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Plasminogen and plasmin can bind to human T cells and generate truncated CCL21 that increases dendritic cell chemotactic responses

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Plasmin is a broad-spectrum protease and therefore needs to be tightly regulated. Active plasmin is formed from plasminogen, which is found in high concentrations in the blood and is converted by the plasminogen activators. In the circulation, high levels of α2-antiplasmin rapidly and efficiently inhibit plasmin activity. Certain myeloid immune cells have been shown to bind plasmin and plasminogen on their cell surface *via* proteins that bind to the plasmin(ogen) kringle domains. Our earlier work showed that T cells can activate plasmin but that they do not themselves express plasminogen. Here, we demonstrate that T cells express several known plasminogen receptors and that they bind plasminogen on their cell surface. We show T cell–bound plasminogen was converted to plasmin by plasminogen activators upon T cell activation. To examine functional consequences of plasmin generation by activated T cells, we investigated its effect on the chemokine, C-C motif chemokine ligand 21 (CCL21). Video microscopy and Western blotting confirmed that plasmin bound by human T cells cleaves CCL21 and increases the chemotactic response of monocyte-derived dendritic cells toward higher CCL21 concentrations along the concentration gradient by increasing their directional migration and track straightness. These results demonstrate how migrating T cells and potentially other activated immune cells may co-opt a powerful proteolytic system from the plasma toward immune processes in the peripheral tissues, where α2-antiplasmin is more likely to be absent. We propose that plasminogen bound to migrating immune cells may strongly modulate chemokine responses in peripheral tissues.

Cells such as endothelial cells, macrophages, and monocytes have been shown to bind plasmin and plasminogen to their cell surface *via* exposed lysines on cell surface proteins that bind to the plasmin(ogen) kringle domains (1). The plasmin precursor plasminogen is found in micromolar range concentrations in the blood and is converted to active plasmin by the plasminogen activators tissue plasminogen activator (tPA) and urokinase plasminogen activator (2, 3).

We have previously shown that T cells upregulate tPA when activated (4), suggesting that localized bursts of immune activation in the tissues might activate plasminogen.

Plasmin is a broad-spectrum protease that is tightly regulated. In the circulation, this is achieved by high levels of α2-antiplasmin that rapidly and efficiently inhibits plasmin activity (5). To explain how T cells might activate plasmin outside of the circulation, we hypothesized that they might bind plasminogen in the circulation and carry it into the tissues. Importantly activation of plasminogen by T cells in the tissues could be especially potent because it would evade the abundant α2-antiplasmin present in the circulation.

Plasmin(ogen)-binding proteins are often referred to as plasminogen receptors, though they do not always signal in response to binding plasmin(ogen). Some of these molecules can often also bind plasminogen activators and facilitate local activation of plasminogen (1, 6). The best-characterized plasminogen receptors are the S100A10-annexin A2 heterotetramer, α-enolase, and PLG-R_{KT} (7). PLG-R_{KT} is of particular interest since it is the only integral membrane protein that binds plasminogen without requiring release from intracellular sites or proteolytic cleavage of membrane proteins to expose C-terminal lysines (8, 9).

We first investigated whether primary human T cells express plasminogen receptors and can bind plasminogen. We found that they express relatively high levels of PLG-R_{KT}, and can bind both plasmin and plasminogen, and facilitate the conversion of plasminogen to plasmin. This *in vitro* evidence supports a model whereby T cells laden with plasminogen from the circulation can convert plasminogen to active plasmin in the peripheral tissues once they encounter their cognate antigen.

We then investigated whether plasmin activation by T cells might play a role in controlling immune cell migration in peripheral tissues.

Our recent work demonstrated that plasmin is one of the proteases that can truncate the chemokine C-C motif chemokine ligand 21 (CCL21), by removing its unstructured C-terminal tail (10). Using end-point migration assays, we also showed that plasmin-mediated truncation of CCL21 induces increased migration of human T cells and dendritic cells (10).

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Guided cellular migration is essential to mounting immune responses, enabling immune cells to interact optimally with each other and with extracellular matrix (11, 12). The chemokines CCL19 and CCL21 both drive homing of dendritic cells (DCs) and T cells to the lymph node and facilitate the presentation of antigens from DCs to T cells (13, 14). CCL21 is also critical in nonlymphoid tissues for the initial recruitment of T lymphocytes to peripheral effector sites (15). CCL19 and CCL21 bind to the same receptor, CCR7, yet they induce different effects in target cells (14, 16). The primary difference between CCL19 and CCL21 is the unstructured C-terminal tail on CCL21 (17). This tail has a net positive charge that allows for binding of CCL21 to extracellular matrix and to glycosaminoglycans. Surface-bound CCL21 facilitates the activation of lymphocyte adhesion molecule LFA-1 and induces *trans*-endothelial migration (18). Bound CCL21 can also provide tracks to guide cell migration both with and without a soluble gradient present (19). However, CCL21 can also be truncated by proteases that remove the C-terminal chain, so that it loses the ability to bind to the extracellular matrix and subsequently behaves very similarly to CCL19 (19, 20).

Since end-point assays cannot always detect subtle changes in chemotaxis (directional migration) and chemokinesis (nondirectional migration) (12), we developed a live-cell microscopy assay that could directly visualize and measure chemokinesis of DCs within a chemokine gradient (21). We have now applied this more sophisticated motility assay to confirm that plasmin bound by T cells, similarly to purified plasmin, upregulates the ability of CCL21 to stimulate chemotaxis of human monocyte-derived DCs (mDCs).

Overall, our results suggest that plasmin is likely to be generated on the cell surface of T cells recognizing cognate antigen within the tissues by activating plasminogen that they have carried in from the circulation. One possible function of the plasmin that is generated is to induce localized tissue-specific cleavage of CCL21 (and potentially other chemokines that have the same tissue-tethering C-terminal tails) to increase recruitment and motility of other immune cells at sites of T-cell activation.

Results

T cells bind plasmin and plasminogen in a lysine-dependent manner

In a previous study, we showed that T cells can activate plasminogen (4). Since plasmin is strongly inhibited in the circulation, we wondered whether lymphocytes could bind plasminogen from plasma and then carry it with them to other tissues, where α2-antiplasmin might not so readily inactivate it. This local activation on the cell surface microenvironment would also allow for significant levels of localized plasmin activity that would not be immediately "mopped up" by plasmin inhibitors.

To test whether T cells had the ability to bind plasmin and/or plasminogen, T cells were incubated in the presence or absence of purified plasmin or plasminogen protein under serum-free conditions and then any excess unbound protein was washed away. Bound plasminogen, if present, was activated with exogenously supplied tPA. Cells were then incubated with a plasmin substrate, which yields a fluorescent signal that can be measured over time when cleaved by plasmin.

This assay revealed high levels of plasmin activity (and therefore plasmin binding) in T cells that had been incubated with plasmin protein (Fig. 1*A*) compared to control T cells that had not been incubated with plasmin or the cell-free negative control (where plasmin was washed away). Similar results were obtained when T cells were preincubated with purified plasminogen followed by activation with tPA (Fig. 1*B*). In this experiment, significant levels of plasmin activity were also observed in T cells that had not been exposed to purified plasminogen compared to protein only controls. This may reflect a carryover of prebound plasminogen from the T-cell culture medium that contains human serum. However, tPA-induced plasmin activity was still significantly increased when cells were preincubated with purified plasminogen (Fig. 1*B*). We also noted that T cells that activated plasminogen bound to their cell surface generated sixfold higher activity than those directly incubated with plasmin. This could reflect the more physiological generation of plasmin, given that free plasmin will never occur like this *in vivo*.

The most recognized mechanism by which plasmin(ogen) binds to cells is *via* interactions between the kringle domains on plasmin with exposed lysines on the cell surface (1). This binding can therefore be disrupted in the presence of excess lysine analogs. When lysine analogs (ε-aminocaproic acid and tranexamic acid) were added during the binding step of our assay, we observed that both plasmin and plasminogen binding to T cells was significantly reduced (see Fig. 1, *C* and *D*), indicative of the predicted lysine-specific binding mechanism.

Next, direct binding of plasmin and plasminogen to the cells was visualized using Western blot (Fig. 1, *E*–*G*). Cells were incubated with 0, 10, or 100 nM plasmin or plasminogen or in medium containing 50% human serum, prior to washing away any unbound plasmin(ogen). Cells were then lysed, and lysates were analyzed by Western blotting.

Preincubation of T cells with plasmin resulted in the detection of a 26 kDa protein, corresponding to the plasmin light chain (Fig. 1*E*). Similarly, preincubation of T cells with plasminogen resulted in the detection of a 100 kDa protein, corresponding to purified plasminogen (Fig. 1*F*). Plasminogen is also observed as a carryover in cells that are precultured in serum-containing media (Fig. 1*E*). This serum-derived effect is enhanced in the presence of increased concentrations of serum, as expected (Fig. 1*G*).

Knowing that T cells can bind plasmin and plasminogen, the ability to cleave the chemokine CCL21 was then tested. T cells that had bound plasmin following preincubation with excess plasmin had a greater ability to cleave CCL21 than those with no preincubation (Fig. 1*H*). The cleavage resulted in a truncated product consistent with tail-less CCL21, as previously reported (10). A similar result was observed with T cells that had been preincubated with plasminogen. However, this effect was only observed when the T cells were activated with

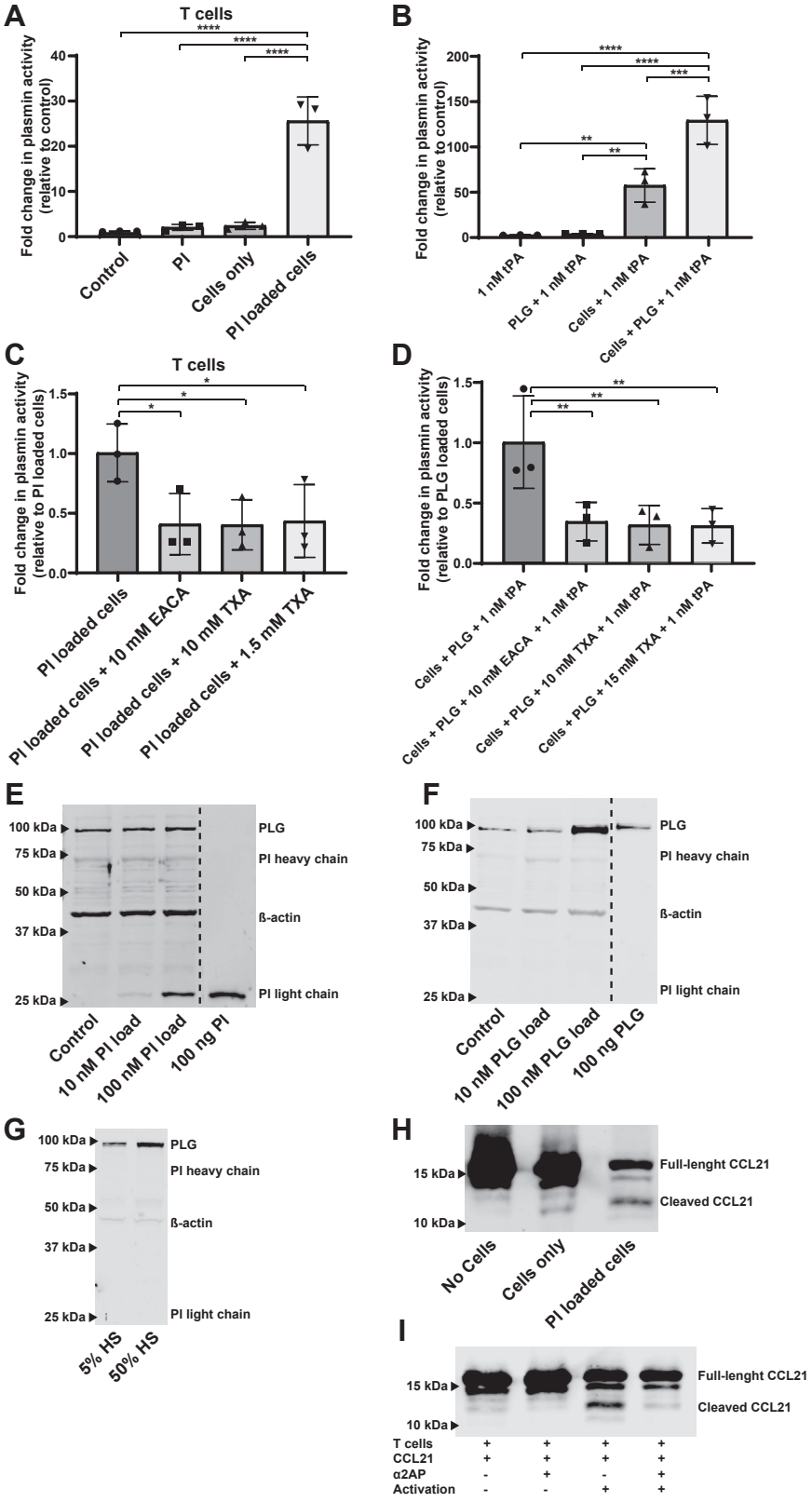

Figure 1. Human T cells bind plasmin and plasminogen in a lysine dependent manner which can induce cleavage of CCL21. *A*, fluorescent signal generated by plasmin activity. Serum-free medium was incubated for 2 h in the presence or absence of T cells and/or purified plasmin (10 nM). Any unbound plasmin was washed away before serum-free medium with a plasmin substrate was added and incubated for 16 h. *B*, fluorescent signal generated by plasmin activity. Serum-free medium was incubated for 2 h in the presence or absence of T cells and/or purified plasminogen (10 nM). Any unbound plasminogen was washed away before plasminogen was activated with tPA (1 nM) in serum-free medium with a plasmin substrate and incubated for 16 h. *C*, fluorescent signal generated by plasmin activity. T cells were incubated in serum-free medium for 2 h in the presence or absence of purified plasmin (10 nM) and/or lysine analogs EACA and TXA. Any unbound plasmin was washed away before serum-free medium with a plasmin substrate was added and incubated for 16 h. *D*, fluorescent signal generated by plasmin activity. T cells were incubated in serum-free medium for 2 h in the presence or absence of

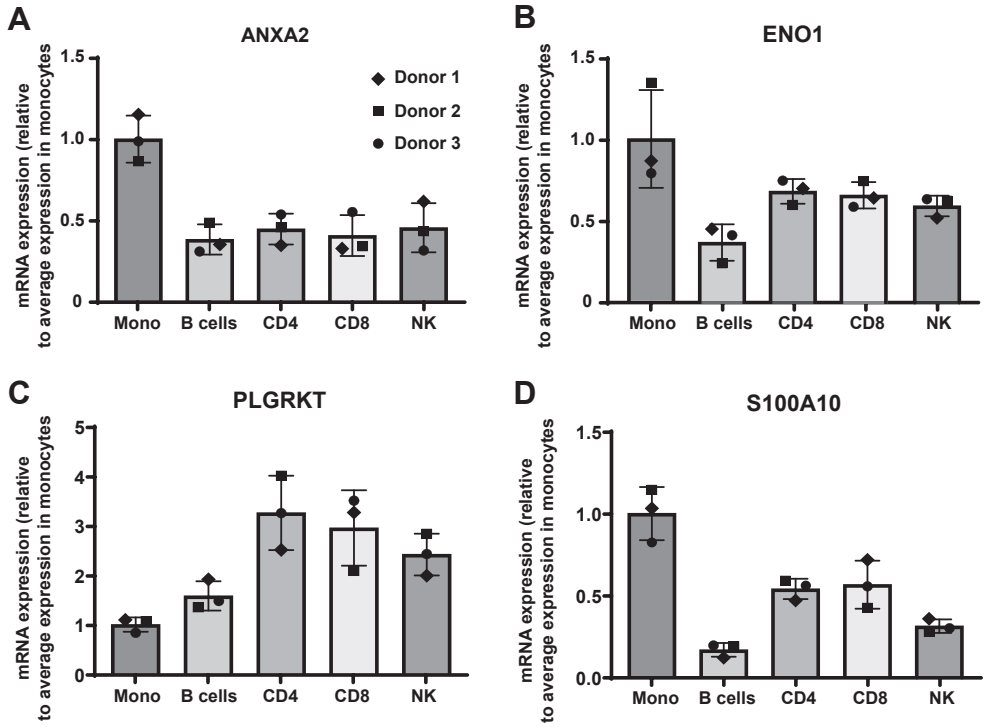

Figure 2. RT-PCR demonstrates that common human lymphocyte subsets express well-characterized plasminogen-binding proteins. *A*, mRNA expression of ANXA2. *B*, mRNA expression of ENO1. *C*, mRNA expression of PLGRKT. *D*, mRNA expression of S100A10. Data are combined from three independent donors and are presented as mean ± SD.

anti-CD3/CD28 beads. This result is consistent with our previous observations that T cells upregulate the plasminogen activator tPA upon activation (4). Therefore, T cell activation can increase the conversion of plasminogen to plasmin, which is then capable of cleaving CCL21. This interpretation is further supported by the observation that the CCL21 cleavage effect was inhibited in the presence of the specific plasmin inhibitor α2-antiplasmin (Fig. 1*I*).

Human lymphocytes express plasmin-binding molecules

We hypothesized that lymphocytes express one or more of the best-documented plasmin-binding receptors. Therefore, reverse transcription quantitative PCR (RT-qPCR) was used to assess the expression of ANXA2, ENO1, PLGRKT, and S100A10 in B cells, CD4+ T cells, CD8+ T cells, monocytes, and natural killer cells derived from three individual human donors. We detected mRNA for all four plasminogen receptors in all of the cell types tested (Fig. 2 and Table S1). In both CD4+ and CD8+ T cells high Ct values for ANXA2 and S100A10 and high expression of PLGRKT relative to that detected in monocytes was observed. These data suggest that T cells have the potential to bind plasminogen from the circulation and carry it into tissues as they migrate, where it could be activated by extracellular plasminogen activators such as tPA.

Plasmin increases the chemotactic response of human DCs to CCL21

We recently demonstrated that an under-agar migration assay used in combination with live-cell microscopy could accurately analyze different parameters of chemotactic and chemokinetic migration of human immune cells in response to CCL19 (21). The number of migrating cells, their migration speed and track straightness, and the total displacement of the cells toward the chemotactic signal can be measured over time, typically up to a 6 h final time point

purified plasminogen (10 nM) and/or lysine analogs EACA and TXA. Any unbound plasminogen was washed away before plasminogen was activated with tPA (1 nM) in serum-free medium with a plasmin substrate and incubated for 16 h. *E*, Western blot showing binding of plasmin to T cells. T cells were incubated in serum-free medium for 2 h in the presence or absence of purified plasmin (10 nM or 100 nM). Any unbound plasmin was washed away before the T cells were lysed. *Dashed line* indicates where the blot image was spliced for presentation purposes. *F*, Western blot showing binding of plasminogen to T cells. T cells were incubated in serum-free medium for 2 h in the presence or absence of purified plasminogen (10 nM or 100 nM). Any unbound plasminogen was washed away before the T cells were lysed. *Dashed line* indicates where the blot image was spliced for presentation purposes. *G*, Western blot showing binding of plasmin and plasminogen from human serum to T cells. T cells were incubated in medium containing 50% human serum over a 24 h time period. Any unbound plasmin and plasminogen was washed away before the T cells were lysed. *H*, Western blot showing cleavage of CCL21. CCL21 (1 ng/μl) was incubated with T cells (100 cells/μl) or T cells that were previously incubated with plasmin (100 nM) for 2 h. *I*, Western blot showing cleavage of CCL21. CCL21 (1 ng/μl) was incubated with T cells (100 cells/μl) or αCD3/CD28–activated T cells, with and without α2-antiplasmin (100 nM), that were previously incubated with plasminogen (100 nM) for 2 h. Data are combined from three independent experiments and are presented as mean ± SD. Results were analyzed using one-way ANOVA with a Fisher's least significant difference (LSD) post hoc test. p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001 (*A–D*). Western blots are a representative of at least three independent experiments (*E–I*). CCL21, C-C motif chemokine ligand 21; EACA, ε-aminocaproic acid; tPA, tissue plasminogen activator; TXA, tranexamic acid.

when chemokine diffusion has annulled the initial gradient (21).

We used this assay to first compare various motility parameters of mDC over time in response to CCL21 alone or CCL21 in the presence of plasmin. Few cells were observed migrating toward CCL21 alone (Fig. 3*A* and Video S1). However, the combination of CCL21 with plasmin led to an increase in the number of migrating cells that showed a statistically significant difference in the response of the cells over the course of the assay and at the 4 to 5 h and 5 to 6 h time points (Fig. 3*A* and Video S2). A significantly increased displacement pattern of mDCs toward CCL21 with plasmin compared to CCL21 alone was observed over the course of the assay ($p \leq 0.01$) and at each hourly time point for the first 5 h of the assay (Fig. 3*B*). At the end of the 6 h time point, this effect was lost, consistent with our previous work in this experimental system that suggested loss of gradient at this time point (21). Similarly, track straightness of mDCs was also significantly increased (Fig. 3*C*) in the presence of plasmin at every time point, except at 5 to 6 h. When the treatment effect in relation to the time factor was analyzed, there was no significant change, indicating that plasmin increases the track straightness in a similar manner at each time point but not in a time-dependent manner. Finally, when analyzing the effect of plasmin on the migration speed of the cells in response to CCL21-mediated migration, no significant increase was observed (Fig. 3*D*). This suggests that the primary impact of exposure to CCL21 with plasmin was on the direction of movement (increased straightness and displacement) rather than overall cell motility.

Plasmin or plasminogen-bound activated T cells increase the CCL21-mediated chemotactic response in DCs

Having demonstrated that plasmin bound to T cells could cleave and truncate CCL21, we then wanted to assess if this was functionally relevant to CCL21-mediated migration of mDCs using the live-cell migration assay. Compared to control cells, plasmin-loaded T cells induced a significantly increased chemotactic response over the measured time course, with a statistically significant increase in the number of migrating cells at the 5 to 6 h time point (Fig. 4*A*) and a significant increase in displacement of mDC toward CCL21 over the complete time course and at the 4 to 5 h time point (Fig. 4*B*). However, neither track straightness (Fig. 4*C*) nor migration speed of the mDCs (Fig. 4*D*) was significantly affected when comparing plasmin loaded or control T cells in this assay.

Having also demonstrated that activated T cells could convert bound plasminogen to active plasmin, which can then cleave CCL21, we hypothesized that activated plasminogen-loaded T cells would increase CCL21-mediated migration of mDCs in this assay in a similar way to plasmin loaded T cells.

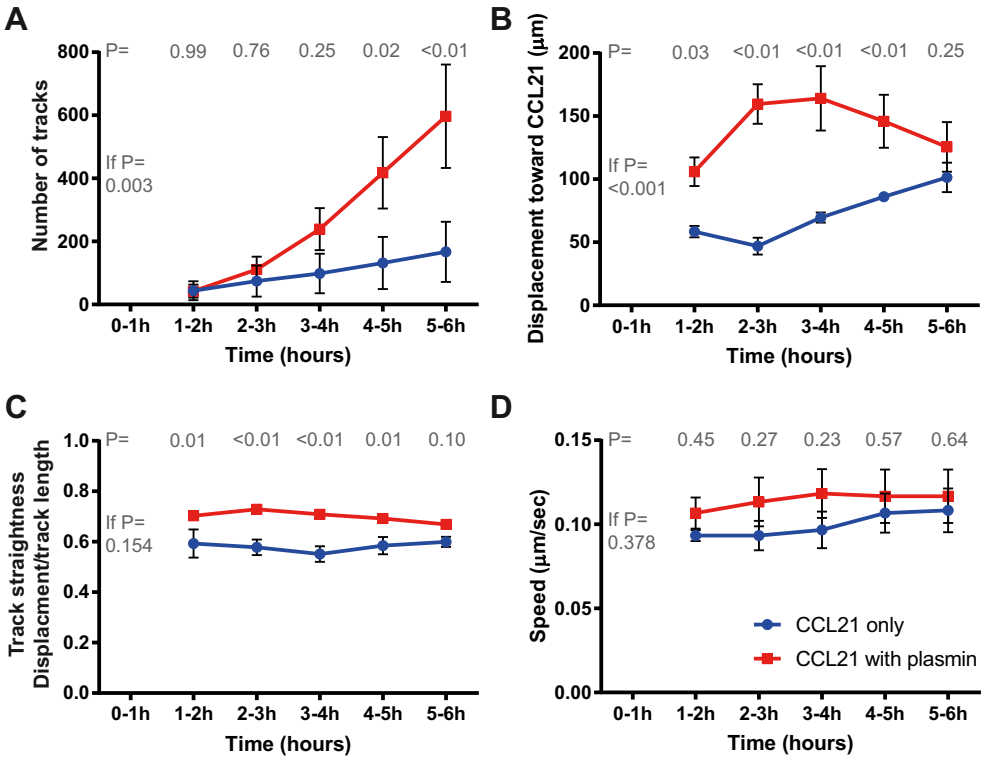

Figure 3. Addition of plasmin in the time-lapse "under-agar" assay leads to increased chemotactic migration of human DCs. *A*, plasmin increased the number of mDCs that respond to CCL21. *B*, plasmin increases the net displacement of the mDCs responding to CCL21. *C*, plasmin increases the track straightness (displacement/total track length) of mDCs in response to CCL21. *D*, effect of plasmin on migration speed of mDCs in response to CCL21. Data are combined from three independent experiments and are presented as mean ± SEM. Results were analyzed using two-way repeated measure ANOVA multiple with *p* values shown for treatment effect over time (interaction factor (If)) and multiple comparisons with Fisher's least significant difference (LSD) post hoc test. CCL21, C-C motif chemokine ligand 21; DC, dendritic cell; mDC, monocyte-derived DC.

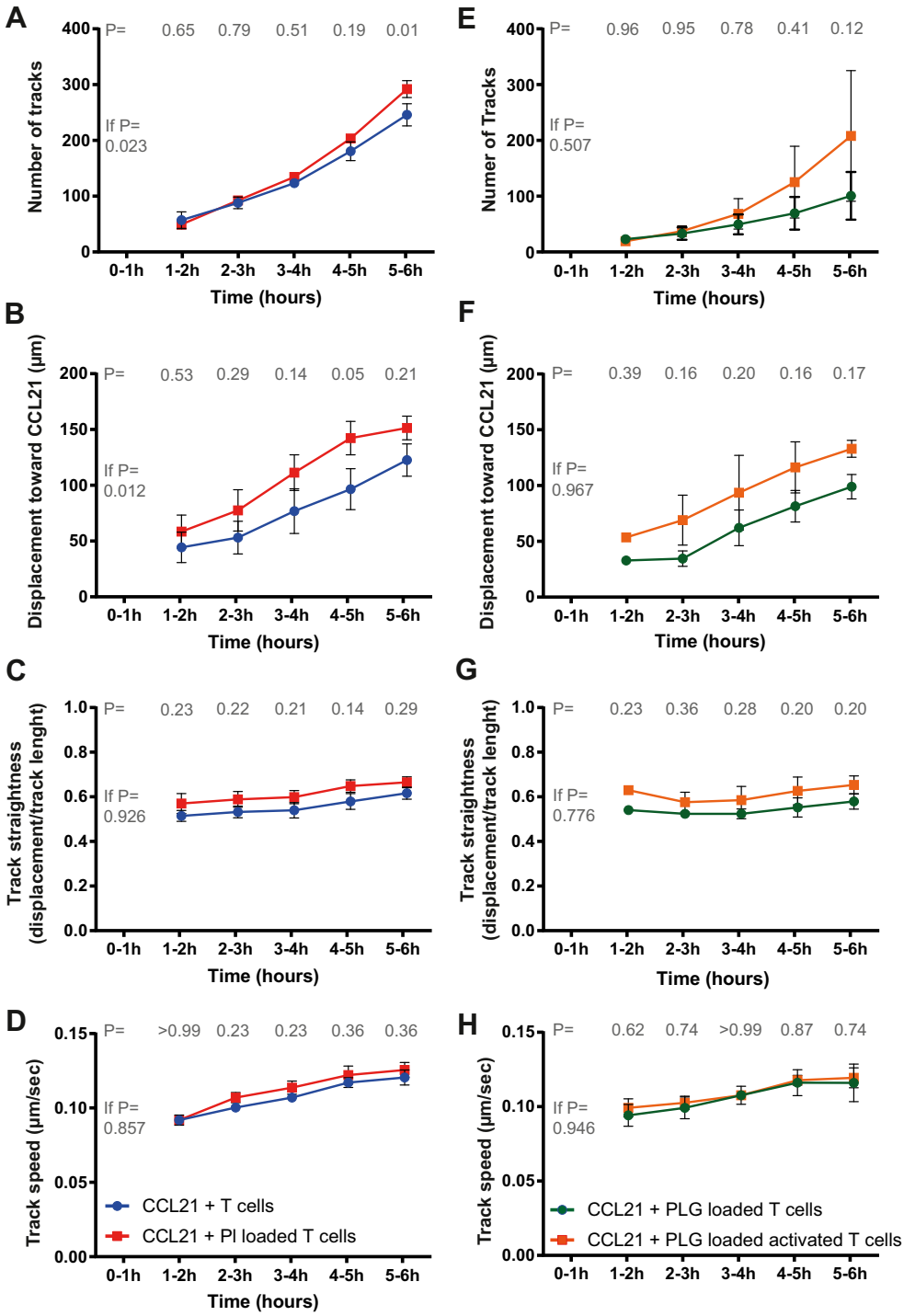

Figure 4. Incubation of CCL21 with plasmin-loaded T cells leads to increased chemotactic migration of DCs. *A*, preloading T cells with plasmin increased the number of mDCs that respond to CCL21. *B*, preloading T cells with plasmin increases the net displacement of the mDCs responding to CCL21. *C*, the effect of preloading T cells with plasmin on the track straightness (displacement/total track length) of mDCs in response to CCL21. *D*, the effect of preloading T cells with plasmin on the migration speed of mDCs in response to CCL21. *E*, the effect of activating T cells preloaded with plasminogen on the number of mDCs that respond to CCL21. *F*, the effect of activating T cells preloaded with plasminogen on the net displacement of the mDCs responding to CCL21. *G*, the effect of activating T cells preloaded with plasminogen on the track straightness (displacement/total track length) of mDCs in response to CCL21. *H*, the effect of activating T cells preloaded with plasminogen on the migration speed of mDCs in response to CCL21. Data are combined from three independent experiments and are presented as mean ± SEM. Results were analyzed using two-way repeated measure ANOVA multiple with *p* values shown for treatment effect over time (interaction factor (If)) and multiple comparisons with Fisher's least significant difference (LSD) post hoc test. CCL21, C-C motif chemokine ligand 21; DC, dendritic cell; mDC, monocyte-derived DC.

Although there was a similar trend as was observed with the plasmin-loaded T cells, when we compared the number of mDCs migrating toward CCL21 in the presence of plasminogen-loaded T cells, the data failed to reach statistical significance on any migration parameter (Fig. 4, *E–G*). The lack of statistical significance may reflect confounding limitations within our assay system, such as the presence of 5% human serum, which is known to contain a complex mixture of plasminogen activators and inhibitors (2).

Discussion

This study demonstrates for the first time that T cells bind plasminogen, potentially enabling them to carry it out of the circulation and convert it to plasmin in tissue environments following T-cell activation through the release of tPA. When incubated with full-length CCL21, plasmin-bound T cells exert an effect similar to purified plasmin alone, that is, they can functionally truncate CCL21 and increase the chemotactic response of mDCs. The increased displacement of mDCs in response to CCL21 cleaved by plasmin was due to increased track straightness in cell motility, without significant increases in migration speed, consistent with increased chemotaxis.

It has been previously reported that monocytes and macrophages express proteins that can bind plasmin and plasminogen on the surface (22–24) and that plasmin activation on the cell surface can affect their function. Considering the broad expression of different plasminogen receptors, it seemed likely that other human immune cells also express these plasminogen receptors and could bind plasmin on their surfaces. Using RT-qPCR, we confirmed that this is indeed the case, as mRNA of four well-characterized plasminogen-binding cell surface proteins was detected on several other immune cell populations. We note that the expression of the PLG-R_{KT} receptor was relatively high on both CD4 and CD8 T cells compared to the other immune cell populations tested. Since PLG-R_{KT} is an integral membrane plasminogen receptor with an extracellular C-terminal lysine, plasmin(ogen) binding is not dependent on preprocessing or other molecular interactions (8). Additionally, both CD4+ and CD8+ T cells showed high expression levels of mRNA for ANXA2 and S100A10. This is of particular interest as the ANXA2 and S100A10 heterotetramer has been identified as a receptor for plasmin generation and plasmin-induced signaling (4, 7, 25). Our data indicate that plasmin(ogen) binding to T cells is lysine dependent, consistent with lysine-dependent binding through the kringle domain of plasmin(ogen). However, we did not directly investigate whether PLG-R_{KT} or ANXA2 and S100A10 heterotetramer were indeed the main cell surface molecules that bound plasmin(ogen).

We have previously reported that plasmin removes the C-terminal peptide of CCL21 (10). The removal of the C-terminal end of CCL21 abolishes its ability to bind to the extracellular matrix or to cell surfaces and induces a shift in the structured domain of CCL21. These changes make CCL21 behave more like the soluble chemokine CCL19, which acts on the same receptor (14, 16). Our results here are consistent with this model of conversion of matrix-bound CCL21 to a soluble molecule similar to CCL19 and expand the possible cellular mechanisms that may induce this conversion to include activated T cells bearing plasmin.

Plasmin bound to T cell surfaces is functional and capable of cleaving CCL21. When plasmin-loaded T cells were coincubated with CCL21, we observed an increase in the chemotactic response of the mDCs consistent with functional cleavage of the CCL21. The effect was not as pronounced compared to coincubation of purified plasmin with CCL21, but significant increases in chemotaxis parameters were observed, including numbers of cells migrating and displacement. The diminished response is probably due in part to a dose reduction, with less plasmin bound to T cells compared to the cell-free purified plasmin control. The presence of T cells may also have reduced the availability of CCL21 to mDCs. This hypothesis aligns with previous observations that migrating leukocytes can actively alter gradients by the removal of chemokines (26).

We have previously reported that expression of the plasminogen activator tPA is increased in activated T cells (4), and this suggests a mechanism whereby T cells, once activated, can convert plasminogen bound to their surfaces to active plasmin. When plasminogen-loaded T cells were coincubated with CCL21 under serum-free conditions, only minimal cleavage of CCL21 was observed. However, when the T cells were stimulated with anti-CD3/CD28 beads, CCL21 cleavage increased in a plasmin-dependent manner. This demonstrates that activated T cells can convert plasminogen to active plasmin that can then cleave CCL21. Although we did not observe a statistically significant increase in CCL21-driven chemotaxis by mDCs when plasminogen-loaded T cells were activated, this may reflect confounding limitations within our assay system. Specifically, the T cells are loaded with plasminogen in the presence of 5% human serum, which is known to contain a complex mixture of plasminogen activators and inhibitors (2) that may interfere with this assay.

This study demonstrates that T cells are a potential source of plasmin in tissues where CCL21 is present and proposes a novel mechanism by which T cells might regulate the localized cleavage and presentation of CCL21 within tissues (Fig. 5). Our data suggest that T cells, while circulating, are likely to bind high amounts of plasminogen present in the plasma. Once the T cells enter the tissue and encounter cognate antigen, activation of the T cell upregulates tPA, which allows for the conversion of plasminogen to active plasmin. This activation may be especially potent in the tissues as the plasmin inhibitors that control and localize plasmin in the circulation are absent. Therefore, in a tissue-specific and localized manner plasmin cleaves CCL21, releasing it from the matrix and generating a gradient of truncated CCL21. The untethered and truncated CCL21 binds to and activates the CCR7 receptor on nearby immune cells, such as DCs, to induce an increased chemotactic response on two levels: (1) compared to bound CCL21, the untethered variant forms a soluble gradient that increases directional movement and (2) soluble CCL21 undergoes a conformational change that increases binding affinity to CCR7 further enhancing directional migration. If

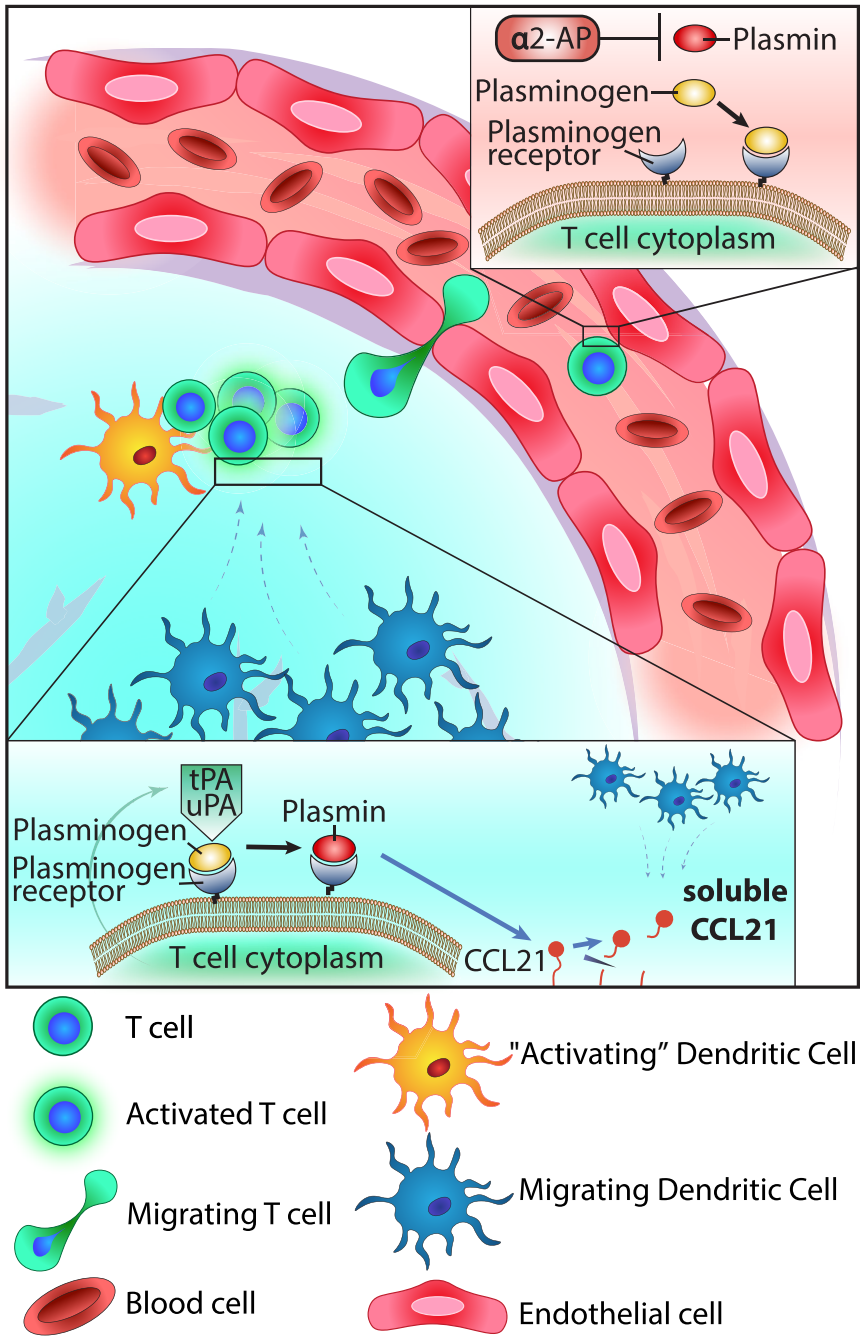

Figure 5. Proposed mechanism by which T cells might regulate the localized plasminogen cleavage and functional effects on CCL21. When T cells are circulating, they likely bind high amounts of plasminogen present in the serum. When the T cells enter tissue and encounter cognate antigen, activation of the T cells upregulates expression of plasminogen activators such as tPA/uPA, which allows for the conversion of plasminogen to active plasmin. Therefore, in a tissue-specific and localized manner plasmin cleaves CCL21, releasing it from the matrix and generating a gradient of truncated CCL21. The untethered and truncated CCL21 binds to and activates the CCR7 receptor on nearby immune cells, such as DCs, inducing chemotaxis. CCL21, C-C motif chemokine ligand 21; DC, dendritic cell; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

this occurred in lymphoid tissue, for example, this could increase migration of both CCR7+ DCs and naïve T cells to locations where T cells are already being activated, accelerating scanning of DCs loaded with immunogenic antigens.

The experiments described here focused on the role of plasmin cleavage in regulating the functional effects on CCL21. However, plasmin is a broad-spectrum protease that has been linked to the regulation of multiple biological activities (27). These include the degradation and modification of extracellular matrix proteins, activation of matrix metalloproteinase zymogens, release of growth factors and cytokines from the stroma, and cleavage of transmembrane molecules to generate cleavage products that induce outside-in signal transduction (28). Some of these plasmin activities have previously been linked to the modulation of chemokine activity. CCL8 is a heparin-binding molecule that can be cleaved by matrix metalloproteinases that are activated by plasmin cleavage (29). Macrophage secretion of CCL20, a chemokine

involved in the recruitment of Th17 cells, was reported to be induced by plasmin (30). Other chemokines with a similar structural motif as CCL21 that are also known to bind extracellular matrix or cell surfaces could potentially be cleaved by plasmin. In support of this possibility, it was previously reported that exposure of stromal cell-derived factor-1α to human serum led to the proteolytic removal of a C-terminal lysine (31).

In the context of the potential clinical relevance of T cells carrying plasminogen into tissues, two immediate fields to contemplate are wound healing and cancer therapy. Plasmin has been shown to play a key role in animal models of wound healing (32, 33), and recent work showed plasminogen can be transported into healing wounds bound to macrophages and neutrophils (32). This additional plasminogen induces the release of cytokines, potentiating the early inflammatory immune response and accelerating healing. In the same way, T cell carriage and activation of plasminogen may aid wound healing, although this effect is likely to be outweighed by myeloid cells, given they are more abundant in most types of wounds. In cancer, T-cell infiltrates are primarily associated with improved clinical outcomes, including improved responses to immunotherapy, such as checkpoint blockade. However, it is currently unknown whether T cells carrying plasminogen into tumors are likely to increase antitumor effects.

On the one hand, increased recruitment of DCs and T cells through the chemokine modulation we observed could be expected to improve the immune attack on cancer cells. Similarly, plasmin can increase the clearance of dying and necrotic cells by antigen-presenting cells (34–36), potentially improving adaptive immune responses. On the other hand, plasmin activation by urokinase plasminogen activator in tumors accelerates invasion, metastasis, and tumor growth (37), so there is the potential that T cells bearing plasminogen might increase some of these protumor activities. Interestingly, T cells infiltrating both wounds and tumors would need to be activated to generate plasmin, suggesting antigen recognition soon after infiltration would be necessary to modulate overall plasmin activity in the tissue.

Overall, our results extend the range of plasmin-regulated activities to include localized regulation of immune cell migration within tissues. Our findings also suggest that immune cells can co-opt this powerful proteolytic system from the circulation and activate it within tissues where it is subject to less regulation and likely to exert wide-ranging functional effects.

Experimental procedures

Recombinant proteins

Human recombinant interleukin (IL)-4 (catalog no.: 200-04), granulocyte macrophage colony-stimulating factor (catalog no.: 300-23), 1β (catalog no.: 200-01B), IL-6 (catalog no.: 200-06), TNF-a (catalog no.: 300-01A), CCL21 (catalog no.: 300-35), IL-7 (catalog no.: 200-07), IL-12 (catalog no.: 200-12p80H), and IL-21 (catalog no.: 200-21) was purchased from PeproTech and are reported to have endotoxin levels less than 0.1 ng/μg of protein (<1 EU μg^{-1}). All CCL21 solutions were prepared in low-bind 1.5 ml Eppendorf tubes. Prostaglandin E_2 (P0409), plasmin from human plasma (catalog no.: P1867), and plasminogen (catalog no.: RSP6518) from human plasma were purchased from Sigma–Aldrich. tPA (Actilyse) was kindly donated by Boehringer Ingelheim Limited. α2-antiplasmin (catalog no.: HA2AP) was purchased from Molecular Innovations.

Cell culture

Human blood was obtained from healthy volunteers after informed consent and with approval by the University of Auckland Human Participant Ethics Committee (Ethics Approval 010558). The human studies reported on in this artcle abide by the Declaration of Helsinki principles.

Peripheral blood mononuclear cells were prepared using Lymphoprep (Axis-Shield) density gradient centrifugation. mDCs were differentiated from CD14+ monocytes based on a previously reported method (38). In short, CD14+ cells were isolated using the MACS human CD14+ isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. One to two × 10^6 CD14+ cells were plated in a 24-well plate with AIM-V medium (Life Technologies) supplemented with 1× GlutaMAX (Life Technologies) and 200 ng/ml IL-4 and 100 ng/ml granulocyte macrophage colony-stimulating factor. Half of the medium was replaced at day 2 or 3. On day 5 nonadherent and mildly adherent cells were resuspended and transferred to a 15 ml conical tube and centrifuged at 350*g* for 5 min. The pellet was resuspended in 1 ml fresh AIM-V containing 100 ng/ml granulocyte macrophage colony-stimulating factor, 10 ng/ml IL-1β, 100 ng/ml IL-6, 250 ng/ml TNF-a, and 1 μg/ml prostaglandin E_2 to mature the cells for a further 48 h.

T cells were expanded and cultured as previously described (4). Human peripheral blood T cells were enriched from isolated peripheral blood mononuclear cells (PBMCs) by negative magnetic bead selection using a MACS human pan T-Cell Isolation Kit (Miltenyi Biotec). All T cells were cultured in RPMI1640 medium containing 5% human serum (One Lambda), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 2 mM GlutaMAX-1 (Life Technologies), supplemented with 5 ng/ml IL-7 (referred to as RS5-IL7) unless stated otherwise. All cytokines were purchased from PeproTech. Expanded T cells, in this article only referred to as T cells, were polyclonally expanded from freshly isolated PBMCs using Dynabeads human T-activator CD3/CD28 beads (Life Technologies) as previously described (39). In brief, 106 PBMCs were activated with Dynabeads at a bead:cell ratio of 1:1 for 3 days in RS5-IL7, supplemented with 10 ng/ml IL-12 and 10 ng/ml IL-21. Following magnetic removal of the beads, the cells were cultured for further 4 days using the same medium, followed by 7 days in RS5-IL7 supplemented with IL-21. Cells were examined daily, and cultures were split once cells were confluent or the medium showed signs of acidification (usually every 2–3 days). Cells were rested for a further 7 to 10 days in

RS5-IL7 prior to use or cryopreservation. Cryopreserved T cells were allowed to recover for at least 24 h in RS5-IL7 (20 ng/ml) before use.

RNA isolation and RT-qPCR

Total RNA was isolated from ~0.5 × 10^6 to 1 × 10^6 cells using RNeasy Mini Columns (Qiagen) and complementary DNA synthesized using oligo(dT) and random hexamer primers and Moloney murine leukemia virus RT, as described by the manufacturer. RT-qPCR was performed using the Kapa Probe Fast kit (Kapa Biosystems) and PrimeTime quantitative PCR assays (Integrated DNA Technologies) for annexin a2, PLGRKT, S100A10, ENO1, cyclophilin A, and TATA box–binding protein. Levels of annexin a2, Plg-Rkt, ENO1, S100A10, cyclophilin A, and TATA box–binding protein transcripts were quantitated by RT-qPCR. ANXA2, PLGRKT, ENO1, and S100A10 expression levels were normalized to cyclophilin A and phosphoglycerate kinase 1. The stability of reference genes across the different samples was determined using the quality control node in the qbase software (Biogazelle) and was found to match the predefined quality control settings. Relative mRNA quantities were calculated using the ΔΔ comparative threshold method by use of qbase software. Any outlying technical replicates were excluded from further analysis (Table S1). The data represented are the mean and SEM of the expression level for each gene, assayed in triplicate.

Plasmin and plasminogen binding to cells and Western blotting

The 0.1 × 10^6 polyclonal T cells were incubated in 100 μl Accell medium containing 2 mM GlutaMAX-1, supplemented with 10 ng/ml IL-7 with 10 nM or 100 nM plasmin or plasminogen for 2 h.

Following incubation, the cells were washed with warm AIM-V medium three times before being centrifuged, and the supernatant was removed. The cell pellet was directly lysed in 62.5 μM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue at 95 °C for 5 min. Protein samples were fractionated on 12% SDS-PAGE gel and analyzed by Western blotting using antiplasminogen (Invitrogen) and anti-b-actin (A00702-100, GenScript) antibodies. Western blots were visualized using 680RD donkey antimouse (Li-Cor Biosciences) and 800 CW donkey anti-rabbit secondary antibodies (Li-Cor Biosciences) and an Odyssey CLx (Li-Cor Biosciences) dual-color imaging system. Quantitation of plasmin(ogen) binding was done using Image Studio v3.1 (Li-Cor Biosciences).

CCL21 cleavage by plasmin system proteins and Western blot analysis

Human T cells were incubated with 100 nM plasmin or plasminogen for 2 h before being washed three times and resuspended in warm AIM-V.

Cleavage of CCL21 by T cells was determined by coincubation of 100 ng CCL21 with 100,000 T cells. T cells were activated using Dynabeads human T-activator CD3/CD28 beads (Life Technologies) with or without 2.5 μg/ml α2-antiplasmin as indicated. 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). Western blot analysis was done as described previously (35), using rabbit polyclonal antihuman CCL19 and CCL21 (catalog. no.: 500-P95B and 300-P109; both PeproTech).

Agar set up

To make 0.5% agar gels, 2 ml 2× RPMI (made from Powder) (Sigma) was mixed with 200 μl human serum (One Lambda) and 800 μl ultrapure H_2O. This was prewarmed to 37 °C in a water bath. About 2% agar was dissolved in ultrapure H_2O by bringing it to a boil in the microwave and mixing it on high speed on a vortex mixer for 20 s. This process was repeated four times. One milliliter of the agar solution was added to the prewarmed mixture to make a 0.5% agar medium solution. Of the solution, 800 μl was added to each well of a 4-well 1.5 polymer tissue culture–treated chambered coverslip (Ibidi) that was precoated with 20% human serum in RPMI for 30 min at 37 °C.

Microscopy

The cells were stained with CytoTrack green or red (Bio-Rad). The dye was diluted 1:500 in PBS. The cells were resuspended in the PBS dye solution at 2 M cell/ml and incubated at room temperature for 15 min. Cells were then centrifuged at 350*g* for 5 min and washed once with their respective culturing medium followed by resuspension in RPMI1640 medium (Life Technologies) containing 5% human serum (One Lambda), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 2 mM GlutaMAX-1 (Life Technologies), supplemented with 5 ng/ml IL-7. The cells, 50,000 mDCs, were added to the middle well in the agar set up. In one of the outside agar wells 100 ng CCL21 was added to generate a gradient by diffusion.

The μslide containing the agar and cells was then placed on an inverted Nikon TI-e (Nikon) and visualized using a 10× 0.4 numerical aperture Nikon lens and an Andor Zyla 5.5 camera (Oxford Instruments). An image was taken every minute for up to the indicated times.

Image analysis with Imaris

Imaris software (Oxford Instruments) was used to analyze the image sequences. Using the spot tracking module, the cells were detected by their respective fluorescence label and tracked in 1 h blocks.

Plasmin activity assay

T cells were cultured in black-walled clear bottom plates (Corning; catalog no.: 3603) before incubation for 2 h with plasmin or plasminogen (10 nM) and either ε-aminocaproic acid (Sigma–Aldrich, catalog no.: A2504), tranexamic acid (Sigma–Aldrich, catalog no.: PHR1812), or vehicle as

indicated. After three washes with prewarmed AIM-V, 10 nM of H-D-Val-Leu-Lys-AMC acetate (I1390, Bachem) with or without 1 nM tPA was added. The plate was then sealed, and fluorescence was measured every 15 min for 16 h on either an EnVison Multilabel plate reader (PerkinElmer) or a Spectramax ID3 plate reader (Molecular Devices).

Statistics

Prism 9.2.0 (GraphPad) was used for all statistical analyses. Graphs were analyzed using a repeated measure two-way ANOVA or a one-way ANOVA with a Fisher's least significant difference as indicated. For the two-way ANOVA the following components were analyzed:

Interaction factor (time × treatment) (treatment effect over time)

The null hypothesis is that there is no interaction between columns (Treatment) and rows (time points). More precisely, the null hypothesis states that any systematic differences between columns are the same for each row and that any systematic differences between rows are the same for each column. This means if this is significant: that time affects the column (in this case, the treatment of the cells) different. An interaction effect means that the effect of one factor depends on the other factor and it is shown by the lines in our profile plot not running parallel. In this case, the effect for time interacts with treatment. That is, time affects plasmin (treated cells) differently than control.

Multiple comparisons (difference between treatments at each time point)

Multiple comparisons were made for each time point, presented in each graph, and corrected with Fisher's least significant difference post hoc test.

Data availability

All data to support our hypothesis have been included. Raw data files are to be shared upon request. Contact: Evert J. Loef (e.j.loef@auckland.ac.nz).

Supporting information—This article contains supporting information.

Author contributions—E. J. L., P. R. D., and N. P. B. conceptualization; E. J. L., P. R. D., and N. P. B. methodology; E. J. L. validation; E. J. L. formal analysis; E. J. L. investigation; H. M. S., P. R. D., and N. P. B. resources; P. R. D. and N. P. B. data curation; E. J. L. writing–original draft; H. M. S. and P. R. D. writing–review & editing; P. R. D. and N. P. B. supervision; P. R. D. and N. P. B. funding acquisition.

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Abbreviations—The abbreviations used are: CCL21, C-C motif chemokine ligand 21; DC, dendritic cell; IL, interleukin; mDC, monocyte-derived DC; PBMC, peripheral blood mononuclear cell; RT-qPCR, reverse transcription quantitative PCR; tPA, tissue plasminogen activator.

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