Enhanced Effector Function of CD8⁺ T Cells From Healthy Controls and HIV-Infected Patients Occurs Through Thrombin Activation of Protease-Activated Receptor 1

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Disruption of vascular integrity by trauma and other tissue insults leads to inflammation and activation of the coagulation cascade. The serine protease thrombin links these 2 processes. The proinflammatory function of thrombin is mediated by activation of protease-activated receptor 1 (PAR-1). We found that peripheral blood effector memory CD4⁺ and CD8⁺ T lymphocytes expressed PAR-1 and that expression was increased in CD8⁺ T cells from human immunodeficiency virus (HIV)–infected patients. Thrombin enhanced cytokine secretion in CD8⁺ T cells from healthy controls and HIV-infected patients. In addition, thrombin induced chemokinesis, but not chemotaxis, of CD8⁺ T cells, which led to structural changes, including cell polarization and formation of a structure rich in F-actin and phosphorylated ezrin-radexin-moesin proteins. These findings suggest that thrombin mediates cross-talk between the coagulation system and the adaptive immune system at sites of vascular injury through increased T-cell motility and production of proinflammatory cytokines.

Keywords. PAR-1; T cells; thrombin; HIV pathogenesis; coagulation; inflammation.

Activation of the coagulation cascade can occur when circulating coagulation factors contact tissue factor. Tissue factor is a type I membrane protein and is expressed on epithelial cells and macrophages in the extravascular space. Disruption of the vascular barrier leads to exposure of tissue factor to circulating coagulation factors, resulting in a series of zymogen conversions of the circulating coagulation factors, with generation of a burst of thrombin and, ultimately, formation of a clot.

This process is accompanied by a host inflammatory response and recruitment of cells, mainly of the innate immune system, into damaged tissue [1]. In pathological conditions characterized by an uncontrolled inflammatory response and microvascular damage, such as systemic lupus erythematosus, rheumatoid arthritis, and sepsis, hypercoagulability may lead to thrombosis and exacerbated tissue damage [2–6]. In addition, patients with human immunodeficiency virus (HIV) infection have elevated biomarkers of both inflammation and coagulation [7–9]. Consistent with these observations was the finding that monocytes from HIV-infected patients showed an increased surface expression of tissue factor that was correlated with viremia, immune activation markers, and D-dimers, which are byproducts of clot formation/fibrinolysis [10].

Thrombin, in addition to activating platelets, induces endothelial cells to secrete proinflammatory...
cytokines such as interleukin 6 and chemokines such as CCL2, leading to recruitment of inflammatory cells [11]. In humans, this activity is mediated, at least in part, by the G-protein–coupled receptors protease-activated receptor 1 (PAR-1) and PAR-3 [12, 13]. Thrombin activates PAR-1 through cleavage of the extracellular domain of the receptor, exposing a unique tethered peptide ligand. PAR-1 is internalized following ligand stimulation through β-arrestin and phosphorylation-dependent mechanisms [14].

Proinflammatory PAR-1 functions have been implicated in experimentally induced lung injury, T-helper 2 (Th2) cell-mediated colitis, antigen-induced arthritis, sepsis, and colon inflammation [15–17]. However, the exact mechanism by which thrombin regulates T-lymphocyte functions is not well understood.

HIV infection is characterized by immune activation of both CD4+ and CD8+ T cells. Chronic immune activation is thought to play a central role in the pathogenesis of HIV infection and is a significant correlate of disease progression [18]. Previously, we observed that T cells from HIV-infected patients and healthy controls express messenger RNA (mRNA) transcripts of PAR-1, which suggested a link between T-cell activation and coagulation [19]. In this study, we characterize the expression of PAR-1 in peripheral blood CD4+ and CD8+ T cells from healthy controls and HIV-infected patients and the functional effects of thrombin on these cell types. Our results suggest that thrombin promotes tissue inflammation by arresting T cells at sites of vascular injury and by enhancing antigen-dependent cytokine production through synergistic PAR-1 and T-cell receptor (TCR) activation. Thus, PAR-1 signaling links coagulation and inflammation mediated by T lymphocytes.

**MATERIALS AND METHODS**

**Patients and Healthy Controls**
Patients and healthy controls consisted of individuals who consented to undergo institutional review board–approved studies conducted by the National Institute of Allergy and Infectious Diseases (NIAID) Clinical Center HIV Program and by the National Institutes of Health Blood Bank, respectively (Table 1, Supplementary Table 1, and Supplementary Materials). The majority of the patients studied had chronic HIV infection.

**Flow Cytometry**

**Detection of PAR-1 in Healthy Controls and HIV-Infected Patients**
Whole-blood samples were stained with a cocktail of the following monoclonal antibodies: anti-human CD3, anti-human CD8, anti-human CD27, and anti-human CD62L (all from BD Biosciences, San Jose, CA); anti-human CD45RA and anti-human CD4 (both from Invitrogen, Grand Island, NY); and anti-thrombin receptor (clone WEDE15; Beckman Coulter, Indianapolis, IN). Samples were acquired with a BD FACSCanto and analyzed with FlowJo software.

**PAR-1 Activation by Thrombin**
CD8+ T cells from healthy controls were isolated using negative selection (Miltenyi Biotech, Auburn, CA). The purity of the cells was analyzed by flow cytometry and ranged between 85% and 95%. For pertussis toxin (PTX) inhibition, cells were pretreated with 100 ng/mL of PTX (Sigma, St. Louis, MO). The PAR-1 antagonist SCH-79797 (Tocris Bioscience, Bristol, United Kingdom) was incubated with the cells for 20 minutes at a previously titrated concentration of 5 μM. A detailed description of the protocol is presented in the Supplementary Materials.

**IFN-γ Detection Following Thrombin Stimulation**

**Detection of C-Terminally Phosphorylated Ezrin-Radixin-Moesin (cpERM) After Thrombin, CCL19, and CXCL12 Stimulation**
Freshly isolated CD8+ T cells were stimulated with either thrombin (10 U/mL), CCL19 (200 ng/mL), or CXCL12 (200 ng/mL). Staining for cpERM was done as described elsewhere [20]. A detailed description of the protocol is presented in the Supplementary Materials.

**Transwell Experiments**

**Microscopy**

**Video Microscopy**
To allow the imaging of individual cells over time, CD8+ T cells from healthy controls were isolated by negative selection and cultured between layers of cross-linked collagen, as described elsewhere [21]. A detailed description of the protocol is presented in the Supplementary Materials.

**Fluorescence Microscopy**
Freshly isolated CD8+ T cells were stimulated with either thrombin (10 U/mL) or CXCL12 (200 ng/mL) for 5 minutes
**Table 1. Analysis of the Correlation Between Patient Characteristics and the Percentage of CD4⁺ and CD8⁺ T-Cell Subsets Expressing Protease-Activated Receptor 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV Status</th>
<th>CD4⁺ T-Cell Subset</th>
<th>CD8⁺ T-Cell Subset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Naive</td>
</tr>
<tr>
<td>Age, y, median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 (35–53) Positive</td>
<td>−0.246, .003</td>
<td>−0.195, .024</td>
<td>−0.287, &lt;.001</td>
</tr>
<tr>
<td>47 (33–54) Negative</td>
<td>0.045, .7606</td>
<td>−0.019, .8959</td>
<td>0.059, .6892</td>
</tr>
<tr>
<td>Viral load, copies/mL, median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 (50–3828) Positive</td>
<td>0.178, .036</td>
<td>−0.071, .412</td>
<td>0.060, .481</td>
</tr>
<tr>
<td>47 (33–54) Negative</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CD4⁺ T-cell count, cells/µL, median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>451 (262–631) Positive</td>
<td>−0.129, .131</td>
<td>0.022, .799</td>
<td>−0.071, .407</td>
</tr>
<tr>
<td>817 (497–1097) Negative</td>
<td>0.081, .583</td>
<td>−0.022, .881</td>
<td>0.059, .692</td>
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<tr>
<td>CD8⁺ T-cell count, cells/µL, median (IQR)</td>
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<tr>
<td>733 (544–992) Positive</td>
<td>0.167, .049</td>
<td>−0.071, .416</td>
<td>0.047, .579</td>
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<tr>
<td>370 (288–523) Negative</td>
<td>0.194, .189</td>
<td>0.066, .656</td>
<td>0.117, .432</td>
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<td>D-dimer level, mg/L, median (IQR)</td>
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<tr>
<td>315 (213–735) Positive</td>
<td>0.118, .226</td>
<td>0.086, .393</td>
<td>0.074, .449</td>
</tr>
<tr>
<td>313 (239–434) Negative</td>
<td>−0.188, .215</td>
<td>−0.164, .278</td>
<td>−0.022, .146</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25% F, 75% M Positive</td>
<td>0.289, .784</td>
<td>0.615, .394</td>
<td>0.652, .739</td>
</tr>
<tr>
<td>53% F, 47% M Negative</td>
<td>3.622, .263</td>
<td>−.898, .991</td>
<td>2.533, .475</td>
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<td>ART therapy</td>
<td></td>
<td></td>
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<tr>
<td>63% yes, 37% no Positive</td>
<td>−0.436, .418</td>
<td>0.626, .322</td>
<td>−0.661, .678</td>
</tr>
<tr>
<td>Virus load (&lt;50–50 copies/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 (50–3828) Positive</td>
<td>1.852, .047</td>
<td>−.71, .408</td>
<td>0.446, .866</td>
</tr>
</tbody>
</table>

Abbreviations: ART, antiretroviral therapy; HIV, human immunodeficiency virus; IQR, interquartile range; n/a: not applicable.

*By the Mann-Whitney U test.
RESULTS

Human CD4+ and CD8+ T Cells With an Effector Memory Phenotype Express PAR-1

We first characterized the pattern of PAR-1 expression in 4 human T-cell subsets from healthy controls: naive T cells (CD45RA+CD62L+), central memory T cells (CD45RA+CD62L+), effector memory T cells (CD45RA-CD62L-), and terminal effector memory T cells (TEM; CD45RA-CD62L-). Overall, median interquartile range (IQR) 21% (10.86–35.95) of CD8+ T cells expressed PAR-1, compared with 7.9% (4.40–13.52) of CD4+ T cells (P < .001; Figure 1A and 1B). Expression of PAR-1 progressively increased with T-cell differentiation (Figure 1B), with the highest expression observed in the effector memory and TEM T-cell subsets, which migrate to peripheral tissues.

Because we noted the expression of PAR-1 mRNA in a previous study of immune activation in HIV-infected patients [19], we compared the expression of PAR-1 in CD4+ and CD8+ T cells from HIV-infected patients with plasma HIV RNA levels of >50 copies/mL to PAR-1 expression in CD4+ and CD8+ T cells from patients in whom viremia had been successfully suppressed to <50 copies/mL by combination antiretroviral therapy (Supplementary Table 1). Relative to T-cell subsets from healthy controls, central memory and effector memory CD8+ T-cell subsets from HIV-infected patients with HIV RNA levels of >50 copies/mL expressed slightly more PAR-1. No difference in PAR-1 expression in the TEM T cells from these patients was observed. T-cell subsets from patients with HIV RNA levels of <50 copies/mL following successful treatment had levels of PAR-1 expression that were similar to those of healthy controls in all subsets (Figure 1C). PAR-1 expression was also comparable in CD4+ T-cell subsets from HIV-infected patients and healthy controls. These results indicate that expression of PAR-1 is enhanced in antigen-experienced CD4+ and CD8+ T cells that traffic through peripheral tissues.

We next analyzed the association between PAR-1 in CD4+ and CD8+ T-cell subsets and a series of variables (Table 1). The expression of PAR-1 in CD8+ T cells from HIV-infected patients significantly correlated with levels of HIV RNA and combination antiretroviral therapy status (Table 1). While serum levels of D-dimers (a byproduct of clot formation/fibrinolysis) [10] showed no significant association with PAR-1 expression in patients with HIV infection, a negative association was observed in healthy controls (Table 1). These observations suggest that there is an alteration in the relationship between CD8+ T-cell PAR-1 expression and coagulation in the setting of HIV infection.

Thrombin Induces PAR-1 Signaling and Internalization in Resting Human CD8+ T cells

Because the results described above suggested that circulating memory T cells might have the capacity to respond to thrombin through PAR-1 during injury and/or an inflammatory process such as infection, we next looked for evidence of thrombin-induced signaling in T lymphocytes. Consistent with published studies using cell lines [23], we found that thrombin activated PAR-1 on T cells, as evidenced by internalization of the receptor (Figure 2). Purified resting human CD8+ T cells were incubated with serial dilutions of active human thrombin for 5 minutes, and the loss of PAR-1 surface expression was measured by flow cytometry, using an antibody directed against the N-terminal domain (amino acid residues 51–64). This reagent allowed detection of both cleaved and uncleaved PAR-1 [17, 24–26]. Thrombin induced a dose-dependent loss of PAR-1 surface staining on CD8+ T cells (Figure 2A). Higher doses of thrombin induced a 70% loss of surface PAR-1 staining within 5 minutes, with no change in total cellular PAR-1 expression (Supplementary Figure 1). Thrombin that underwent heat denaturing at 65°C for 1 hour was unable to induce PAR-1 internalization (data not shown), indicating that the protease activity of thrombin was required for PAR-1 activation. The PAR-1 antagonist SCH-79797, which binds to the thrombin cleavage site of PAR-1 [27], blocked internalization of PAR-1 on freshly isolated CD8+ T cells stimulated with thrombin (Figure 2B). In contrast, PAR-1 internalization was insensitive to treatment with PTX, which catalyzes the ADP ribosylation of α subunits of the Gαq/o family of heterotrimeric G proteins [28]. Neither thrombin antagonist nor PTX treatment alone had any effect on PAR-1 expression (Figure 2B). These results demonstrate that thrombin activates PAR-1 expressed on resting human CD8+ T cells in a Gαq-independent manner [1, 28].

Thrombin Enhances IFN-γ Secretion by CD8+ T Cells From Healthy Controls and HIV-Infected Patients

In other cell types, PAR-1 is thought to couple promiscuously to various G proteins, including Gαq and Gα12/13, to induce Ca2+ mobilization, cytoskeletal changes, and gene expression [29]. In Jurkat T cells, PAR-1 activation by thrombin elicited Ca2+ mobilization and activation of nuclear factor of activated T cells (data not shown). These results suggested that PAR-1 signaling enhanced cytokine gene expression in T cells. Accordingly, we found that although thrombin alone elicited only a slight increase in the frequency of IFN-γ-secreting CD8+ T cells, pretreatment of CD8+ T cells with thrombin significantly enhanced IFN-γ secretion induced by anti-CD3/CD28 stimulation (Figure 3A and 3B).
Thus, PAR-1 activation by thrombin may induce a more robust antigen response in effector memory T cells. CD8+ T cells from patients with HIV infection and viral loads of >50 copies/mL showed increased production of IFN-γ, as did cells from noninfected healthy controls. These results suggest a possible contribution of this pathway to the heightened immune activation/inflammation seen in patients with HIV infection.
Thrombin-Induced Polarization and Chemokinesis of Resting Human CD8+ T cells

Our results thus far indicated that thrombin activates human T cells and can enhance antigen-induced IFN-γ secretion. In contrast, thrombin does not activate Gαi, as indicated by the resistance of these responses to PTX, and Gαi-dependent pathways are generally required for chemotaxis [30]. Therefore, we next determined whether thrombin could act as a chemoattractant for CD8+ T cells, using a transwell system. Compared with CXCL12, thrombin induced a very weak level of

Figure 2. Thrombin induces protease-activated receptor 1 (PAR-1) activation and internalization in CD8+ T cells. A, CD8+ T cells from healthy controls were incubated with medium and serial dilutions of thrombin ranging from 0.002 to 50 U/mL for 5 minutes. The graph depicts changes in PAR-1 expression as a function of thrombin concentration; the dark histograms represent the FMO control (full minus one control). The percentage loss of median fluorescence intensity (MFI) was calculated with regard to PAR-1 expression in cells incubated with medium. Data are presented as the average of 5 independent experiments. B, CD8+ T cells were pretreated with either pertussis toxin (PTX; 100 ng/mL for 2 hours) or PAR-1 antagonist (SCH-79797; 5 μM for 20 minutes) prior to incubation with serial dilutions of thrombin. The left panel represents the histograms of PAR-1 expression in thrombin-treated or untreated cells preincubated with medium, SCH-79797, or PTX. The right panel represents the average of 4 independent experiments (mean ± standard error of the mean).
chemotaxis among CD8\(^+\) T cells from healthy controls (Supplementary Figure 2) and patients with HIV infection (Figure 4A). In addition, there was no difference between CD8\(^+\) T cells from HIV-infected patients and those from healthy controls in their capacity to migrate in response to CXCL12 or thrombin (Figure 4A). The weak chemotactic effect of thrombin was consistent with the rapid kinetics of thrombin-receptor internalization. Within the first 30 minutes of thrombin stimulation, 80% of PAR-1 surface staining was lost, and it remained low after 24 hours. In contrast, chemokines induced a much more gradual loss of CXCR4 and CCR5 in response to CXCL12 and CCL5, respectively (Supplementary Figure 3), suggesting a more sustained response to a chemotactic gradient. The rapid desensitization of PAR-1 is more consistent with chemokinesis as opposed to chemotaxis.

Although thrombin is not a potent chemoattractant, it could be biochemical inducer of cell movement (chemokinesis). To investigate whether thrombin induces chemokinesis and could thereby “arrest” memory T cells at sites of vascular injury in order to “survey” tissue damage, resting human CD8\(^+\) T cells were cultured between 2 layers of cross-linked collagen, and individual cells were imaged over time. This system allowed us to visualize cell polarization and movement of individual cells [21]. Following addition of thrombin, CD8\(^+\) T cells developed an asymmetric body consistent with polarization and increased cell motility (Figure 4B). Responding cells (26% of the population) left a track of the distance covered over the time studied (Figure 4C). A video (Supplementary Video 1) of the imaged cells before and after thrombin stimulation provides clear evidence that thrombin induces chemokinesis of resting human CD8\(^+\) T cells. Signs of polarization were observed 6–8 minutes after addition of thrombin, likely reflecting the time needed for reorganization of the cytoskeleton (Figure 4C).

Figure 3. Thrombin enhances interferon \(\gamma\) (IFN-\(\gamma\)) secretion in response to T-cell receptor stimulation in CD8\(^+\) T cells from healthy controls and human immunodeficiency virus (HIV)-infected patients. A, CD8\(^+\) T cells from healthy controls and HIV-infected patients with viral loads >50 copies/mL were cultured with plate-bound CD3/CD28 monoclonal antibodies in the presence or absence of 10 U/mL of thrombin. The cells were pregated on the basis of CD3 and CD8 expression. Gates for IFN-\(\gamma\) were set up on the basis of the unstimulated controls. B, IFN-\(\gamma\) secretion in CD8\(^+\) T cells from healthy controls (n = 13) and HIV-infected patients (n = 10). Comparisons between unstimulated and stimulated cells in HIV-infected patients and healthy controls were performed by the Wilcoxon matched pairs test. The red line represents the \(P\) value for the HIV group, and the black line represents the \(P\) value for the healthy control group. Comparisons between HIV-infected patients and healthy controls showed significant differences for CD3/CD28 stimulation (\(P = .008\)) and CD3CD28\(^+\) in the presence of thrombin (\(P = .04\)). Comparisons between HIV-infected patients and healthy controls were performed using Mann-Whitney \(U\) test.
Figure 4. Thrombin induces chemokinesis of human CD8+ T cells. A, Transwell experiments were performed with isolated CD8+ T cells from healthy controls (n = 12) and HIV-infected patients (n = 15) to study the chemotaxis to thrombin (10 U/mL) or CXCL12 (200 ng/mL). P values were calculated by the Wilcoxon matched pairs test and represent differences between responses to thrombin or CXCL12 and their respective controls. The P values represent the combine P value of both groups. B, CD8+ T cells were cultured in a cross-linked collagen layer to image individual cells over time (10 seconds/frame). Frames of the same video before and after thrombin addition (50 U/mL) are shown. The arrow indicates a resting CD8+ T cell polarized following addition of thrombin. C, A larger field of the same video shown in panel B. A total of 75 frames (750 seconds in total time) are overlaid either before thrombin (T1 upper panel) or after thrombin (T2 lower panel). Note that overlay shows numerous tracks of cells after thrombin but few such tracks before thrombin. To facilitate comparison, white circles indicate the same cells before and after thrombin. Percentage of the motile cells was calculated from the videos by comparing a frame just before thrombin addition (T1) and a frame at 800 seconds later (T2). Immobile cells were marked with circles at T1. The same circles were overlaid on the T2 frame, and the empty regions (representing cells that moved away) were counted to determine the percentage of motile cells. An average of 24% ± 8% mean ± standard error of the mean (SEM) motile cells after thrombin stimulation was observed in 3 independent experiments. Cells incubated only with media showed an average of 5.9% ± 0.6% mean ± SEM in 3 independent experiments.
Figure 5. Thrombin-induced chemokinesis of CD8\(^+\) T cells is protease-activated receptor 1 (PAR-1) dependent. A, Experimental design for cells (in cross-linked collagen layers) pretreated with the PAR-1 antagonist (SCH-79797; 5 \(\mu\)M). Before addition of thrombin, immotile cells were counted (T\(_0\)). Thrombin (50 U/mL) was added, and the motile cells were counted at T\(_1\). As control for the specificity of the antagonist, CCL19 (50 ng/mL) was added to the same plates, and motile cells were counted at T\(_2\). The percentage of mobile cells was calculated as described in the Supplementary Material and Methods. B, The average of 3 independent experiments after incubation with media alone or sequential addition of thrombin (T\(_1\)) and CCL19 (T\(_2\)) in collagen layers pretreated with the PAR-1 antagonist is shown. C, Experimental design for cells (in cross-linked collagen layers) pretreated with the inhibitor pertussis toxin (PTX; 100 ng/mL). Immotile cells were counted at T\(_0\), CCL19 (50 ng/mL) was then added to the plates, and motile cells were counted at T\(_1\). As a control for the specificity of the inhibitor, thrombin (50 U/mL) was added after T\(_1\), and motile cells were counted at T\(_2\) as described above and in the Supplementary Methods. D, The graph represents an average of 3 independent experiments after incubation with media alone or sequential addition of CCL19 (T\(_1\)) and thrombin (T\(_2\)) in collagen layers. E, Motile T cells in collagen layers stimulated with thrombin or CCL19 were tracked in videos and analyzed using Image J Software. The top panel shows trajectory plots created by Chemotaxis and Migration Tool (Ibidi, WI). The initial point for each track is set to zero. Movements of cells stimulated with thrombin are more constrained than those stimulated by CCL19. The bottom panel shows plots of velocity, accumulated distance, and directionality index (defined as Euclidean distance/accumulated distance). The point values on the graphs represent single cells tracked in 5 independent experiments (mean ± standard error of the mean). Comparisons were performed using Mann-Whitney U tests.

Thrombin-Induced Chemokinesis of Resting CD8\(^+\) T Cells Is Mediated Through PAR-1 Activation

To determine whether the thrombin-induced chemokinesis was mediated by PAR-1 activation, we pretreated the cross-linked collagen layers containing CD8\(^+\) T cells with the PAR-1 antagonist SCH-79797 and tracked the cells by video microscopy, as before. Only 9\% of the SCH-79797–treated population became motile in the presence of thrombin, whereas SCH-79797 had no effect on motility induced by subsequent addition of the chemokine CCL19 (Figure 5A and 5B). In addition, PTX pretreatment had no effect on thrombin-induced motility, although it efficiently blocked CCL19-induced chemokinesis.
(Figure 5C and 5D). Collectively, these results demonstrate that thrombin elicits chemokinesis of resting CD8+ T cells in a PAR-1–dependent but Gαi-independent manner.

We next characterized the cell motility induce by thrombin or CCL19 by measuring velocity, accumulated distance, and directionality. While thrombin and CCL19 evoked similar increases in velocity and total distance traveled by resting human CD8+ T cells (Figure 5E), there were striking differences in the directionality of the cells in response to these factors. The movement induced by thrombin stimulation was more constrained, and the cells were able to change direction very rapidly, consistent with a “searching or scanning” mode (Figure 5E). In contrast, CCL19 elicited significantly increased directionality relative to thrombin (directionality index, 0.26 vs 0.47; \( P < .001 \)). These results are consistent with the data described above, showing that thrombin induces chemokinesis but has little chemotactic effect.

**Thrombin Stimulation and Phosphorylation of ERM Proteins**

During migration from circulation into inflamed tissues, lymphocytes transition from a “rigid” to a “flexible” form that allows them to transit through small spaces and cross the endothelial barrier [31]. This process is mediated by reorganization of the cytoskeleton in which ERM proteins play an important role. ERM proteins cross-link cortical actin filaments with the cytoplasmic tail of membrane-bound proteins. Stimulation of T cells with CXCL12 leads to dephosphorylation of ERM proteins, actin polymerization, and polarization of the migrating cell [31]. ERM phosphorylation was quantified by flow cytometry, using a monoclonal antibody specific for the phosphorylated form of ERM proteins [20]. As expected, CXCL12 and CCL19 induced dephosphorylation of ERM proteins in the naive T-cell subset (Figure 6A and 6B). Consistent with the minimal PAR-1 expression we observed in naive T cells (Figure 1), thrombin stimulation had a negligible effect on ERM protein dephosphorylation in this subset (Figure 6A and 6B). In the memory CD8+ T-cell subset, however, thrombin induced a transient slight increase in ERM protein phosphorylation, followed by modest and slow dephosphorylation (5% decrease from basal levels). The response to CCL19 in this subset was less marked than in the naive cells, consistent with the more heterogeneous expression of CCR7 by the memory CD8+ T-cell subset.

In response to chemokine signals, T cells undergo cytoskeletal changes in order to migrate into tissues. Polarized T cells develop a front side that is rich in F-actin yet excludes CD3 molecules. Other molecules, such as CD44 and CD43, are localized at the rear of the cell (ie, in the uropod), and cpERM molecules undergo dephosphorylation [31, 32]. We characterized thrombin-mediated cytoskeletal changes by microscopy (Figure 6C). After stimulation of resting purified CD8+ T cells for 5 minutes with thrombin, a mean percentage (mean ± standard error of the mean [SEM]) of 28% ± 1.8% of the cells had a thrombin-induced membrane protrusion that was enriched with F-actin filaments and cpERM proteins and contained CD3. Other molecules, including CD43 and CD44, also localized with cpERM in this structure (data not shown). By contrast, the majority of cells (mean percentage [mean ± SEM], 74% ± 2.6%) stimulated with CXCL12 developed a protrusion enriched for F-actin in which cpERM proteins (the presence of which yielded a very dull signal because of dephosphorylation; Figure 6A and 6B) and CD3 were uniformly excluded, consistent with the presence of a lamellipodium [32]. These data demonstrate that thrombin induces polarization of CD8+ T cells with the development of a structure rich in F-actin and cpERM.

**DISCUSSION**

The current study provides direct evidence that thrombin couples the coagulation cascade with the adaptive immune system through stimulation of memory T cells. Thrombin is capable of activating memory CD8+ T-cell subsets through PAR-1–dependent signaling. These subsets include long-lived, pathogen-specific cells that seek out antigen-presenting cells in peripheral tissues. Our data support a physiological mechanism of immune surveillance, inflammation, and tissue repair in which thrombin functions as an early biochemical messenger or “danger signal” [33–35] that may allow for the accumulation of memory T cells at the site of tissue injury.

For migrating lymphocytes to extravasate into inflamed tissues in response to chemokine signaling, they transition from a “rigid” to a “flexible” structure, which facilitates transit through endothelial barrier. Dephosphorylation of ERM proteins [31] and cell polarization mediate these shape changes. We found that thrombin, in contrast to chemokines, could promote the “flexible” state through PAR-1 activation and formation of structures (ie, thrombin-induced protrusions) with concentrated levels of F-actin, resulting in a flexible cell body. In patients with systemic lupus erythematosis, formation of polar caps in T cells have been described and suggested as promoters of lymphocyte extravasation [36]. In addition, consistent with the constrained (ie, nondirectional) movements induced by thrombin, this structure could facilitate rapid change in directionality, allowing the localization of patrolling T cells at the site of vascular injury and promoting increased surveillance of the nearby tissue.

Links between inflammation and coagulation have primarily been reported to involve cells of the innate immune system [15, 16]. For example, it is well recognized that PAR-1 activation of dendritic cells induces interleukin 1β (IL-1β) secretion [17, 27, 37]. In addition, in vivo PAR-1–knockout mice models of inflammation of human diseases, such as lung injury, sepsis, Th2 cell–mediated colitis, antigen-induced arthritis, and colon inflammation, have shown a delayed inflammatory...
Figure 6. Thrombin induces rapid phosphorylation-dephosphorylation of ezrin-radixin-moesin (ERM) proteins in CD8+ T cells. A. As a positive control, CD8+ T cells from healthy controls were treated with the phosphatase inhibitor calyculin A (CA; 50 nM) to induce maximum phosphorylation of ERM proteins (light gray histograms). As a negative control, cells were treated with the kinase inhibitor staurosporine (STA; 500 nM) to prevent protein phosphorylation (dark gray histograms). The black lines in the top 2 histograms show the basal levels of C-terminally phosphorylated ERM (cpERM) proteins in naive and memory CD8+ T cells incubated with medium. The changes over time are represented for cells stimulated with thrombin or CXCL12. Cells were stained with CD45RA and CD27 markers to define naive (CD45RA+CD27+) and memory (CD45RA-CD27+) CD8+ T-cell subsets. B. The kinetics of cpERM changes on stimulation with CXCL12 or CCL19 as a percentage of maximal phosphorylation (due to CA) in 5 independent experiments. C. CD8+ T cells were stimulated for 5 minutes with thrombin (10 U/mL), CXCL12 (200 ng/mL) or media alone and stained for F-Actin (Phalloidin-Alexa 488), and cpERM-Alexa 647 and CD3-Alexa 568. Arrows indicate the thrombin-induced protrusion or chemokine-induced polarization. The average percentage of polarized T cells was 28% ± 1.8% mean ± standard error of the mean (SEM) following thrombin stimulation and 74% ± 2.6% mean ± SEM after CXCL12 stimulation in 4 independent experiments. Cells treated in medium alone did not show signs of polarization. The values represent mean ± SEM.
response in several types of cells [17, 25]. The role of PAR-1 in primary human T lymphocytes has not been investigated. Prior work in T cell lines has shown that PAR-1 activation induces calcium mobilization, activation of protein kinase C [38], and possible synergy with TCR cross-linking, leading to enhance proliferation [39]. In a separate study, secretion of IL-6 by peripheral blood mononuclear cells was observed after thrombin stimulation [39, 40]. We have shown that thrombin induced chemokinesis in CD8⁺ T cells through a PTX-resistant pathway. Preliminary studies of the signaling routes activated by thrombin in T cells indicate that thrombin-mediated Ca²⁺ mobilization induces NFAT activation through both Gαq and Gα12/13 activity, which could account for the enhanced TCR-evoked cytokine (ie, IFN-γ) production in cells costimulated with thrombin. These findings highlight the role of thrombin as a potential regulator of T-lymphocyte activation.

Human infectious or chronic inflammatory diseases are often associated with continual activation of the coagulation cascade, which can exacerbate disease pathology [41, 42]. In HIV infection, serious non–AIDS-defining events, including cardiovascular disease, liver disease and renal failure, are now the leading causes of death. The incidences of these complications are increased among patients with elevated levels of interleukin 6 and D-dimers, which correlate with higher HIV viral loads [7, 9, 43]. Although levels of PAR-1 expression are similar on CD8⁺ T cells from patients and controls, the increased numbers of CD8⁺ T cells expressing PAR-1 in patients with HIV infection and the association of CD8⁺ T cells with levels of HIV RNA (R = 0.365; P < .001) suggest that PAR-1 may contribute to increase immune activation. How increased levels of PAR-1 on CD8⁺ T cells may enhance the immunopathology of HIV infection remains unclear. The substantial expansion of the CD8⁺ T-cell pool in the setting of HIV infection creates a scenario whereby any increase in thrombin could be immediately accompanied by increase in CD8 activation and accumulation at the sites of thrombin deposition. This could lead to further enhancement of endogenous tissue damage. The biomarker D-dimer is a degradation byproduct of fibrin and its serum levels increase following fibrinolysis. Of note we observed, a negative association between levels of D-dimers in serum and PAR-1 expression in the CD8⁺ T-cell subsets from healthy controls suggesting a relationship between in vivo levels of coagulation and PAR-1 expression. In contrast, this association was not observed in HIV-infected patients, suggesting a disruption in the physiological relationship between these parameters. In the context of HIV infection, monocytes express higher levels of tissue factor than those from healthy controls, suggesting that the coagulation cascade could be activated directly by HIV replication [10]. How these subsets of cells contribute to the inflammation-coagulation process during health and disease needs additional studies. The present data extend previous findings by linking an arm of the adaptive immune system (T cells) with the coagulation system. This interface may play an important role in host defense and the immunopathogenesis of inflammatory diseases.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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