Cytoskeletal confinement of CX$_3$CL1 limits its susceptibility to proteolytic cleavage by ADAM10

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**ABSTRACT** CX$_3$CL1 is a unique chemokine that acts both as a transmembrane endothelial adhesion molecule and, upon proteolytic cleavage, a soluble chemoattractant for circulating leukocytes. The constitutive release of soluble CX$_3$CL1 requires the interaction of its transmembrane species with the internal membrane metalloprotease ADAM10, yet the mechanisms governing this process remain elusive. Using single-particle tracking and subdiffraction imaging, we studied how ADAM10 interacts with CX$_3$CL1. We observed that the majority of cell surface CX$_3$CL1 diffused within restricted confinement regions structured by the cortical actin cytoskeleton. These confinement regions sequestered CX$_3$CL1 from ADAM10, precluding their association. Disruption of the actin cytoskeleton reduced CX$_3$CL1 confinement and increased CX$_3$CL1–ADAM10 interactions, promoting the release of soluble chemokine. Our results demonstrate a novel role for the cytoskeleton in limiting membrane protein proteolysis, thereby regulating both cell surface levels and the release of soluble ligand.

**INTRODUCTION**

The recruitment of leukocytes to sites of inflammation involves a complex series of coordinated signaling cascades mediating interactions between leukocytes and the injured endothelium. Two critical components of this process are chemokines, responsible for attracting and activating of leukocytes, and endothelial adhesion molecules, responsible for firm adhesion of leukocytes to endothelial cells (ECs; Huang et al., 2009). Among the >40 chemokines described, CX$_3$CL1, also known as fractalkine, is one of only two that can exist both as a soluble chemokine and a transmembrane, endothelial adhesion molecule (Bazan et al., 1997; Fong et al., 1998, 2000; Imai et al., 1997). Of importance, CX$_3$CL1 binds only one known receptor, CX$_3$CR1, expressed by various leukocytes, including monocytes, subpopulations of T lymphocytes, and natural killer (NK) cells (Fong et al., 1998). In recent years, CX$_3$CL1 and its receptor have been implicated in the pathogenesis and progression of many inflammatory disorders, including organ transplant rejection, renal inflammation, rheumatoid arthritis, and, most notably, atherosclerosis (Robinson et al., 2000; Haskell et al., 2001; Ruth et al., 2001; Combadiere et al., 2003; Lesnik et al., 2003; Lucas et al., 2003; Nakatani et al., 2010).

Conversion of CX$_3$CL1 from the transmembrane to the soluble species occurs through proteolytic cleavage that can be mediated by two metalloproteases: a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10), a constitutively active protease, and the tumor necrosis factor-α–converting enzyme (TACE, also called ADAM17), a protease with inducible activity (Garton et al., 2001; Tsou et al., 2001; Hundhausen et al., 2003). The mechanisms controlling these processes, however, have not been well defined. Whereas TACE remains inactive in the absence of stimulation, ADAM10 is constitutively active, yet, paradoxically, cleaves...
only a small fraction of CX3CL1 in the basal state (Hundhausen et al., 2003). Thus it is conceivable that either ADAM10 has limited proteolytic activity or is physically restricted from accessing the entire pool of cell surface CX3CL1.

Molecules in the plasma membrane can be compartmentalized by the cytoskeleton or segregated into lipid microdomains, which restrict and regulate their lateral diffusion (Kusumi et al., 2005a,b; Suzuki et al., 2007; Andrews et al., 2008; Goswami et al., 2008; Treanor et al., 2010, 2011). Whether membrane compartmentalization can regulate proteolysis by limiting protease–substrate interactions has not been explored. To address these questions, we used a combination of single-particle tracking, immunochemistry, and subdiffraction imaging to study both CX3CL1 and ADAM10. Our results indicate that cortical actin confinement regions restrict plasmalemmal CX3CL1 mobility. We further demonstrate that this confinement limits interactions between ADAM10 and CX3CL1, regulating the amount of transmembrane chemokine cleaved by the protease. Taken together, our results reveal an unprecedented regulatory role of the cytoskeleton in protecting substrates from proteolytic cleavage, thereby controlling the availability of ligand on the plasma membrane, as well as the release of ligand from the plasma membrane.

RESULTS

CX3CL1 experiences confined motion on the surface of endothelial cells

To investigate the dynamics of CX3CL1 in living cells, we assessed the mobility of individual molecules using single-particle tracking (SPT). This system entailed labeling surface CX3CL1 at an intermediate density with primary antibody, followed by biotinylated secondary Fab fragments, and, finally, streptavidin-coated quantum dots. Caution was taken to limit the formation of CX3CL1 clusters by blocking excess binding sites on the quantum dots with soluble biotin (see Materials and Methods). Using this approach, we were able to limit photobleaching, record videos at a frame rate of 30 Hz for 300 frames, and reconstruct CX3CL1 trajectories using a multiparticle tracking algorithm (Jaqaman et al., 2011). This algorithm uses moment scaling spectrum (MSS) analysis, identifying both mode of diffusion and diffusion coefficient.

Whereas primary ECs express minimal levels of CX3CL1 under noninflammatory conditions, robust levels of the chemokine appear on the cell surface after exposure to tumor necrosis factor α (TNF-α; unpublished data). Using TNF-α-stimulated ECs, we tracked the mobility of endogenous cell surface CX3CL1 and found that 81.0 ± 1.5% experienced confined motion, whereas 18.0 ± 1.0% experienced free motion (Figure 1, A–D, and Supplemental Movie S1). These results were surprising, as this level of confinement is much greater than what has been described for other membrane-spanning proteins (Treanor et al., 2010; Jaqaman et al., 2011; Jaumouille et al., 2014). Furthermore, we found that the confined population moved with a diffusion coefficient of 0.028 ± 0.005 μm²/s and was restricted to a mean confinement area of 0.036 ± 0.003 μm² (Figure 1, D and E). CX3CL1, however, experienced a broad range of confinement areas, indicating a heterogeneous population (Figure 1, E and F). To confirm that we were visualizing only surface CX3CL1 and that labeling with antibody did not cause internalization of the chemokine, we exposed cells to an acid wash to dissociate extracellular antibody while preserving cell integrity. Less than 10% of the labeled CX3CL1 remained after the acid wash, indicating that surface rather than internalized CX3CL1 was being detected (unpublished data).

It is conceivable that the quantum dot probes used may cause artefactual confinement of CX3CL1 molecules. To address this concern, we performed SPT of the chemokine using secondary Fab fragments conjugated to the small fluorophore Cy3. Although the signal-to-noise ratio of Cy3 is much less than that of a quantum dot, we found similar fractions of CX3CL1 undergoing confined and free motion (77.0 ± 1.4% and 21.0 ± 1.2%, respectively) and measured comparable diffusion coefficients (Supplemental Figure S1, A and B). These findings demonstrate that the detected confinement of CX3CL1 is not due to artificial clustering or cross-linking but is in fact an inherent property of the chemokine itself.

Although confined, CX3CL1 molecules were clearly mobile (diffusion coefficient of 0.028 μm²/s), suggesting that they are not anchored to a fixed structure but instead diffusing within a membrane corral. To validate the restricted mobility of CX3CL1, we performed SPT using fixed samples. We reasoned that if the detection limit of our experimental system had not been reached, fixation would further reduce the mobility of CX3CL1. Accordingly, fixation increased confined motion from 81.0 ± 1.5 to 95.0 ± 0.5% (p < 0.0001), decreased free motion from 18.0 ± 1.0 to 4.6 ± 0.6% (Supplemental Figure S1D; p < 0.0001), and, of importance, decreased the diffusion coefficient of the confined molecules from 0.028 ± 0.005 to 0.005 ± 0.001 μm²/s (Supplemental Figure S1E; p < 0.0001). Fixation also resulted in a decrease in confinement area from 0.036 ± 0.003 to 0.007 ± 0.002 μm² (Figure 1, E and F, p < 0.0001). Taken together, these results suggest that in live cells, CX3CL1 is not completely immobile but instead moves within confined regions of the plasma membrane.

CX3CL1 is not confined by caveolae or membrane rafts

The preceding results led us to question how CX3CL1 might be confined within regions of the plasma membrane. We reasoned that CX3CL1 could be associated with cholesterol-enriched membrane domains, such as rafts or caveolae, which have been shown to restrict membrane protein diffusion (Thomsen et al., 2002; Pelkmans et al., 2004; Sinha et al., 2011). Caveolae are particularly abundant in ECs, and thus we assessed the colocalization of CX3CL1 and the caveolar protein, caveolin-1, using single-molecule subdiffraction imaging (Figure 2A; see Materials and Methods for details). This approach, termed spatial apposition analysis (SAA), involved labeling both proteins with saturating concentrations of primary antibody, followed by fluorescent secondary Fab fragments, and has been validated (Heit et al., 2013). The relative distances between CX3CL1 and caveolin-1 particles were measured next using a nearest-neighbor approach and compared with a randomly generated distribution of the same particles (Figure 2A). We then compared the fraction of particle pairs within a predetermined colocalization distance criterion (CDC) in both measured and random populations. As shown in Figure 2B, the amount of colocalization between CX3CL1 and caveolin-1 did not differ significantly from a predicted random distribution, indicating that the two proteins are not associated on the surface of endothelial cells. We verified the ability of the SAA to detect colocalization by using a Fab2 to cross-link CX3CL1 and TACE, two proteins with limited interactions under basal conditions (Tole et al., 2010). As reflected in our analysis, the amount of colocalization between these proteins initially did not differ significantly from that of a predicted random distribution (Supplemental Figure S2, A and B). However, upon cross-linking, we detected significant colocalization between CX3CL1 and TACE, thereby supporting the use of our system to detect protein interactions (Supplemental Figure S2, C and D).

To confirm that CX3CL1 is not confined by caveolae, we treated ECs with methyl-β-cyclodextrin (MβCD) to deplete cholesterol, thus disassembling caveolae, and assessed the distribution of the chemokine. As expected, treatment with MβCD depleted 60% of
cell cholesterol (Supplemental Figure S2, E and F) and disassembled caveolae, causing caveolin-1 to become uniformly distributed (Figure 2C). CX₃CL1 distribution, however, remained unchanged, further supporting the notion that it is not associated with caveolae (Figure 2C).

The possibility of rafts and caveolae confining CX₃CL1 was finally discounted by performing SPT after treatment with MβCD. We hypothesized that if these membrane domains confined CX₃CL1, depleting cholesterol would yield a measurable increase in mobility.

After depletion of cholesterol, however, we were unable to detect a significant change in either mode of diffusion or confinement area (Figure 2, D and E). In fact, treatment with MβCD actually decreased both CX₃CL1 confined and free diffusion coefficients (Figure 2F), similar to what has been reported for other membrane proteins (Fujisawa et al., 2002; Murase et al., 2004). Taken together, these results demonstrate that CX₃CL1 is not confined by caveolae or cholesterol-enriched membrane microdomains.

**CX₃CL1 is confined by the actin cytoskeleton**

Emerging evidence indicates that the cortical actin meshwork plays a role in regulating the mobility and distribution of membrane-associated proteins (Goswami et al., 2008; Chung et al., 2010; Treanor et al., 2010; Jaqaman et al., 2011; Gowrishankar et al., 2012). To determine whether the organization of actin affects the confinement of CX₃CL1, we treated cells with calyculin A (Cal A), which stimulates myosin II activity by inhibiting the myosin phosphatase. By stimulating myosin-mediated contractility, Cal A treatment induced the compaction of the actin cytoskeleton to the center of the cell, generating membrane blebs devoid of cortical actin (Figure 3A; Henson et al., 2003). Incubation of cells with blebbistatin, an inhibitor of myosin IIa, greatly reduced the formation of membrane blebs, confirming that Cal A compaction of the cytoskeleton is indeed myosin dependent (Supplemental Figure S3A). We hypothesized that if CX₃CL1 was associated with actin through direct or indirect interactions, incubation with Cal A would cause the chemokine to accumulate near the center of the cell, along with the contracted cytoskeleton. Accordingly, we found that Cal A caused CX₃CL1 to concentrate near the center of cells, colocalizing with the actin cytoskeleton (Figure 3B and Supplemental Figure S3B). To verify that this was not a general effect of Cal A on all membrane proteins, we examined its effects on the localization of a protein associated with the plasma membrane outer leaflet, glycosyl-phosphatidylinositol–linked green fluorescent protein (GPI-GFP), as well as a protein associated electrostatically with the inner leaflet, the tail of K-Ras (K-Ras-GFP; Yeung et al., 2006). In contrast to CX₃CL1, Cal A treatment caused both GPI-GFP and K-Ras-GFP to enter membrane blebs devoid of cortical actin (Figure 3, A and C). Of importance, GT-46–GT-46–yellow fluorescent protein (YFP), a chimeric transmembrane protein that is not associated with lipid rafts (Kenworthy et al., 2004), also localized to membrane blebs (Figure 3B). These results demonstrate that association with the cytoskeleton is a unique feature of CX₃CL1 rather than a property shared by all membrane proteins.
FIGURE 2: CX3CL1 is not confined by caveolae or cholesterol-rich membrane domains. (A) Single-molecule SAA of cell surface CX3CL1 and Cav-1. ECs were incubated with TNF-α and labeled with primary antibody, followed by fluorescent Fab fragments, detecting CX3CL1 and caveolin-1 at low densities. Experimentally measured particle distribution (solid black line) was compared with a predicted random distribution (dashed red line). Representative histogram depicting the spatial association between CX3CL1 and Cav-1 at the subdiffraction level. Reconstructed image of CX3CL1 (blue) and Cav-1 (red) after subdiffraction localization. Scale bar, 3 μm. (B) Comparison of the spatial association between CX3CL1 and caveolin-1 particles. The fraction of particle pairs within a predetermined colocalization cutoff was compared between the experimentally measured population (measured coincidence) and the predicted, random population (simulated random coincidence). The CDC was determined as described in Materials and Methods. For A and B, analysis was based on 3192 particle pairs from four independent experiments, combining >10 fields per experiment. (C) Spinning-disk confocal fluorescence images of cell surface CX3CL1 and caveolin-1 before and after depleting cholesterol with MβCD. Images are representative of three independent experiments. Insets, magnified view of indicated area. Scale bar, 15 μm. (D) Fraction of CX3CL1 particles experiencing confined and free motion. (E) Diffusion coefficients of confined and free CX3CL1 after MβCD treatment. (F) CX3CL1 confinement area after treatment with MβCD determined by SPT. Individual data points represent the median value of particles from a single field of view. Data are mean ± SEM of the medians from four independent experiments with 5–10 fields of view analyzed per experiment. *p < 0.05, **p < 0.001.
When combined with the SPT analyses, these findings suggest that CX3CL1 is fenced within actin-delimited “corrals” yet not firmly bound to them. To test this notion, we performed membrane extractions using nonionic detergents in a cytoskeleton-stabilizing buffer (see Materials and Methods for details). As shown in Figure 3, E and F, a large fraction (63%) of CX3CL1 was lost from the membrane upon extraction ($p < 0.0001$ vs. control). This confirms that CX3CL1 is not stably associated with the cytoskeleton and suggests that the observed mobility of the chemokine is a reflection of its free diffusion within corrals rather than motion restricted by a flexible,
CX₃CL1 confinement in the plasma membrane depends on the actin cytoskeleton. (A) Cells were incubated with either Lat B or Toxin B, and SPT of cell surface CX₃CL1 was performed as in Figure 1. Representative tracks of surface CX₃CL1 in Lat B–treated cells. Scale bar, 3 μm. (B) Fraction of CX₃CL1 particles experiencing confined and free motion after treatment with Lat B or Toxin B. (C) Confined and free diffusion coefficients for CX₃CL1 after disruption of actin using Lat B or Toxin B. (D) CX₃CL1 confinement area following treatment with Lat B or Toxin B. Individual data points represent the median value of particles from a single field of view. Data are mean ± SEM of the medians from at least three independent experiments with 5–15 fields of view analyzed per experiment. * p < 0.05, **p < 0.001, ***p < 0.0001.

CX₃CL1 mobility is restricted by the actin cytoskeleton
If CX₃CL1 is indeed trapped within cytoskeletal corrals, disruption of actin filaments should decrease the chemokine’s confinement. To this end, we treated ECs with either latrunculin B (Lat B), which sequesters actin monomers, or Clostridium difficile toxin B (Toxin B), which inhibits Rho-family GTPases, particularly Rac and Cdc42 (Supplemental Figure S3C; Just et al., 1994). Minimum concentrations and incubation times were used to prevent the detachment of cells from the coverslip. After incubation with either Lat B or Toxin B, we performed SPT of CX₃CL1 and found a considerable decrease in confined motion and an accompanying increase in free motion (Figure 4, A and B, and Supplemental Movie S2; p < 0.0001 vs. control). Lat B also greatly increased the free diffusion coefficient to 0.094 ± 0.012 μm²/s (Figure 4C; p < 0.01 vs. control), whereas Toxin B increased both the confined and free diffusion coefficients to 0.043 ± 0.005 s and 0.085 ± 0.010 μm²/s (Figure 4C; p < 0.05 vs. control), respectively, as well as the confinement area to 0.120 μm² (Figure 4D, p < 0.0001 vs. control). The more pronounced effect of Toxin B on CX₃CL1 confinement likely reflects the fact that it disrupted the cortical cytoskeleton to a greater extent than Lat B (unpublished data). Of note, higher concentrations of Lat B resulted in gross morphological changes, causing many ECs to collapse or detach from the coverslip. Toxin B, on the other hand, induced a stable tether. However, the possibility that the gentle nonionic detergent used dislodged CX₃CL1 from a cytoskeletal attachment site cannot be discounted.

The cytoplasmic domain of CX₃CL1 influences confinement by the cytoskeleton
The intracellular domains of immune receptors, such as the B-cell receptor (BCR), have been shown to influence their diffusion dynamics (Treonor et al., 2010). Thus we asked whether the intracellular domain of CX₃CL1 contributed to its confinement by the cytoskeleton. To investigate this possibility, we expressed a truncated allele of CX₃CL1, lacking the intracellular domain of the chemokine (CX₃CL1-360), and tracked its mobility using SPT (Figure 5, A and B, and Supplemental Movie S3; Huang et al., 2009). To account for changes in mobility that could result from overexpression, we transfected ECs with a full-length CX₃CL1 plasmid in parallel. Of importance, ECs were not treated with TNF-α, to avoid confounding effects of the endogenous chemokine. Under these conditions, full-length CX₃CL1 demonstrated similar diffusion characteristics compared with endogenous CX₃CL1 in TNF-α-treated EC (Figures 1, A–E, and 5, C and D). In sharp contrast, CX₃CL1-360 experienced a marked decrease in confined motion, from 78.0 ± 3.0 to 33.0 ± 2.0%, and an increase in free motion, from 20.0 ± 3.0 to 60.0 ± 2.0% (Figure 5C, p < 0.0001 vs. full-length CX₃CL1). CX₃CL1-360 also displayed a significant increase in its confined diffusion coefficient, from 0.025 ± 0.002 to 0.050 ± 0.005 μm²/s (Figure 5D; p < 0.0001 vs. full-length CX₃CL1), as well as in its confinement area, from 0.050 to 0.166 μm² (Figure 5, E and F; p = 0.0001 vs. full-length CX₃CL1), suggesting that it could more readily hop between actin corrals. These results demonstrate that the intracellular domain of CX₃CL1 plays a critical role in its confinement by the actin cytoskeleton.

CX₃CL1 is segregated from ADAM10 within the plasma membrane
The confined distribution of CX₃CL1 in the plasma membrane might explain why the chemokine is not readily accessible and fully cleaved by the plasmalemmal, transmembrane metalloprotease ADAM10, which is constitutively active (Hundhausen et al., 2003). To investigate this possibility, we performed SPT of ADAM10 using a similar labeling procedure to that of CX₃CL1 and found that 56.0 ± 2.0% of the protease experienced confined motion, moving with a diffusion coefficient of 0.012 ± 0.001 μm²/s, whereas 41.0% ± 2.0% experienced free motion, with a diffusion coefficient of 0.051 ± 0.003 μm²/s (Supplemental Movie S4 and Figure 6, A–D). Furthermore, the confined population of ADAM10 was restricted to a mean area of 0.034 ± 0.002 μm² (Figure 6, E and F). These results greatly differ from the SPT measurements of CX₃CL1, indicating that the mobility of these two transmembrane proteins is dictated by different determinants (Figure 1, A–F).
would severely limit the chance of detecting colocalization. In the presence of G1254023X, active segregation between CX3CL1 and ADAM10 was recapitulated. Remarkably, after treatment with Lat B, we detected significant colocalization between CX3CL1 and ADAM10 compared with that predicted for a random distribution (Figure 7, A and B; *p < 0.05 vs. random). These results demonstrate that the cortical actin cytoskeleton physically limits interactions between CX3CL1 and ADAM10.

Functionally, increased CX3CL1 and ADAM10 interactions should decrease the amount of transmembrane chemokine present on the cell surface and increase the amount of soluble chemokine released. To this end, we again took advantage of Toxin B to disrupt the cortical actin and examined the effects on the proteolytic cleavage of CX3CL1 (Figure 7, C and D). Strikingly, exposure to Toxin B reduced levels of CX3CL1 present at the cell surface by 40% (Figure 7D; *p < 0.05 vs. control). A similar 47% decrease was obtained using Lat B (Supplemental Figure S4, A and B; *p < 0.05 vs. control). To determine whether the decrease in cell surface CX3CL1 was in fact due to proteolytic shedding of the chemokine, we incubated cells with the metalloprotease inhibitor TAPI-2, which inhibits ADAM10 (Tole et al., 2010). Exposure to TAPI-2 prevented the observed decreases in cell surface CX3CL1 induced by both Toxin B and Lat B (Figure 7, C and D, and Supplemental Figure S4, A and B; *p < 0.0001 vs. Toxin B or Lat B alone). Most important, treatment with Toxin B also increased the release of soluble CX3CL1 (Figure 7E; *p < 0.05 vs. untreated), an effect prevented by metalloprotease inhibition (Figure 7E; *p < 0.0001 vs. Toxin B alone).

We next sought to confirm that the increased cleavage of CX3CL1 after disruption of the actin cytoskeleton was specifically due to ADAM10. Thus we performed gene silencing of ADAM10 using

Our data imply that there are distinct confined and free subpopulations of ADAM10. We initially questioned whether CX3CL1 and ADAM10 coexist in the same confinement regions, possibly accounting for the modest, yet significant basal cleavage of CX3CL1. To test this possibility, we used single-molecule imaging and the SAA to assess the localization of both proteins. These results demonstrate that within the plasma membrane, CX3CL1 and ADAM10 are not significantly associated or coconfined. Instead, it appears that at small distances, ADAM10 is physically restricted from interacting with CX3CL1.

The actin cytoskeleton controls shedding of cell surface CX3CL1

Given that ADAM10 is constitutively active and a large fraction is mobile, we asked what factors limited its accessibility to the majority of CX3CL1, preventing complete shedding of the latter from the cell surface. We hypothesized that the CX3CL1 confinement regions, which depend on the integrity of actin, may limit access of ADAM10 to the chemokine. To test this notion, we disrupted the actin cytoskeleton with Lat B and again used single-molecule SAA to assess the localization of both proteins. Cells were initially pretreated with the ADAM10 inhibitor G1254023X to prevent excess shedding of CX3CL1 that could result from disrupting the cytoskeleton, since this would severely limit the chance of detecting of colocalization. In the presence of G1254023X, active segregation between CX3CL1 and ADAM10 was recapitulated. Remarkably, after treatment with Lat B, we detected significant colocalization between CX3CL1 and ADAM10 compared with that predicted for a random distribution (Figure 7, A and B; *p < 0.05 vs. random). These results demonstrate that the cortical actin cytoskeleton physically limits interactions between CX3CL1 and ADAM10.

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small interfering RNA (siRNA), followed by treatment of the ECs with Toxin B (Figure 7F). Similar to effects seen with TAPI-2, knockdown of ADAM10 prevented the decrease in cell surface CX3CL1 induced by Toxin B, an effect not seen in cells treated with scrambled siRNA as a control (Figure 7, F and G; p < 0.0001 scrambled siRNA vs. ADAM10 siRNA).

Previously, we reported that CX3CL1 contained two putative adaptor protein-2 (AP-2)-binding domains and that inhibition of clathrin-mediated endocytosis enhanced ADAM-dependent shedding of the chemokine (Huang et al., 2009). Thus, it is conceivable that disrupting the actin cytoskeleton may disrupt clathrin-mediated endocytosis of CX3CL1 and increase proteolytic cleavage by ADAM10. This concern was addressed by assessing the internalization of transferrin, as well as of CX3CL1, in the presence of Lat B. Disrupting the actin cytoskeleton had little effect on internalization of both proteins (Supplemental Figure S5, A–C). Furthermore, we performed SPT of a mutant CX3CL1 (CX3CL1-Y362A-Y392A) in which alanine residues were substituted for the tyrosine residues of both AP-2-binding motifs previously shown to be critical for endocytosis (Huang et al., 2009). Unlike what was observed with CX3CL1-360, CX3CL1- Y362A-Y392A displayed no significant changes in lateral mobility compared with control, full-length CX3CL1 (Figure 5C and Supplemental Figure SSD). Therefore our results cannot be explained by disruption of clathrin-mediated endocytosis but instead support the notion that shedding of the chemokine is regulated by the actin cytoskeleton.

If actin confinement regions limit interactions between CX3CL1 and ADAM10, then CX3CL1-360, which is only 33.0 ± 2.0% confined, should be cleaved significantly more than full-length CX3CL1 (Figure 5C). To test this hypothesis, we initially compared the amount of cell surface CX3CL1 in EC expressing full-length or truncated CX3CL1. As expected, after normalization for expression levels, significantly less chemokine (~60% less) was detected at the plasma membrane in cells expressing CX3CL1-360 (Figure 7, H and I; p < 0.0001 vs. full-length CX3CL1). However, these results do not account for potential differences in trafficking that truncated CX3CL1-360 may experience. To control for this possibility, we performed an enzyme-linked immunosorbent assay (ELISA) to measure the release of soluble chemokine in ECs again expressing either full-length or truncated CX3CL1. In parallel, cells were treated with G1254023X, which allowed us to calculate the fraction of CX3CL1 cleaved by ADAM10 and normalize for possible changes in trafficking to the plasma membrane (see Materials and Methods). As Figure 7J illustrates, CX3CL1-360 was shed approximately twofold more by ADAM10 than with full-length CX3CL1 (p < 0.0001). This implies that less confinement does indeed result in more ADAM10-dependent proteolytic cleavage. Overall these results demonstrate that disrupting the actin cytoskeleton leads to ADAM10-mediated cleavage of cell surface CX3CL1 and the concomitant release of the soluble form of the chemokine.

**DISCUSSION**

Our results indicate that 1) CX3CL1 is confined in regions of the plasma membrane by the cortical actin meshwork, and 2) this confinement results in segregation of CX3CL1 from ADAM10, limiting basal proteolytic cleavage and the release of soluble chemokine. Membrane proteins can be influenced by the cytoskeleton in two distinct ways. The first involves direct or indirect anchoring or tethering to the cytoskeleton (Haggie et al., 2006; Chen et al., 2009; Jaqaman and Grinstein, 2012). The second involves the creation of cytoskeletal barriers, which limit protein diffusion (Kusumi et al., 2005a). Our observation that confined CX3CL1 displays measurable diffusion and that a large fraction of CX3CL1 is lost from the cell surface after membrane extraction does not support the first scenario. Instead, our data suggest that the cortical actin cytoskeleton compartmentalizes the membrane, restricting CX3CL1 diffusion to corral of varying size. Note, however, that the confinement of CX3CL1 is observed throughout acquisitive periods of ~10 s, differing from the short-term, hop diffusion events observed by Kusumi et al. (2005b) at the microsecond time scale. Because a fraction of CX3CL1 remained associated with the insoluble fraction after membrane extraction, we cannot rule out that the plasma-membrane pool of the chemokine is heterogeneous, with a small subpopulation stably tethered to actin, whereas the majority diffuses isotropically within actin corals.

Given the time scale used for SPT, we are unable to distinguish whether our observations represent total confinement of a small population of CX3CL1 molecules or temporary confinement of the majority of CX3CL1. However, our data do indicate that CX3CL1 molecules are strongly trapped within the actin network. Indeed, the chemokine colocalizes with the compacted cytoskeleton upon treatment with Cal A, unable to escape the dense filamentous meshwork. This suggests that the majority of CX3CL1 may experience transient interactions with actin or actin-associated molecules, leading to its recruitment to the cortical cytoskeleton by a diffusion and capture mechanism (Rudner et al., 2002). Although the exact mechanism of confinement remains to be determined, it is likely that trapping of CX3CL1 within corral occurs through interactions with another membrane protein complex directly linked to the cytoskeleton. The identity of this protein complex remains to be determined; however, CX3CL1 has previously been suggested to interact with the endothelial adhesion molecule VCAM-1 to support leukocyte recruitment (Kerfoot et al., 2003). Furthermore, on leukocytes, the receptor for VCAM-1, VLA-4, has been shown to exist in a complex with the receptor for CX3CL1, CX3CR1, implying that the two adhesion molecules may be associated (Fujita et al., 2012). Of importance, VCAM-1 is reported to anchor to the actin cytoskeleton through ezrin-radixin-moesin (ERM) proteins (Heiska et al., 1998; Barreiro et al., 2002). Thus, it is conceivable that CX3CL1 interacts with the actin cytoskeleton through transient associations with VCAM-1 and ERM protein complexes.

The restricted diffusion of transmembrane CX3CL1 correlates with limited release of its soluble species. This raises the possibility that confinement influences the ability of CX3CL1 to interact with ADAM10, regulating its rate of proteolytic cleavage. Of interest, recent Monte Carlo simulations demonstrated that cytoskeleton-based confinement of plasma membrane proteins could, under certain conditions, restrict protein collisions (Jaumouille et al., 2014). Thus, in principle, protein confinement could reduce the rate of collisions between CX3CL1 and ADAM10, altering the frequency of CX3CL1 cleavage. The effect of confinement, however, depends on several factors, including the surface densities of the protease and substrate, their respective diffusion coefficients, the time that the proteins are confined, and, finally, the size of the confinement regions. Thus, whereas at low surface densities membrane confinement would reduce the number of collisions (Jaumouille et al., 2014), at higher densities collision frequency may remain unaltered or even increase (Kalay et al., 2012). It is also noteworthy that not every collision between the protease and its substrate will result in a productive proteolytic event. If multiple collisions are required, as is likely the case, the effects of confinement on proteolysis would be greatly magnified: reduced collision rates would require much longer times to yield proteolysis and vice versa. In our studies, disruption of the cytoskeleton reduced CX3CL1 confinement, increased
**FIGURE 6:** ADAM10 has limited interaction with CX3CL1 on the surface of ECs. (A) ECs were incubated with TNF-α and surface ADAM10 detected by quantum dot labeling. SPT was performed as in Figure 1. Representative tracks of ADAM10. Scale bar, 1 μm. (B) Magnified confined and free trajectories from A (insets) color coded based on time (green to blue to red, ~10 s). Scale bar, 0.5 μm. (C) Fraction of ADAM10 particles experiencing confined and free motion. (D) Diffusion coefficient of ADAM10 was calculated as in Figure 1D. (E) ADAM10 confinement area values and (F) distribution of confinement area. For C–E, individual data points represent the median value of particles from a single field of view. Data are mean ± SEM of the medians from four independent experiments with 10 fields of view analyzed per experiment. (G) Single-molecule SAA of CX3CL1 and ADAM10. ECs were treated with TNF-α and labeled with primary antibody, followed by fluorescent Fab fragments, detecting CX3CL1 and ADAM10 at low densities. Experimentally measured particle distribution (solid black line) was compared with a predicted random distribution (dashed red line). Representative histogram depicting the spatial association between CX3CL1 and ADAM10 at the
FIGURE 7: The actin cytoskeleton sequesters CX3CL1 and limits its interactions with ADAM10. (A) Single-molecule SAA of CX3CL1 and ADAM10. ECs were incubated with TNF-α, followed by treatment with the ADAM10 inhibitor G1254023X. Cells were incubated with Lat B, fixed, and labeled as in Figure 6. Experimentally measured particle distribution (solid black line) in Lat B–treated cells was compared with a predicted random distribution (dashed red line). Representative histogram depicting the spatial association between CX3CL1 and ADAM10 at the subdiffraction level. Reconstructed image of CX3CL1 (blue) and ADAM10 (red) after subdiffraction localization. Scale bar, 3 μm. (B) The fraction of CX3CL1 and ADAM10 particle pairs within a predetermined colocalization cutoff after treatment with Lat B was compared as in Figure 6H. For A and B, analysis was based on either 8539 particle pairs for G1254023X or 4435 particle pairs for G1254023X + Lat B from three independent experiments, combining 20 fields per experiment. Data are mean ± SEM. (C) ECs were incubated with TNF-α, exposed to Toxin B in the presence or absence of the metalloprotease inhibitor TAPI-2, fixed, and immunofluorescently labeled with Ab detecting CX3CL1 (red) and with Draq5 nuclear stain (blue). Representative spinning-disk confocal images. Scale bar, 15 μm. (D) Cell surface CX3CL1 was quantified as described in Materials and Methods. Data are mean values ± SEM of four independent experiments, each with 10 fields of view and at least 50 cells. (E) Experiments were performed as in C, and the amount of soluble CX3CL1 was measured using an ELISA. Data represent the ratio between soluble CX3CL1 detected in conditioned medium and CX3CL1 detected in cell lysates. Data are mean values ± SEM from four independent experiments. (F) ECs were transfected with siRNA targeting ADAM10 or with scrambled nontargeting siRNA. Top, representative immunoblot performed using Ab detecting ADAM10. Bottom, cells were incubated with toxin B and cell surface CX3CL1 detected as described in C. (G) Cell surface CX3CL1 was measured as in C. Data are mean values ± SEM of three independent experiments each with 10–15 fields of view and at least 50 cells per experiment. (H) ECs were transfected with plasmids encoding full-length CX3CL1 or truncated CX3CL1-360. Representative XY and XZ optical slices were obtained using spinning-disk confocal microscopy. Scale bar, 15 μm. (I) Quantification of plasma membrane CX3CL1 in H normalized for varying levels of expression between cells (see Materials and Methods). Data are mean values ± SEM of three independent experiments each with 10 cells quantified. ***p < 0.0001. (J) ECs were transfected as in H. Cells were incubated with DMSO vehicle control or with G1254023X, and the amount of soluble CX3CL1 was measured using an ELISA. Cells were normalized for level of expression, and data are reported as the amount of ADAM10-dependent shedding (see Materials and Methods). *p < 0.05, ***p < 0.0001.

subdiffraction level. Reconstructed image of CX3CL1 (blue) and ADAM10 (red) after subdiffraction localization. Scale bar, 3 μm. (H) The fraction of CX3CL1 and ADAM10 particle pairs within a predetermined colocalization cutoff was compared between the experimentally measured population (measured coincidence) and the predicted, random population (simulated random coincidence). For G and H, analysis was based on 2291 particle pairs from three independent experiments, combining 20 fields per experiment. Data are mean ± SEM. ***p < 0.0001.
the diffusion coefficient of CX₃CL1, and promoted interactions with ADAM10, facilitating the proteolytic release of soluble chemokine. To this extent, our data seem most consistent with a model in which confinement reduces the likelihood of ADAM10 and CX₃CL1 converging and colliding.

Given the fact that ADAM10 plays a critical role in regulating the availability of adhesion molecules and ligands to various receptors expressed by circulating leukocytes, it is not surprising that such a spatial and temporal regulatory mechanism exists (Dreymueller et al., 2012). The cytoskeleton was previously shown to restrict diffusion of membrane proteins, such as the BCR, Fcγ receptor, and CD36, thereby modulating their signaling. To our knowledge, this is the first demonstration that the actin cytoskeleton regulates physical contact between a membrane protease and its substrate (Treanor et al., 2010; Jaqaman et al., 2011; Jaumouille et al., 2014). Several factors could regulate such an exclusion mechanism. Because ADAM10 has both a large confined and freely diffusing population, we envision three potential, yet not exclusive models for regulating cleavage of membrane substrates. In the first scenario, physiological stimuli may release or mobilize a confined substrate through reorganization of the cytoskeleton, greatly enhancing the accessibility of mobile ADAM10. Second, the release of a confined substrate may promote its interactions with confined ADAM10. Finally, the confined population of ADAM10 may be mobilized in response to various stimuli, increasing its likelihood of encountering target substrate (Figure 8). Of interest, similar regulatory mechanisms involving differential compartmentalization of functionally associated membrane proteins have been described in other cell systems. For example, our results parallel what has been described for the BCR, which is confined by the actin cytoskeleton, and its coreceptor CD19. In this case, after disruption of the cytoskeleton, mobility of the BCR increases, favoring interactions with CD19, resulting in tonic or antigen-induced signaling (Treanor et al., 2010; Mattila et al., 2013). Therefore it would seem that membrane compartmentalization represents a general, biological phenomenon critical for regulating a wide variety of cellular functions. It is tempting to speculate that other ADAM10 substrates may be similarly protected from cleavage by the actin cytoskeleton.

It has been unclear how ADAM10 is regulated and whether its catalytic activity can be altered. Of interest, many of the pharmacologic agents that have been described to increase ADAM10 activity also drastically alter actin cytoskeleton dynamics. These agents include the calcium ionophore ionomycin, the RhoA activator thrombin, and the calmodulin inhibitor W7 (Hundhausen et al., 2007; Schulz et al., 2008; Prasain and Stevens, 2009; Beckers et al., 2010). Thus the results obtained using these agents may reflect mobilization of confined substrates, such as CX₃CL1, or the mobilization of confined ADAM10. Both scenarios are consistent with our proposed

**FIGURE 8:** Model for cytoskeletal-regulated cleavage of plasma membrane CX₃CL1. (A) Schematic of the structure of CX₃CL1. (B) Schematic of the structure of ADAM10. (C) Proposed model for cytoskeletal-mediated exclusion between CX₃CL1 and ADAM10. CX₃CL1 diffusion is restricted due to actin confinement regions, which limit the access of ADAM10 to the chemokine. (D) Disruption of the cytoskeleton promotes convergence between CX₃CL1 and ADAM10, and the subsequent release of soluble chemokine.
model of membrane compartmentalization. However, we cannot
discount the possibility that disrupting actin filaments may inhibit an
actin-sensitive protein that regulates ADAM10 activity or shields
CXCL1 from cleavage. Note that although we found that choles-
terol depletion did not affect the confinement of CXCL1, it has
been reported to increase ADAM10-mediated shedding of the
chemokine, as well as of other substrates (Matthews et al., 2003;
Muray et al., 2011; Dreymueller et al., 2012). It is possible that these
results reflect mobilization of confined ADAM10 rather than a
change in its catalytic activity.

What physiological stimuli could modulate cytoskeletal confine-
ment regions? It is highly conceivable that in states of vascular in-
flammation, such as atherosclerosis, signals that cause reorganiza-
tion of the endothelial cytoskeleton may affect CXCL1–ADAM10
interactions and thus influence the progression of disease. These
signals could include secreted inflammatory molecules, physical in-
teractions between leukocytes and ECs, or perhaps changes in me-
chanical force exerted on the endothelium. Indeed, atherosclerotic
lesions develop in areas where fluid shear flow shifts from a laminar
to a disturbed pattern (Cunningham and Gotlieb, 2005; Chiu and
Chien, 2011). This transition in shear flow causes changes in both
cytoskeletal orientation and cytoskeletal tension of vascular ECs
(Chiu and Chien, 2011; Ting et al., 2012). The random cytoskeletal
orientation and decreased tension produced by disturbed shear
flow may disrupt actin corrals within the EC membrane and increase
the proteolytic release of soluble CXCL1. This in turn would pro-
mote recruitment of CXCR1-positive monocytes to the injured vas-
cular wall, a cardinal feature of atherogenesis (Combadiere et al.,
2003; Lesnik et al., 2003). Further work is required to test this
hypothesis.

In summary, we reported here that the cortical actin cytoskeleton
plays a critical role in regulating the diffusion of CXCL1 in the
plasma membrane and in protecting the chemokine from prote-
olytic cleavage. We demonstrated a new mode of regulating prote-
olysis, in which the actin cytoskeleton acts as a barrier limiting physi-
cal contact between integral membrane proteases and their
substrates. This novel cytoskeletal mechanism for controlling the
availability of ligand on the surface of ECs could potentially be gen-
eralized to other metalloprotease substrates and may greatly con-
tribute to the release of soluble inflammatory molecules. How such
a regulatory mechanism is modulated by physiological or pathologi-
cal stimuli in vivo remains a highly novel and exciting area of biology
warranting further investigation.

MATERIALS AND METHODS
Cell culture and transfections
Primary human umbilical vein endothelial cells (HUVECs) were cul-
tured in endothelial cell basal medium-2 (EBM-2; Clonetics, La Jolla,
CA). For all experiments, only cells cultured for fewer than six pas-
tages were used.

Transient transfection of HUVECs was performed using Amaxa
(Gaithersburg, MD) nucleofection or the NEON transfection system
(Life Technologies, Burlington, Canada) as per the manufacturer’s
instructions. The expression plasmids used in this study include
CXCL1-mCherry, CXCL1-Y362A-Y392A, CXCL1-LifeAct-GFP and RFP,
GPI-GFP, GT-46 YFP, and the tail of K-Ras-GFP (Kenworthy et al.,
2004; Yeung et al., 2006; Dunkan et al., 2007; Riedl et al., 2008;
Huang et al., 2009; Tole et al., 2010). For gene silencing, HUVECs
were electroporated with siRNA directed against human
ADAM10 or nontargeting, scrambled siRNA (Santa Cruz Biotechnol-
ogy, Santa Cruz, CA) on days 0 and 2. Knockdown was confirmed by
immunoblotting. Experiments were performed on day 4.

Antibodies and reagents
The primary antibodies used in this study included goat anti-human
CXCL1 immunoglobulin G (IgG) and mouse anti-human ADAM10
IgG from R&D Systems (Minneapolis, MN), rabbit anti-human
ADAM10 IgG (Abcam, Toronto, Canada), and rabbit anti-human ca-
veolin-1 IgG (BD Transduction Laboratories, San Jose, CA). All fluo-
rescent and biotin-conjugated secondary antibodies were obtained
from Jackson ImmunoResearch Laboratories (Bar Harbor, ME) and
include DyLight 549–conjugated donkey anti-goat IgG, Cy5-conju-
gated donkey anti-goat IgG, DyLight 488–conjugated donkey anti-
mouse IgG, DyLight 488–conjugated donkey anti-rabbit IgG, biotin-
conjugated rat anti-mouse Fab fragment, and biotin-conjugated
rabbit anti-goat Fab fragment. Streptavidin Qdot 655 was obtained
from Invitrogen (Burlington, Canada).

The following reagents were used: MBcD, Lat B, and Toxin B
from Sigma-Aldrich (Oakville, Canada), Cal A (Calbiochem, Gibb-
stown, NJ), DramaS (Biostatus, Shepshed, United Kingdom), fibronect-
in (Roche, Mississauga, Canada), filipin from Streptomyces filipin-
ensis (Polysciences, Warrington, PA), wheat germ agglutinin (WGA;
Life Technologies), TAPI-2 (Peptides International, Louisville, KY),
and G1254023X (Tocris, Minneapolis, MN).

Single-particle tracking and diffusion analysis
HUVECs were grown to confluence on glass coverslips coated with
fibronectin (100 μg/ml). In unstimulated conditions, these cells ex-
press a negligible amount of CXCL1 protein on their cell surface.
HUVECs were next incubated with TNF-α (0.02 μg/ml) for 4 h to up-
regulate endogenous levels of cell surface CXCL1 (Bazan et al.,
1997). Cells were incubated with anti-CXCL1 (0.2 μg/ml) or anti-
ADAM10 antibody (Ab; 0.01 μg/ml) diluted in EBM-2 for 10 min at
10°C, washed with cold Hank’s balance salt solution (HBSS), and then
incubated with biotinylated secondary Fab fragment (0.375 μg/ml)
for 10 min at 10°C. Cells were washed further and then incubated
with Qdot 655–conjugated streptavidin (0.1 nm) diluted in HBSS for
4 min at 10°C. To block unengaged streptavidin, cells were incu-
bated with cold medium containing biotin for 20 s. After washing,
the coverslip was transferred to a Leiden chamber, warmed to 37°C,
and placed on the heated stage of an Axiovert 200 M inverted epi-
fluorescence microscope (Carl Zeiss, Toronto, Canada) equipped
with a custom 2.5× lens and a 100× (numerical aperture [NA] 1.45)
ioil-immersion objective for live-cell imaging (Jaqaman et al., 2008).

SPT was performed using an Exfo X-Cite 120 light source for il-
illumination and a Hamamatsu C9100-13 deep-cooled electron-multi-
plying charge-coupled device camera for recording (Hamamatsu
Photonics, Bridgewater, NJ). Image acquisition was controlled using
Velocity software taking 300 frame videos at a frame rate of 30 Hz.
Imaged molecules of CXCL1 or ADAM10 were detected and
tracked as described by Jaqaman et al. (2008). In brief, subdiffra-
tion particle positions and intensities were estimated by 1) detect-
ing significant local intensity maxima and 2) fitting Gaussian kernels ap-
proximating the two-dimensional point spread function of the mi-
croscope. The detected particles were tracked using a two-step
single-particle tracking algorithm that could follow dense particle
fields and generate complete trajectories by closing gaps and cap-
turing merging and splitting events. Diffusion rates and diffusion
types were then extracted using MSS analysis of particle displac-
ements (Ferrari et al., 2001; Ewers et al., 2005; Flannagan et al.,
2010; Jaqaman et al., 2011).

Typically, MSD(r2) versus Δt is used to analyze a particle trajectory.
For normal or free diffusion, the MSD is expected to be a linear func-
tion of time (r2 = 4DΔt). However, many molecules will exhibit a non-
linear relationship to time. The resulting anomalous diffusion can be
described as superdiffusion (directed) or subdiffusion (confined). Thus a measure of nonlinearity can be expressed by the parameter \( \alpha \), resulting in \( r^2 = 4D\Delta t^\alpha \). This measurement can be further enhanced for a nonnegative integer \( \nu \) introduced by Ferrari et al. (2001) such that \( r^2 = \Delta t^{1+\nu} \). The plot of \( \nu \) versus \( \nu \) is called the MSS, and its slope, \( S_{\text{MSS}} \), represents a value associated with a mode of diffusion: \( S_{\text{MSS}} = 0.5 \), free motion; \( <0.5 \), confined motion; \( >0 \), directed motion; 0, im-
mobile. The \( S_{\text{MSS}} \) offers two major advantages over the MSD: 1) a smaller error in measurement due to greater linearity in the MSS and 2) a clearer distinction between modes of diffusion (Ferrari et al., 2001; Ewers et al., 2005; Jaqaman et al. 2007). For the purpose of this study, only confined and free motions were reported, as di-
rected motion was minimal.

In parallel experiments, cells were incubated with M5CD (10 mM) for 30 min to deplete cholesterol and either Toxin B (50 ng/ml) for 105 min or Lat B (1 \( \mu \)M) for 10 min to disrupt actin. Cells were sub-
sequently imaged live for SPT.

**Single-molecule colocalization**

HUVECs were incubated with TNF-\( \alpha \), and cells were fixed using 4% paraformaldehyde, blocked with 5% donkey serum for 45 min, incubated with anti-CL1 Ab (0.2 \( \mu \)g/ml) for 10 min, washed, and incubated with a DyLight 549–conjugated anti-goat Fab fragment (0.375 \( \mu \)g/ml) for 10 min. Cells were washed and then incubated with either anti–caveolin-1 Ab (0.036 \( \mu \)g/ml) for 10 min or anti-
ADAM10 Ab (0.01 \( \mu \)g/ml) for 20 min, followed by DyLight 488–conjugated anti-rabbit (0.4 \( \mu \)g/ml) or anti-mouse Fab fragment (0.4 \( \mu \)g/ml) for 20 min. Coverslips were placed in a Leiden chamber, and single molecules were detected as described.

To investigate colocalization between CL1 and ADAM10, or CL1 and caveolin-1, the spatial relationship between the two molecules was assessed using a nearest-neighbor approach termed the SAA, as described in Heit et al. (2013; also see Dunne et al., 2009; Lachmanovich et al., 2003). Initially, the positional precision of DyLight 549 (\( \sigma_{549} \)) and DyLight 488 (\( \sigma_{488} \)) particles was calculated individually for every image, yielding values of \(-30 \) nm. These values were then used to calculate the cross-channel positional precision (\( \sigma_{\text{MSS}} \)), determined by calculating the root mean square of the po-
nitional precisions for DyLight 549 and DyLight 488:

\[
\sigma_{\text{MSS}} = (\sigma_{549}^2 + \sigma_{488}^2)^{1/2}
\]

To determine a CDC, the distance below which molecules are considered to be colocalized, the result of the foregoing equation was multiplied by a 90% probability cut-off (1.65 SDs), followed by the addition of the image registration accuracy of the micro-
scope (\( l_{\text{reg}} \)), by calculating the root mean square of the po-
nitional precisions for DyLight 549 and DyLight 488:

\[
\text{CDC} = 1.65\sigma_{\text{MSS}} + l_{\text{reg}}
\]

In this study, the \( l_{\text{reg}} \) was experimentally determined to be 105 nm, resulting in a CDC of 165–175 nm. Therefore a conservative CDC of \(-180 \) nm was used for consistency in all analyses.

The Euclidean distance between each DyLight 549–labeled partic-
le and nearest DyLight 488 particle was next measured. This method is nonsymmetrical, however, and thus all distances were measured relative to DyLight 549 (CL1) particles. This allowed for the coincidence of colocalization to be determined based on the fraction of DyLight 549 and 488 pairs separated by a distance \( \Delta t \) CDC. A Monte Carlo approach was then used to determine whether the observed colocalization differed from a randomly distributed population of particles (Metropolis and Ulam, 1949). In brief, a random population containing the same number of observed DyLight 549 and 488 particles was generated using a uni-
formly distributed pseudorandom number generator. The random population was then positioned on a virtual image with the same dimensions as the observed image, and colocalization was assessed by the nearest-neighbor approach. This process was repeated 2000 times, allowing for the frequency of colocalization in a random population to be compared with the observed population.

In some instances, cells were pretreated with either a dimethyl sulfoxide (DMSO) vehicle control or the ADAM10 inhibitor G1254023X (10 \( \mu \)M) for 3 h, followed by treatment with Lat B (1 \( \mu \)M) for 10 min. Cells were then fixed and labeled for single-molecule imaging and SAA as described.

**Fluorescence microscopy**

To deplete cholesterol, cells were incubated with methyl-\( \beta \)-
cyclodextrin (10 mM) for 30 min. Cholesterol content was evaluated by incubating cells with filipin (0.5 mg/ml) for 1 h using a DMIRE2 microscope equipped with 63× oil immersion objective. Detection of cell surface CXCL1 was performed using spinning-disk confocal microscopy after fixing cells in 4% paraformaldehyde, blocking with 5% donkey serum, and incubating with anti-CXCL1 Ab (0.2 \( \mu \)g/ml) for 1 h. Cells were washed and incubated with DyLight 549–conju-
gated anti-goat IgG (0.75 \( \mu \)g/ml) for 1 h before permeabilization with 0.1% Triton-X. Cells were then incubated with anti–caveolin-1 Ab (0.25 \( \mu \)g/ml) for 1 h, washed, and incubated with DyLight 488–conjugated anti-rabbit IgG (0.8 \( \mu \)g/ml) for 1 h.

To disrupt actin, cells were incubated with Toxin B (50 ng/ml) for 105 min or Lat B (2 \( \mu \)M) for 10 min. Detection of cell surface CXCL1 was performed as described.

To perturb myosin, cells were treated with blebbistatin (60 \( \mu \)M) for 20 min or Cal A (200 nM) for 5 min. Detection of CXCL1 in live cells was performed by spinning-disk microscopy after incubation of cells with anti-CXCL1 Ab (1 \( \mu \)g/ml) for 25 min at 10°C, followed by incubation with DyLight 549–conjugated anti-goat IgG (0.75 \( \mu \)g/ml) for 25 min at 10°C.

The spinning-disk confocal systems (Quorum Technologies, Guelph, Canada) were used with an Axiovert 200 M microscope (Carl Zeiss) equipped with 63× (NA 1.4) or 100× (NA 1.45) oil immersion objectives. These microscopes were equipped with diode-pumped solid-state lasers (440, 491, 561, 638, and 655 nm; Spectral Applied Research, Richmond Hill, Canada) and a motor-
zied XY stage (Applied Scientific Instrumentation, Eugene, OR). Images were acquired using a back-thinned, electron-multiplied camera (model C9100-13 ImagEM; Hamamatsu Photonics) controlled using Volocity software, version 6.0.1 (PerkinElmer, Waltham, MA).

**Membrane extractions**

HUVECs were incubated with TNF-\( \alpha \), incubated with anti-CXCL1 Ab (1 \( \mu \)g/ml) for 25 min at 10°C, washed, and incubated with DyLight 549–conjugated anti-goat Fab fragment (1.5 \( \mu \)g/ml) for 25 min at 10°C. To label nuclei, cells were incubated with Drag5 (5 \( \mu \)M). Mem-
brane extractions were performed in a cytoskeleton buffer containing 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.1, 138 mM KCl, 3 mM MgCl\(_2\), 2 mM ethylene glycol tetraacetic acid, and 0.32 M sucrose. Extractions were performed for 2 min at 4°C using 0.1% Triton X-100, and cells were fixed with 4% paraformaldehyde (Schaefer et al., 2002; Medeiros et al., 2006). Cells were then washed and im-
aged using spinning-disc confocal microscopy. The average amount of surface CXCL1 per cell was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD), measuring summed fluorescence intensity through entire z-projections per field of view,
subtracting background, and dividing by the number of nuclei per field of view (Collins, 2007; Waters, 2009).

**Endocytosis assays**

HUVECs were treated with DMSO or Lat B for 5 min and then incubated with transferrin-488 at 37°C for 10 min (Lat B was not washed out). Cells were then placed on ice, washed with cold phosphate-buffered saline, and acid washed to removed surface-bound transferrin (Jaqaman et al., 2011). After fixation, cells were incubated with Draq5, and transferrin fluorescence was quantified by spinning-disk confocal microscopy as described. To assess CX3CL1 internalization, HUVECs were stimulated with TNF-α and incubated with anti-CX3CL1 Ab (1 μg/ml) for 25 min at 10°C. Cell were then stained with Draq5 and treated with latrunculin B (2 μM) or DMSO at 37°C for 15 min. For qualitative analysis, cells were fixed, and cell surface CX3CL1 was labeled with Alexa 488–conjugated anti-goat IgG. Cells were then permeabilized, and total CX3CL1 was labeled with a DyLight 549–conjugated secondary. For quantitative analysis, cells were fixed and labeled with a DyLight 549–conjugated anti-goat IgG (0.75 μg/ml) to label cell surface CX3CL1 and, in parallel, permeabilized to label total CX3CL1. CX3CL1 fluorescence was determined for both permeabilized and nonpermeabilized conditions by spinning-disk confocal microscopy. Internalized CX3CL1 was determined by calculating CX3CL1permeabilized – CX3CL1non-permeabilized for cells incubated with LatB or DMSO.

**CX3CL1 shedding**

HUVECs were incubated with TNF-α, followed by incubation with Toxin B (50 ng/ml) or Lat B (2 μM). In some experiments, cells were first incubated with the metalloprotease inhibitor TAPI-2 (20 μM) for 1 h at 37°C or electroporated with siRNA targeting ADAM10. Cells were incubated with Draq5 (5 μM) and fixed in 4% paraformaldehyde, and cell surface CX3CL1 was immunofluorescently labeled. Soluble CX3CL1 was detected in conditioned medium using a CX3CL1 ELISA (R&D Systems, Minneapolis, MN), as per the manufacturer’s instructions (Tole et al., 2010).

To compare shedding between full-length CX3CL1 and truncated CX3CL1-360, HUVECs were electroporated with the corresponding plasmids. Cells were fixed and incubated with the plasma membrane marker WGA-488. Using the free-hand tool in ImageJ, highly magnified regions of plasma membrane were delineated using WGA-488 as a reference, and the mean CX3CL1 fluorescence per pixel was determined. After background subtraction, plasma membrane CX3CL1 was divided by the mean total fluorescence (CX3CL1surface/CX3CL1total), allowing for comparison between cells of varying levels of expression (Huang et al., 2009). Soluble CX3CL1 was measured by performing a CX3CL1 ELISA (R&D Systems) after incubation with a DMSO vehicle control or the ADAM10 inhibitor G1254023X (10 μM) for 3 h. The absence of inhibitor was treated as the maximum amount of CX3CL1 cleaved, and the presence of inhibitor was treated as the amount of CX3CL1 cleaved in the absence of ADAM10. After normalization for expression levels, CX3CL1shed/CX3CL1shed + CX3CL1cell-associated, these values were used to determine the fraction of chemokine specifically cleaved by ADAM10 in cells expressing full-length CX3CL1 or truncated CX3CL1-360.

**Statistics**

Statistics was performed using Prism 5 software (GraphPad, La Jolla, CA). For multiple comparisons, p values were calculated by one-way analysis of variance using Bonferroni’s multiple comparison test. In all other cases, p values were calculated using Student’s t test. Mathematical and statistical analyses for SPT and subdiffraction imaging experiments were performed using custom scripts written in Matlab software (MathWorks, Natick, MA; Jaqaman et al., 2008, 2011). Data are presented as mean ± SEM unless otherwise stated.

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