditions through cleavage of these substrates (reviewed in references 19–21). Plasmin has previously been shown to activate immune cell migration.[22] It can cleave the constitutively expressed chemokine CCL14 to activate broad-spectrum chemokine activity.[23] Plasmin also regulates chemokine-mediated neutrophil transendothelial migration by regulating levels of surface-bound IL-8 through cleavage of syndecan-1, a proteoglycan that complexes with IL-8.[24]

The plasmin precursor plasminogen circulates in plasma and interstitial fluids with levels increasing upon inflammation or injury.[25] Active plasmin is generated when the inactive zymogen plasminogen is cleaved by tPA or uPA. The circulating native form of plasminogen, Glu-plasminogen, can be directly activated by either tPA or uPA.[26–28] Plasmin activity is also tightly regulated through direct inhibitors such as α_2-antiplasmin and through the upstream regulation of the activating proteases tPA and uPA.[29] PAI-1 (serpin E1) and neuroserpin (serpin I1), inhibitors of tPA, are both members of the serine protease inhibitor (serpin) family, a group of proteins defined by their conserved structure and their ability to inhibit serine proteases.[30–32] Neuroserpin strongly inhibits tPA and, to a lesser extent, uPA.[33,34]

In the present study we show that plasmin cleaves the C terminus of CCL21 to convert it from a surface-bound to a soluble form and that plasmin, tPA and α_2-antiplasmin modulate CCL21-mediated cell migration and CCL21-dependent cell tethering. We also identify a regulatory role for the tPA inhibitor neuroserpin, which we have previously shown to be expressed in human T cells and DCs.[35,36]

RESULTS

The plasmin inhibitor α_2-antiplasmin inhibits CCL21-mediated human immune cell migration

To test whether plasmin might have a role in modulating CCL21 function, we measured the effects of α_2-antiplasmin on T-cell migration using transwell migration assays. T-cell motility in response to CCL21 was reduced by a maximum of ~50% by 50 nM α_2-antiplasmin (Figure 1a); treatment with 100 and 250 nM α_2-antiplasmin did not result in any further inhibition (data not shown). Importantly, the migratory response to CCL19 was not affected (Figure 1a). To investigate the generation of soluble CCL21 more directly, we analysed cell migration using under-agarose migration assays. In this assay, responding migratory cells and chemoattractants with or without DCs or T cells and α_2-antiplasmin are placed in separate wells in agarose. This allows the diffusion of soluble attractant and the migration of cells along the generated gradient.[37] Cleavage of CCL21 by a cell-derived protease would allow the formation of a diffusible chemotactic gradient. Consistent with this postulation, human DCs showed an approximately sixfold increase in migration towards the well that contained both CCL21 and DCs when compared with CCL21 alone (Figure 1b), similar to a previous report on murine DCs.[15] We found that the enhanced migratory response towards

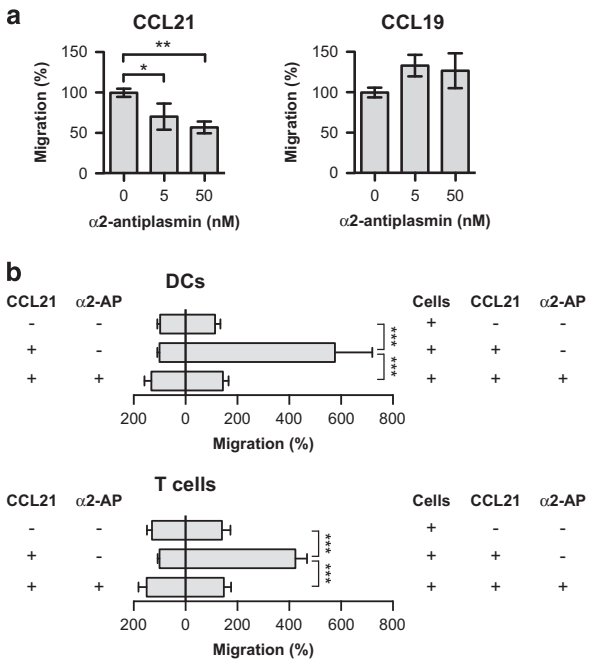


Figure 1 CCL21-stimulated migration of DCs and T cells is inhibited by α_2-antiplasmin. (**a**) T-cell migration was assessed in transwell chambers 3 h after addition of CCL21 or CCL19 to the lower chamber in the presence or absence of α_2-antiplasmin. Data are from three independent experiments using two donors and are normalised to specific migration with 0 nM α_2-antiplasmin. Data are presented as mean ± s.e.m. and were analysed by one-way analysis of variance (ANOVA) with Kruskal–Wallis and Dunn's multiple comparison test. $^*P<0.05$; $^{**}P<0.01$. (**b**) DC or T-cell migration determined using an under-agarose assay. DCs or T cells were labelled with CFSE and the migratory responses of these cells were measured towards wells containing medium alone, cells alone (either T cells or DCs), α_2-antiplasmin (α_2-AP) alone or combinations of these treatments after incubation for 16 h at 37 °C. Migration responses were normalised to the migration towards the CCL21 only control. Data are combined from three independent experiments with cells from three separate donors presented as mean ± s.e.m. and were analysed by one-way ANOVA with Bonferroni's multiple comparison test. $^{***}P<0.0001$.

CCL21 in the presence of DCs was reduced to the response levels seen in wells with CCL21 alone when α_2-antiplasmin was added to the target well (Figure 1b). T cells showed comparable responses, with an approximately fourfold increase in migration towards a mixture of CCL21 and T cells when compared with CCL21 alone, and complete inhibition of this cell-enhanced migration by α_2-antiplasmin.

Plasmin specifically cleaves CCL21

To evaluate CCL21 cleavage by plasmin, CCL21 was incubated with increasing amounts of plasmin and analysed by western blotting. Plasmin cleaved CCL21 in a concentration- and time-dependent manner (Figures 2a and b). Intact CCL21 was detected as an ~17.2-kDa immunoreactive band. Partial digest of CCL21 resulted in two immunoreactive bands of ~13.0 and ~11.7 kDa. With higher levels of plasmin and longer digest times a single band at ~11.7 kDa was seen. The two ~13.0- and ~11.7-kDa forms were also generated when CCL21 was incubated with the plasmin precursor plasminogen and tPA, but not with plasminogen or tPA alone (Figure 2c). Plasmin-mediated cleavage of CCL21 was inhibited by the plasmin inhibitor α_2-antiplasmin (Figure 2d). Importantly, incubation of CCL21 with DCs or T cells also resulted in cleavage to an ~11.7-kDa peptide (Figure 2e), which was inhibited by α_2-antiplasmin (Figure 2e) showing that CCL21 cleavage can be facilitated by cell-associated plasmin. We confirmed that plasmin activity was associated with T cells by measuring enzymatic activity in three donors using the established plasmin substrate Val-Leu-Lys-pNA (Figure 2f). No plasminogen mRNA was detected in T cells or DCs (data not shown), suggesting that the measured plasmin activity was extracellular plasmin(ogen) from serum that had associated with plasmin receptors on the cell surface.[38] We saw no evidence of cell-mediated cleavage of CCL19, suggesting that it is not a substrate for plasmin cleavage (Figure 2g).

We identified the plasmin-cleavage sites within CCL21 using mass spectrometry. LC-MS/MS analysis confirmed that intact CCL21 alkylated with iodoacetamide had a molecular mass of 12 592.87 Da, consistent with recombinant human CCL21 lacking the 23-amino-acid signal peptide sequence as supplied by PeproTech (Fragment A, Figures 3a–c; amino acids 24–134 in Genbank CAG29322, described henceforth as $CCL21_{1–111}$). Following plasmin digestion three major new bands with apparent molecular weights of ~13.0 and ~11.7 kDa (Fragments C–E) were observed (Figure 3a), the same as seen following incubations of CCL21 with DCs or T cells (Figure 2e). LC-MS/MS analysis revealed three products (Fragments C–E) corresponding to $CCL21_{1–81}$ (9522.85 Da), $CCL21_{1–88}$ (10 136.82 Da) and $CCL21_{1–91}$ (10 423.22 Da), consistent with cleavages following Arg_{81}, Lys_{88} and Lys_{91} (Figures 3b–d). Analysis of the peptide fragment intensities indicated that levels of fragments B–E increased relative to intact CCL21 over time. The cleavage products for each observed mass were validated by MS/MS with limited sequence-specific ions generated from the termini (Supplementary Table 1). These data confirmed that all plasmin-cleavage sites follow lysine or arginine residues consistent with the known specificity of this protease.[18] Together, our data reveal plasmin-specific C-terminal cleavage of recombinant CCL21, consistent with a role for this protease in the conversion of surface-bound to soluble CCL21.

As an incidental finding, in the undigested sample we also detected small amounts of a second peptide (Fragment B) as well as $CCL21_{1–111}$. LC-MS/MS analysis identified this peptide as $CCL21_{1–104}$, indicating loss of a seven-amino-acid C-terminal fragment (Figures 3a–d). This peptide, detected on western blots as a minor 15.5-kDa band (Figures 2 and 3a) likely represents a form of CCL21 that the manufacturer had previously identified during purification of the recombinant protein from *Escherichia coli* (personal communication with Peprotech, Rocky Hill, NJ, USA).

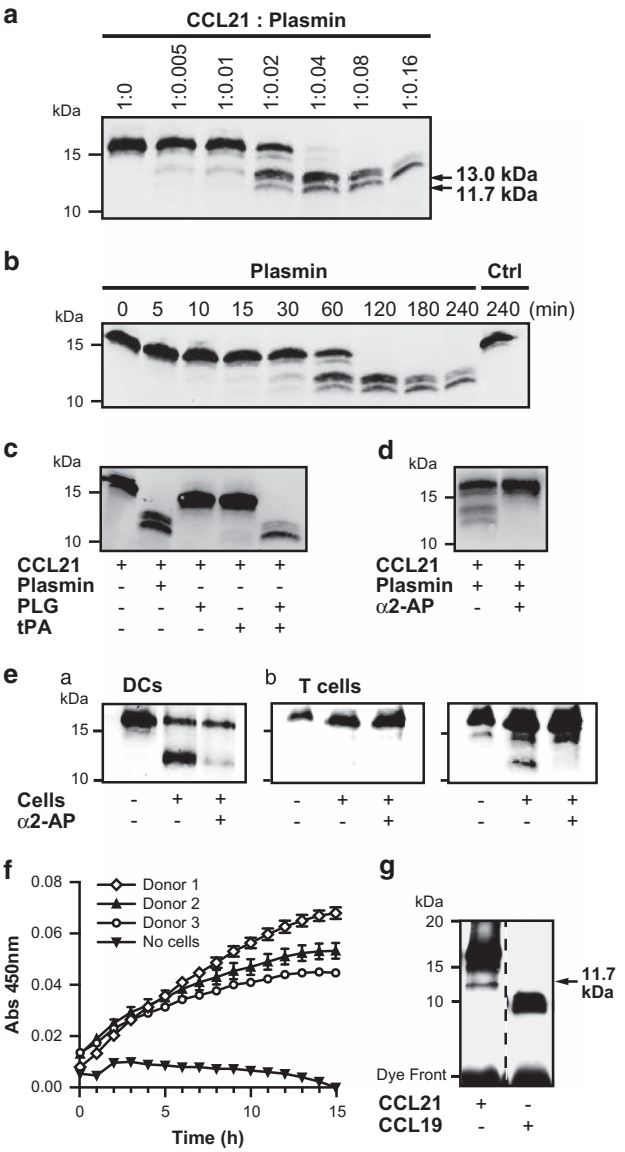
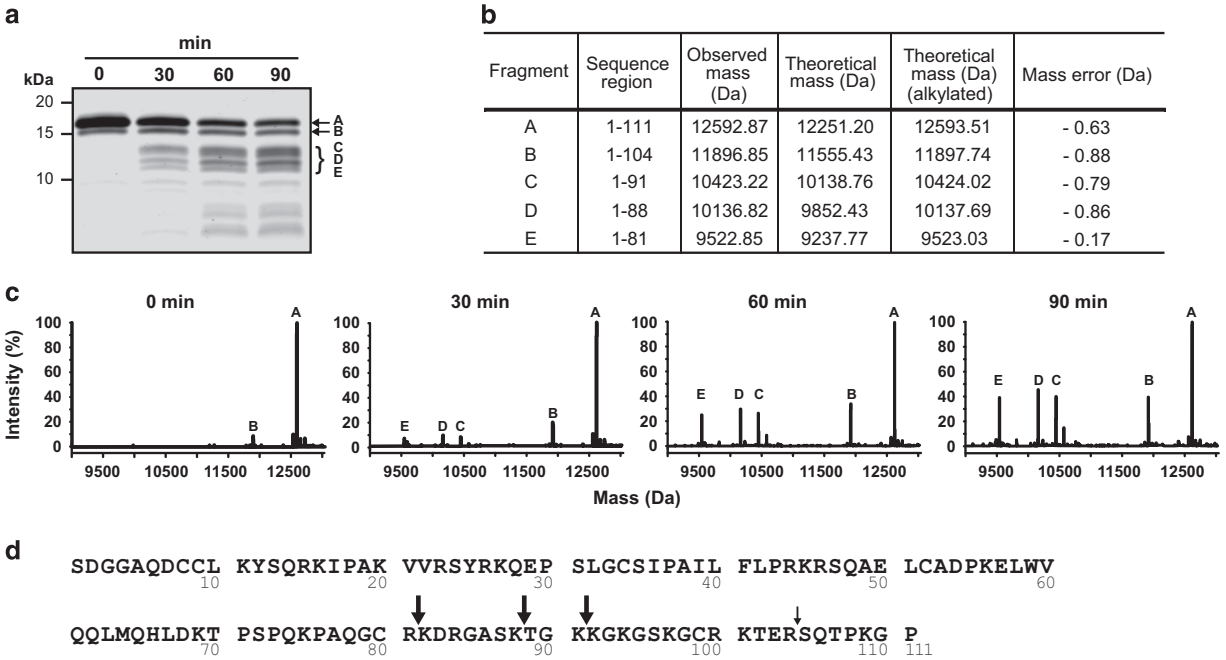


Figure 2 Plasmin cleaves recombinant CCL21. (**a**) Western blot analysis of CCL21 cleavage after incubation with plasmin at indicated molar ratios for 4 h at 37 °C or (**b**) a fixed molar ratio (1:0.08) for the times indicated. Ctrl, CCL21 incubated for 240 min in the absence of plasmin. (**c**) CCL21 cleavage after incubation with plasmin, plasminogen (PLG) and tPA at a fixed molar ratio (1:0.08) for 4 h at 37 °C. (**d**) CCL21 cleavage after incubation with plasmin (Sigma) at a fixed molar ratio (1:0.08) with and without α_2-antiplasmin for 4 h at 37 °C. (**e**) DCs and T cells were incubated with CCL21 in the presence and absence of α_2-antiplasmin for 3 h and culture supernatants analysed by western blotting. Exposure settings for **a** and the left hand blot in **b** are matched, whereas exposure of the right hand blot in **b** has been increased to more clearly visualise the cleaved CCL21 product. (**f**) Plasmin activity detected in T-cell culture supernatants after incubation of cells for 96 h in a serum-free medium. Results are shown from three separate donors. Data presented as mean ± s.e.m. from three biological replicates. (**g**) T cells were incubated with CCL21 or CCL19 for 3 h before western blot analysis. All blots are representative of three independent experiments.



Fragment	Sequence region	Observed mass (Da)	Theoretical mass (Da)	Theoretical mass (Da) (alkylated)	Mass error (Da)
A	1-111	12592.87	12251.20	12593.51	- 0.63
B	1-104	11896.85	11555.43	11897.74	- 0.88
C	1-91	10423.22	10138.76	10424.02	- 0.79
D	1-88	10136.82	9852.43	10137.69	- 0.86
E	1-81	9522.85	9237.77	9523.03	- 0.17

Figure 3 Plasmin cleavage sites within CCL21. SDS-PAGE and mass spectrometry analysis of CCL21 cleaved by plasmin (Sigma). (**a**) Coomassie blue-stained SDS-PAGE gel showing intact and plasmin-digested CCL21 after incubation for 0, 30, 60 and 90 min at 37 °C. (**b**) Mass spectrometric analysis of CCL21. Predicted molecular weights were calculated using the ExPASy Compute pI/MW tool. (**c**) Mass spectrometry profiles showing the major cleavage products of CCL21 after incubation with plasmin for 0, 30, 60 and 90 min, deconvoluted into intact molecular weight profiles. The levels of each peptide are presented relative to intact CCL21 at each time point. (**d**) CCL21 sequence with arrows showing plasmin-cleavage sites. Arrows identify sites cleaved by plasmin. Small arrow indicates cleavage seen in purified CCL21 as provided by the supplier. As recombinant CCL21 lacks the 23-amino-acid signal peptide, the sequence numbering of the recombinant $CCL21_{1–111}$ corresponds to amino acids 24–134 of human CCL21 (Genbank sequence CAG29322).

Plasmin cleavage of surface-bound CCL21 modulates immune cell interactions

To investigate plasmin cleavage of cell surface-bound CCL21 and its influence on cell interactions, we treated CCL21-pulsed B cells with plasmin, α_2-antiplasmin or tPA and plasminogen. CCL21 readily bound to B cells and was dependent on heparin-like GAGs, as pretreatment of B cells with heparinase to remove surface GAGs decreased CCL21 binding in a concentration-dependent manner (Figure 4a). Levels of surface-bound CCL21 were also reduced following tPA and plasminogen or plasmin treatment but remained unchanged with simultaneous addition of plasmin and α_2-antiplasmin (Figure 4b). To quantify CCL21-mediated immune cell interactions, we used flow cytometry to monitor cell:cell adhesion over time. $CCR7^+$ T-cell clones rapidly tethered to CCL21-pulsed B cells, with ~20% of T cells coupled after 5 min, decreasing to ~10% after 45 min (Figure 4c). Addition of plasmin reduced cell tethering, and this effect was neutralised through the addition of α_2-antiplasmin (Figure 4c).

The tPA inhibitor neuroserpin regulates CCL21 cleavage

To investigate the regulatory role of the plasmin system proteins on CCL21 function, we analysed the effects of the tPA inhibitor neuroserpin on CCL21-mediated migration, tethering and cleavage. Neuroserpin inhibited CCL21-mediated T-cell migration in a concentration-dependent manner, but had no effect on CCL19-mediated cell migration (Figure 5a). Inhibition of migration was directly linked to the capacity of neuroserpin to inhibit tPA, as the neuroserpin mutant R362A, which is unable to inhibit tPA,[39] had no effect (Figure 5b). Similarly, neuroserpin inhibited DC and T-cell migration in under-agarose assays (Figures 5c and d). Neuroserpin delayed the proteolytic cleavage of CCL21 by tPA and plasminogen (Figure 5e) and inhibited cleavage of CCL21 in culture supernatants of DCs and T cells (Figure 5f). Finally, the reduction in cell tethering mediated by plasminogen and tPA together was rescued with the addition of neuroserpin (Figure 5g).

DISCUSSION

In this study we report a new role for plasmin in controlling immune cell migration and immune cell interactions by modulating levels of surface-bound CCL21. Our results demonstrate that plasmin and two of its regulators, tPA and neuroserpin, modulate CCL21-mediated migratory and adhesion responses through targeted cleavage of its carboxyl-terminus anchoring peptide. No effect of cell-associated plasmin activity on the cleavage of CCL19 and the lack of an effect of plasmin or neuroserpin on the chemotactic responses of T cells to CCL19 further support the specificity of the plasmin effect for CCL21 function.

Plasmin cleaved CCL21 after Arg_{81}, Lys_{88} and Lys_{91}. All three of these cleavage sites remove the C-terminal peptide that distinguishes CCL21 from other chemokines and, on the basis of the structure–activity relationships of homologous chemokines, are expected to be chemotactic. Direct evidence for the chemotactic activity of C terminally truncated CCL21 was recently reported with a recombinant form of mouse CCL21. This CCL21 truncated at Lys_{75} was shown to stimulate DC migration in under-agarose migration assays, behaving like soluble CCL19, rather than full-length CCL21.[15] Our results suggest that the endogenous cleavage site may be Arg_{81}. The $CCL21_{1–91}$ peptide we identified by MS is likely to correspond to the ~13.0-kDa immunoreactive band seen on western blots and may represent an intermediate in the generation of soluble CCL21. tPA

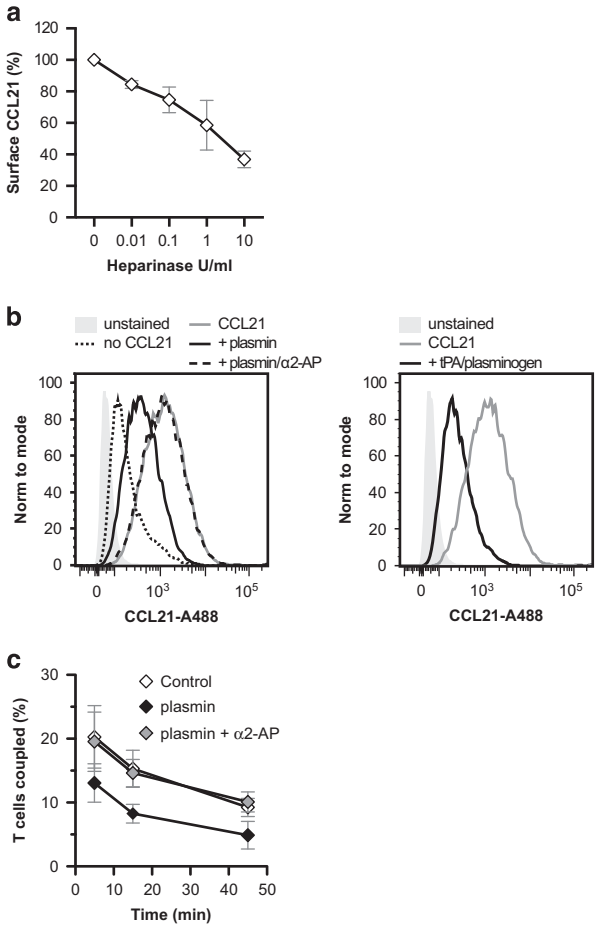


Figure 4 Cleavage of CCL21 on the surface of B cells reduces B-cell to T-cell coupling. (**a**) B cells were incubated with varying concentrations of heparinase for 1 h at 37 °C and then incubated with CCL21 for a further hour before analysis using flow cytometry. (**b**) B cells were pulsed with CCL21 for 1 h at 37 °C and then incubated with indicated combinations of plasmin, α_2-antiplasmin, tPA and plasminogen (PLG) for a further 30 min before analysis using flow cytometry. Unstained or cells incubated in the absence of CCL21 were run as controls. Data are representative of three independent experiments. (**c**) The interaction of T-cell clones with CCL21-loaded B cells treated with plasmin or plasmin and α_2-antiplasmin as described in **a** was determined using flow cytometry. Data combine six independent experiments, and results are shown as mean ± s.e.m.

alone was unable to cleave CCL21, but CCL21 was cleaved when incubated with both tPA and plasminogen. This is consistent with tPA acting as an activator of plasminogen, rather than cleaving CCL21 directly.

Both T cells and DCs cleaved full-length CCL21 to release the C-terminal anchoring peptide. We found that the proportion of cleaved CCL21 was greater in DC compared with T-cell culture supernatants, which is analogous to previously reported differences in relative CCL21 cleavage between murine DCs and T cells.[15] Importantly, this cellular CCL21-cleaving activity was sensitive to both α_2-antiplasmin and neuroserpin, suggesting that cell-associated plasminogen would be activated by tPA produced by T cells or DCs. The failure to detect plasminogen transcripts in T cells or DCs and the presence of plasmin activity associated with T cells support this view. Taken together, our results are consistent with the idea of immune cell-mediated activation of cell-surface-bound plasminogen, with the resulting plasmin mobilising CCL21 by cleaving it from its cell- or matrix-bound anchoring peptide.

Cleavage of full-length CCL21 by plasmin resulting from tPA activation of plasminogen explains the results of our migration assays. Recombinant CCL21 induced T-cell migration in both transwell chambers and in under-agarose migration assays, and altering the generation of plasmin in both systems modulated its effects. Under the conditions used in these assays, it is likely that a proportion of the available CCL21 binds to serum components that coat the tissue culture surfaces, effectively forming surface-bound depots. Our assays show that cells can release CCL21 from these depots in a plasmin-dependent manner. In under-agarose assays, the presence of T cells or DCs with CCL21 increases cell migration, and this increase is inhibited by specific inhibitors of both plasmin (α_2-antiplasmin) and tPA (neuroserpin). In contrast, a non-inhibitory mutant neuroserpin had no effect. These data strongly indicate that cell-derived plasminogen activators, such as tPA, could activate plasminogen sourced from the culture medium and present on the cell surface. The resulting plasmin mediates cleavage of CCL21 from surface-bound depots to enhance cell migration. Plasmin generation is localised to the cell surface through the action of a family of plasminogen receptors that bind plasminogen and form a ternary complex with tPA on the cell surface.[38] Crucially, these effects are not seen for CCL19, which lacks CCL21's high-affinity binding domain for extracellular matrix components.[40]

Our results point to a potential role for the plasmin system in regulating the balance between cell- or surface-bound CCL21 in several important contexts in the generation of immune responses. Within lymphoid tissue, it has been proposed that DCs switch between haptokinetic movement driven by CCL21 bound to fibroblastic reticular cells and chemotactic migration driven by soluble CCL21 gradients that they can themselves generate by cleaving CCL21 from its binding peptide.[15,41] Local activation of plasmin by DCs is a feasible molecular mechanism for this type of localised CCL21 release. Previous studies have also shown that activated DCs are able to condition lymph nodes to recruit additional cells into the lymph nodes.[42] Increased conversion of surface-bound to soluble CCL21 could explain this conditioning by creating microenvironments that attract further immune cells into reactive lymph nodes.

Other than its prominent functions in DC and T-cell homing to and colocalisation within the lymph node, CCL21 has been shown to also promote priming of T cells for antigen-specific interactions with APCs.[9] In CCL21 knockout mice, such interactions still occur, but in altered locations and with diminished efficiency.[1,43] Surface-bound CCL21 on APCs tethers T cells and promotes extensive, localised scanning of the presented antigens.[9] It is thought that T cells captured in this manner have a higher probability to form a stable antigen-dependent interaction, as CCL21 also lowers the T-cell activation threshold *in vitro*.[44] We therefore investigated whether plasmin system proteins regulate T-cell tethering using a lymphoblastic B-cell line and a $CD8^+$ T-cell clone. CCL21 binding to the surface of B cells was GAG-dependent, consistent with other studies reporting the importance of heparin sulphate for the binding of CCL21 to cell surfaces.[14,45] In our studies, plasmin as well as tPA with plasminogen reduced cell surface-associated CCL21, and the plasmin-mediated decrease was inhibited by α_2-antiplasmin. This reduction in surface CCL21 decreased coupling between T and B cells. These findings support a role for plasmin system proteins in influencing the binding and activation of T cells to APCs via modulation of CCL21 bound to APC surfaces. Altered CCL21 levels on the APC surfaces will alter expression of adhesion molecules on $CCR7^+$ T cells, especially active

forms of LFA-1[46]. Given that T-cell engagement with APCs is dynamic, and necessarily involves release from the APCs after initial binding, it is also possible that dynamic modulation of plasmin at sites of T-cell binding helps control the kinetics of CCL21-driven T-cell adhesion mechanisms, ultimately negating CCL21 to enable T-cell release. Our previous results showing a large increase in tPA expression and reduced neuroserpin expression following T-cell activation[36] suggest a mechanism to inactivate T cell:APC tethering following activation of T cells by APCs. Hence, plasmin-mediated control of CCL21 availability on APC cell surface has the potential to influence the kinetics of both T-cell binding and release, with potential impact on the sensitivity to presented antigen.[9] As tethering of T cells to stromal cells bearing CCL21 via engagement of the CCR7 receptor has been proposed to have a role in T-cell retention within lymph nodes,[47] modulation of plasmin activation by T cells could regulate their retention or egress from lymph nodes accordingly. In the absence of a plasmin-resistant form of CCL21 we cannot exclude a role for plasmin to also cleave other proteins involved in T-cell tethering.

In conclusion, our data support a role for plasmin to operate as a controllable switch to modulate the function of CCL21. Localised activation of cell surface-bound plasminogen by tPA secreted by T cells or DCs could convert surface-bound CCL21 on fibroreticular cells, extracellular matrix or APCs to its soluble form. Precise control of CCL21 cleavage would be maintained by serpins including neuroserpin to inhibit tPA-mediated activation of plasminogen to plasmin and α_2-antiplasmin, which will inhibit plasmin. This suggested mechanism might regulate CCL21-mediated effects on cell activation, migration and T-cell homing and egress from lymph nodes.

METHODS

Recombinant proteins

Wild-type neuroserpin and the mutant (NS.R362A) were expressed in *Escherichia coli* BL21(DE3) pRIL cells (Stratagene, La Jolla, CA, USA) and purified by Talon affinity chromatography (Clontech, Mountain View, CA, USA). Human Glu-plasminogen and α_2-antiplasmin were purchased from Molecular Innovations (Novi, MI, USA). Most experiments used plasmin from Molecular Innovations. Some later experiments used plasmin sourced from Sigma (St Louis, MO, USA)—these are noted in the relevant figure legends. tPA (Actilyse) was provided by Boehringer Ingelheim (NZ) Limited (Auckland, New Zealand). All cytokines were purchased from Peprotech and have endotoxin levels less than 0.1 ng μg^{-1} of protein (<1 EU μg^{-1}).

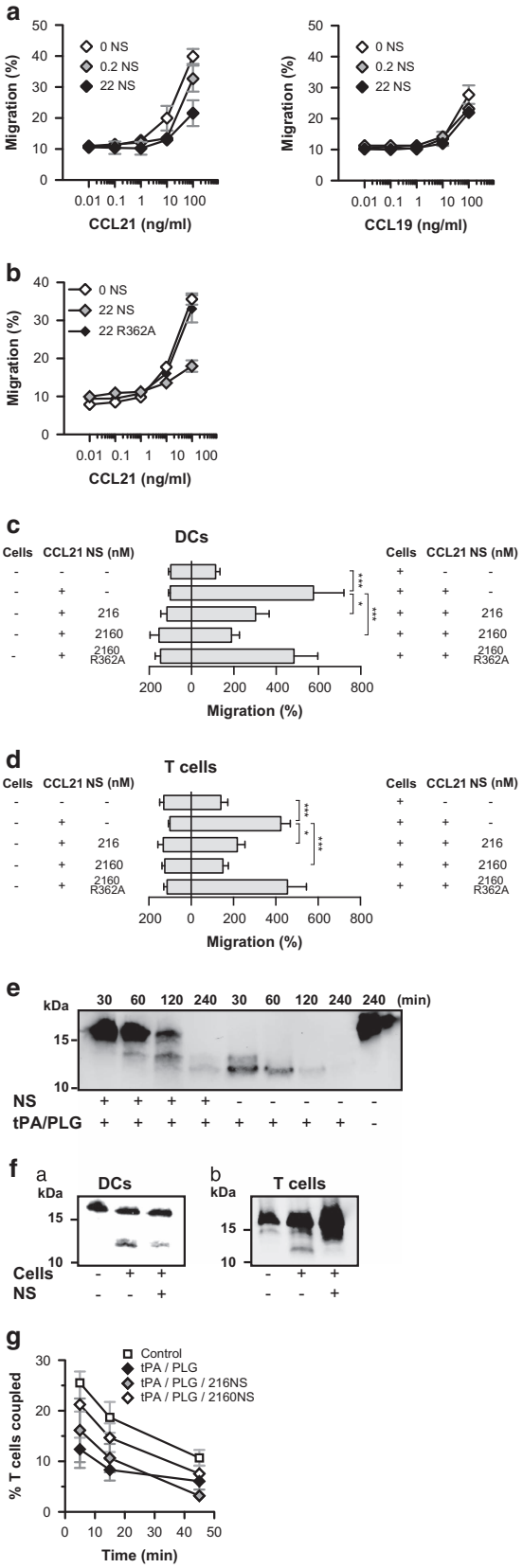


Figure 5 Neuroserpin regulates CCL21 cleavage by inhibiting tPA and thereby plasminogen to plasmin conversion. (**a**) T cells were preincubated with 0.2 nM (0.2 NS) and 22 nM neuroserpin (22 NS) for 15 min at 37 °C and T-cell migration assessed in transwell chambers after 3 h in response to CCL21 and CCL19. (**b**) T cells were preincubated with 22 nM wild-type (NS) or mutant (R362A) NS for 15 min at 37 °C and T-cell migration assessed in transwell chambers after 3 h in response to CCL21. Migratory response of DCs (**c**) and T cells (**d**) in under-agarose assays towards CCL21 following preincubation with 216 or 2160 nM wild-type NS or 2160 nM mutant (R362A) NS and cells. Data are presented as mean ± s.e.m. from three (**c**) and four (**d**) independent experiments and were analysed by one-way ANOVA with Bonferroni's multiple comparison test. $*P<0.05$; $***P<0.0001$. (**e**) Western blot analysis of CCL21 cleavage after incubation with tPA and plasminogen (PLG) for up to 4 h at 37 °C in the presence or absence of 1.5 μM NS. (**f**) DCs (**a**) and T cells (**b**) were incubated with CCL21 in the presence and absence of NS for 3 h and culture supernatants analysed by western blotting. Image brightness and contrast were adjusted to optimise visualisation of cleavage products. (**g**) The interaction of T-cell clones with CCL21-loaded B cells treated with tPA and PLG in the presence or absence of 216 and 2160 nM wild-type NS (216 NS and 2160 NS). Data combine three independent experiments and results are shown as mean ± s.e.m.

Cell isolation and culture

Blood was taken from healthy volunteers after informed consent and approval by the University of Auckland Human Participant Ethics Committee (Ethics approval 010558). Polyclonal T cells were expanded from freshly isolated peripheral blood mononuclear cells as previously described[36] and are referred to as T cells in this manuscript. All T-cell experiments were performed in RPMI-1640 medium containing 5% human serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM GlutaMAX-1 (Life Technologies, Carlsbad, CA, USA), supplemented with 5 ng ml^{-1} IL-7. The Epstein–Barr-Virus-lymphoblastic B-cell line M31-LCL was generated as previously described[36] and is referred to as B cells in this manuscript. In tethering assays the CCR7^{+}CD8^{+} T-cell clone U22-1C5 was used and is referred to as T-cell clone in this manuscript. This clone recognises the HLA-A2-restricted epitope Melan-A/MART-1$_{26–35\ A27L}$ and was isolated as previously described for clone U22-1G11.[36] DCs were differentiated from monocytes as previously described[48] and are referred to as DCs in this manuscript. All DC experiments were performed in RPMI-1640 medium containing 2% human serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM GlutaMAX-1.

CCL21 cleavage by plasmin system proteins and western blot analysis

Human CCL21 (125 nM) was incubated in phosphate-buffered saline with plasmin at molar ratios between 1:0 and 1:0.16 for 4 h at 37 °C or at a 1:0.08 molar ratio for 0–4 h at 37 °C. Incubation of CCL21 with other plasmin system proteins was at a 1:0.08 molar ratio ± 1.5 μM neuroserpin or 1.5 μM α_2-antiplasmin for 4 h at 37 °C. Cleavage of CCL21 or CCL19 by T cells or DCs was determined by co-incubation of 150 ng CCL21 with 500 000 cells in the presence or absence of 100 μg ml^{-1} α_2-antiplasmin or 100 μg ml^{-1} neuroserpin for 3 h. Cells were pelleted by centrifugation for 3 min at 500 *g* and supernatant used for analysis. Cleavage of CCL21 was stopped with reducing tricine sample loading buffer before separation on 16.5% Tris-tricine SDS-PAGE gels (Bio-Rad, Hercules, CA, USA). Western blot analysis was carried out as described previously[36] using rabbit polyclonal antihuman CCL19 and CCL21 (Cat. No 500-P95B and 300-P109; both from Peprotech).

Plasmin activity in serum-free culture supernatants

T cells were cultured under conditions outlined above for 48 h before transfer into Accell siRNA Delivery Media (Dharmacon, Lafayette, CO, USA) with 2 mM GlutaMAX-1 and 10 ng ml^{-1} IL-7 and seeded at 200 000 cells per well in 200 μl in a 96-well plate. The cells were incubated for 96 h and plasmin activity determined in the supernatants using chromogenic plasmin substrate S-2251 at a final concentration of 170 μM (Chromogenix by Instrumentation Laboratory, Bedford, MA, USA). Cleavage was quantitated at 405 nm on an EnVision Multilabel Reader (PerkinElmer, Waltham, MA, USA) continuously for 15 h. Values were corrected for background measured for medium with substrate alone.

Mass spectrometry

CCL21 (15.35 μM) and plasmin (62 nM) were incubated in 20 mM ammonium acetate buffer (pH 7.2) for up to 90 min at 37 °C. The reaction was stopped with 10 mM dithiothreitol (15 min at 56 °C) and the reaction quenched by alkylation with iodoacetamide for 20 min at room temperature in the dark. For the time zero sample, dithiothreitol was added, the sample heated to 56 °C, which will inactivate plasmin activity, and purified plasmin added. Before analysis, the dithiothreitol concentration was increased to 40 mM, 0.5% formic acid was added and samples were placed on ice. Half of each sample was mixed with reducing tricine sample loading buffer before separation on 16.5% Tris-tricine SDS-PAGE gels and stained with Coomassie R250. The other half of the samples was diluted 100-fold in 0.1% formic acid, and 10 μl was injected into a 150-μm-diameter Protecol C8 H trap column at 7.5 μl min^{-1} using an Eksigent Ekspert NanoLC 425 UPLC (Sciex, Framingham, MA, USA). After 5 min of desalting, the flow rate was dropped to 700 nl min^{-1} and the flow switched to the analytical column eluting with the following gradient applied: 0–2 min 10% B, 20 min 60% B, 21 min 90% B, 24 min 90% B, 26 min 10% B and 30 min 10% B, where the mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The column effluent was directed into the nanospray source of a TripleTOF 6600 mass spectrometer (Sciex) operating in a positive ionisation mode using a spray voltage of 2300 V. For the time-course measurements of intact molecular weights, a TOF-MS scan was performed from *m/z* 350 to 2000 for 250 ms. The resulting TOF-MS data were deconvoluted into intact molecular weight profiles using the Reconstruct Protein Tool within the BioTool kit add-on to PeakView version 2.1 (Sciex), whereas theoretical molecular masses were calculated using the ExPASy Compute pI/MW tool (Swiss Institute of Bioinformatics, Lausanne, Switzerland). The identities of the cleavage products assigned to each observed mass were confirmed by subsequent 'top-down' MS/MS, with manual verification of the limited sequence-specific ions that were generated from the protein termini.

In vitro migration assays

Transwell migration assays were performed as previously described.[49] In brief, CCL21 and CCL19 (0.01–100 ng ml^{-1}) and α_2-antiplasmin (5–50 nM), neuroserpin wt (0.22–22 nM) or R362A mutant (22 nM) were added into HTS-Transwell receiver plates (Corning, Corning, NY, USA). T cells (50 000 per well) were added to a 96-well HTS-Transwell permeable support (3-μm-pore polycarbonate membrane, Corning) and preincubated with corresponding treatments for 15 min at 37 °C before insertion into the acceptor plate to create upper and lower chambers. Cell migration was quantified after 3 h at 37 °C with the ATPlite Luminescence Assay System (PerkinElmer) according to the manufacturer's instructions. Typically, ~15 000 cells responded to a CCL19 stimulus and ~20 000 cells to CCL21. Specific cell migration was determined by correcting for the number of migrating cells in the absence of chemoattractant (consistently <1000 cells). T cells and DCs used in under-agarose assays were labelled with 2 μM CellTrace carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) according to the manufacturer's instructions. Assays were set up as previously described,[37] with human serum replacing fetal calf serum. Briefly, three wells were cut into 1% (w/v) agarose in a straight line, each 3.5 mm in diameter and 2.2 mm apart. Plates were equilibrated for 1 h at 37 °C, 5% CO_2 before use in experiments. Outside wells were filled with 600 ng ml^{-1} CCL21 in the presence of appropriate treatments with or without T cells or DCs (60 000 per well). Dye-labelled, type-matched responder cells (60 000 per well) were added to the centre well and were allowed to migrate for 16 h at 37 °C before quantification of migration through manual cell counts. Whereas soluble chemokine triggered a migration of cells towards the edge of the well, only cells that had migrated under the agarose were counted. Typical counts were ~450 DCs and ~100 T cells migrating towards CCL21 and cells compared with only ~75 DCs and ~30 T cells migrating in a medium-only control. Responses were normalised to the migration towards CCL21 without the addition of cells.

Surface cleavage of CCL21 and flow cytometry-based coupling assay

Experiments were run on a BD SORP FACS Aria II (Becton Dickson, Franklin Lakes, NJ, USA) and data analysed using FlowJo V10 OSX (FlowJo LLC, Ashland, OR, USA). B cells were pulsed with 2 μg ml^{-1} CCL21 for 1 h at 37 °C in serum-free media before incubation with 640 nM plasmin, plasmin with 2.5 μg ml^{-1} α_2-antiplasmin or 640 nM tPA and 640 nM plasminogen for 30 min. Surface-bound CCL21 was detected with anti-CCL21 (cat# 500-P109, Peprotech) followed by anti-rabbit-Alexa488 (Molecular Probes, Eugene, OR, USA). 4,6-Diamidino-2-phenylindole was used to exclude dead cells from analysis. To test the effect of heparinase on CCL21 binding to the cell surface B cells were incubated with 0.01–10 U ml^{-1} of heparinase II (Sigma-Aldrich, St Louis, MO, USA) in serum-free media for 1 h at 37 °C before pulsing with 4 μg ml^{-1} CCL21 for a further hour at 37 °C. For the detection of surface-bound CCL21, cells were stained as outlined above and analysis was performed on the BD Accuri C6 cytometer and the associated software. Data were corrected for background staining of unpulsed B cells and normalised to CCL21-pulsed, untreated cells.

To access cell coupling, T-cell clones and B cells were stained with CellTracker Violet BMQC and CellTrace CFSE (both from Life Technologies) in serum-free media before pulsing the B cells with CCL21 (see above). B cells were preincubated with 640 nM plasmin, 2.5 μg ml^{-1} α_2-antiplasmin, 640 nM tPA, 640 nM plasminogen and 216–2160 nM neuroserpin for 15 min at 37 °C.

After addition of T cell, cells were centrifuged for 1 min at 228 *g* and incubated at 37 °C for times indicated. Cell couples were fixed with 2% (v/v) paraformaldehyde for 10 min at 37 °C and analysed with flow cytometry on a BD SORP FACS Aria II. Couple formation was quantified as the proportion of $BMQC^+CFSE^+$ T cells compared with the number of $BMQC^+$ T cells. Results for individual experiments were corrected for time-matched coupling in the absence of CCL21. Coupling in the absence of CCL21 ranged from 2 to 5% at time zero, increasing to a maximum of 8% at 45 min.

Statistical analysis

Data represent at least three independent experiments. Combined data are shown as mean ± s.e.m. Using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) one-way analysis of variance with Bonferroni's or Kruskal–Wallis and Dunn's multiple comparison test were performed whenever applicable, with $P<0.05$ considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank E Miranda for providing the neuroserpin WT plasmid; O Strauss, J Kühne and J McIntosh for help with phlebotomy, the Centre for Genomics and Proteomics for mass spectrometry analysis and D Christie for valuable comments on the manuscript. Financial support for this research came from a Marsden Grant from the Royal Society of New Zealand and a University of Auckland Doctoral Scholarship (to EJL). Boehringer Ingelheim (NZ) Ltd., kindly provided tPA.

1 Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci USA* 1998; **95**: 258–263.
2 Luther SA, Tang HL, Hyman PL, Farr AG, Cyster JG. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc Natl Acad Sci USA* 2000; **97**: 12694–12699.
3 Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E *et al.* CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999; **99**: 23–33.
4 Zidar DA, Violin JD, Whalen EJ, Lefkowitz RJ. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc Natl Acad Sci USA* 2009; **106**: 9649–9654.
5 Bardi G, Lipp M, Baggiolini M, Loetscher P. The T cell chemokine receptor CCR7 is internalized on stimulation with ELC, but not with SLC. *Eur J Immunol* 2001; **31**: 3291–3297.
6 Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, Struthers RS. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J Biol Chem* 2004; **279**: 23214–23222.
7 Hauser MA, Legler DF. Common and biased signaling pathways of the chemokine receptor CCR7 elicited by its ligands CCL19 and CCL21 in leukocytes. *J Leukoc Biol* 2016; **99**: 869–882.
8 Shamri R, Grabovsky V, Gauguet J-M, Feigelson S, Manevich E, Kolanus W *et al.* Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat Immunol* 2005; **6**: 497–506.
9 Friedman RS, Jacobelli J, Krummel MF. Surface-bound chemokines capture and prime T cells for synapse formation. *Nat Immunol* 2006; **7**: 1101–1108.
10 Hirose J, Kawashima H, Swope Willis M, Springer TA, Hasegawa H, Yoshie O *et al.* Chondroitin sulfate B exerts its inhibitory effect on secondary lymphoid tissue chemokine (SLC) by binding to the C-terminus of SLC. *Biochim Biophys Acta* 2002; **1571**: 219–224.
11 Nagira M, Imai T, Hieshima K, Kusuda J, Ridanpää M, Takagi S *et al.* Molecular cloning of a novel human CC chemokine secondary lymphoid-tissue chemokine that is a potent chemoattractant for lymphocytes and mapped to chromosome 9p13. *J Biol Chem* 1997; **272**: 19518–19524.
12 Hromas R, Kim CH, Klemsz M, Krathwohl M, Fife K, Cooper S *et al.* Isolation and characterization of Exodus-2, a novel C-C chemokine with a unique 37-amino acid carboxyl-terminal extension. *J Immunol* 1997; **159**: 2554–2558.
13 Love M, Sandberg JL, Ziarek JJ, Gerarden KP, Rode RR, Jensen DR *et al.* Solution structure of CCL21 and identification of a putative CCR7 binding site. *Biochemistry* 2012; **51**: 733–735.
14 Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DF *et al.* Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* 2013; **339**: 328–332.
15 Schumann K, Lammermann T, Bruckner M, Legler DF, Polleux J, Spatz JP *et al.* Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity* 2010; **32**: 703–713.
16 Wilkins-Port CE, Higgins SP, Higgins CE, Kobori-Hotchkiss I, Higgins PJ. Complex regulation of the pericellular proteolytic microenvironment during tumor progression and wound repair: functional interactions between the serine protease and matrix metalloproteinase cascades. *Biochem Res Int* 2012; **2012**: 454368.
17 Yoshida R, Nagira M, Kitaura M, Imagawa N, Imai T, Yoshie O. Secondary lymphoid-tissue chemokine is a functional ligand for the CC chemokine receptor CCR7. *J Biol Chem* 1998; **273**: 7118–7122.
18 Weinstein MJ, Doolittle RF. Differential specificities of the thrombin, plasmin and trypsin with regard to synthetic and natural substrates and inhibitors. *Biochim Biophys Acta* 1972; **258**: 577–590.
19 Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 1997; **91**: 439–442.
20 Plow EF, Ploplis VA, Carmeliet P, Collen D. Plasminogen and cell migration *in vivo*. *Thromb Haemost* 1999; **13**: 49–53.
21 Syrovets T, Lunov O, Simmet T. Plasmin as a proinflammatory cell activator. *J Leukoc Biol* 2012; **92**: 509–519.
22 Li X, Syrovets T, Genze F, Pitterle K, Oberhuber A, Orend K-H *et al.* Plasmin triggers chemotaxis of monocyte-derived dendritic cells through an Akt2-dependent pathway and promotes a T-helper type-1 response. *Arterioscler Thromb Vasc Biol* 2010; **30**: 582–590.
23 Vakili J, Standker L, Detheux M, Vassart G, Forssmann WG, Parmentier M. Urokinase plasminogen activator and plasmin efficiently convert hemofiltrate CC chemokine 1 into its active [9-74] processed variant. *J Immunol* 2001; **167**: 3406–3413.
24 Marshall LJ, Ramdin LSP, Brooks T, DPhil PC, Shute JK. Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. *J Immunol* 2003; **171**: 2057–2065.
25 Deryugina EI, Quigley JP. Cell surface remodeling by plasmin: a new function for an old enzyme. *J Biomed Biotechnol* 2012; **2012**: 564259.
26 Gong Y, Kim SO, Felez J, Grella DK, Castellino FJ, Miles LA. Conversion of Glu-plasminogen to Lys-plasminogen is necessary for optimal stimulation of plasminogen activation on the endothelial cell surface. *J Biol Chem* 2001; **276**: 19078–19083.
27 Zhang L, Gong Y, Grella DK, Castellino FJ, Miles LA. Endogenous plasmin converts Glu-plasminogen to Lys-plasminogen on the monocytoid cell surface. *J Thromb Haemost* 2003; **1**: 1264–1270.
28 Miles LA, Castellino FJ, Gong Y. Critical role for conversion of glu-plasminogen to Lys-plasminogen for optimal stimulation of plasminogen activation on cell surfaces. *Trends Cardiovasc Med* 2003; **13**: 21–30.
29 Schaller J, Gerber SS. The plasmin-antiplasmin system: structural and functional aspects. *Cell Mol Life Sci* 2011; **68**: 785–801.
30 Miranda E, Lomas DA. Neuroserpin: a serpin to think about. *Cell Mol Life Sci* 2006; **63**: 709–722.
31 Huntington JA. Serpin structure, function and dysfunction. *J Thromb Haemost* 2011; **9** (Suppl 1): 26–34.
32 Lee TW, Tsang VWK, Birch NP. Physiological and pathological roles of tissue plasminogen activator and its inhibitor neuroserpin in the nervous system. *Front Cell Neurosci* 2015; **9**: 396.
33 Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. *EMBO J* 1996; **15**: 2944–2953.
34 Hastings GA, Coleman TA, Haudenschild CC, Stefansson S, Smith EP, Barthlow R *et al.* Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. *J Biol Chem* 1997; **272**: 33062–33067.
35 Kennedy SA, van Diepen AC, van den Hurk CM, Coates LC, Lee TW, Ostrovsky LL *et al.* Expression of the serine protease inhibitor neuroserpin in cells of the human myeloid lineage. *Thromb Haemost* 2007; **97**: 394–399.
36 Lorenz N, Loef EJ, Verdon DJ, Chen C-JJ, Mansell CJ, Angel CE *et al.* Human T cell activation induces synaptic translocation and alters expression of the serine protease inhibitor neuroserpin and its target protease. *J Leukoc Biol* 2015; **97**: 699–710.
37 Heit B, Kubes P. Measuring chemotaxis and chemokinesis: the under-agarose cell migration assay. *Sci STKE* 2003; **2003**: PL5.
38 Godier A, Hunt BJ. Plasminogen receptors and their role in the pathogenesis of inflammatory, autoimmune and malignant disease. *J Thromb Haemost* 2013; **11**: 26–34.
39 Lee TW, Coates LC, Birch NP. Neuroserpin regulates N-cadherin-mediated cell adhesion independently of its activity as an inhibitor of tissue plasminogen activator. *J Neurosci Res* 2008; **86**: 1243–1253.
40 Fernandez EJ, Lolis E. Structure, function, and inhibition of chemokines. *Annu Rev Pharmacol Toxicol* 2002; **42**: 469–499.
41 Murphy PM. Double duty for CCL21 in dendritic cell trafficking. *Immunity* 2010; **32**: 590–592.
42 Martín-Fontecha A, Sebastiani S, Höpken UE, Uguccioni M, Lipp M, Lanzavecchia A *et al.* Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* 2003; **198**: 615–621.

43 Förster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* 2008; **8**: 362–371.
44 Gollmer K, Asperti-Boursin F, Tanaka Y, Okkenhaug K, Vanhaesebroeck B, Peterson JR *et al.* CCL21 mediates $CD4^+$ T-cell costimulation via a DOCK2/Rac-dependent pathway. *Blood* 2009; **114**: 580–588.
45 Bao X, Moseman EA, Saito H, Petryniak B, Petryanik B, Thiriot A *et al.* Endothelial heparan sulfate controls chemokine presentation in recruitment of lymphocytes and dendritic cells to lymph nodes. *Immunity* 2010; **33**: 817–829.
46 Kinashi T. Intracellular signalling controlling integrin activation in lymphocytes. *Nat Rev Immunol* 2005; **5**: 546–559.
47 Pham THM, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity* 2008; **28**: 122–133.
48 Dauer M, Obermaier B, Herten J, Haerle C, Pohl K, Rothenfusser S *et al.* Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *J Immunol* 2003; **170**: 4069–4076.
49 Grimsey NL, Moodley KS, Glass M, Graham ES. Sensitive and accurate quantification of human leukocyte migration using high-content Discovery-1 imaging system and ATPlite assay. *J Biomol Screen* 2012; **17**: 386–393.

The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)