CD8⁺ T-Cell–Derived Tumor Necrosis Factor Can Induce Tissue Factor Expression on Monocytes

Michael L. Freeman,^{1,2} Soumya Panigrahi,^{1,2} Bonnie Chen,^{1,2} Steven Juchnowski,³ Scott F. Sieg,^{1,2} Michael M. Lederman,^{1,2} Nicholas T. Funderburg,⁵ and David A. Zidar^{1,3,4}

¹Center for AIDS Research and ²Division of Infectious Diseases and HIV Medicine, Department of Medicine, Case Western Reserve University/University Hospitals Cleveland Medical Center, ³Harrington Heart and Vascular Institute, University Hospitals Cleveland Medical Center, and ⁴Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, and ⁵Division of Medical Laboratory Sciences, School of Health and Rehabilitation Sciences, Ohio State University, Columbus, Ohio

Circulating CD8⁺ T cells and monocytes are activated during human immunodeficiency virus (HIV) infection and colocalize in the aortas of simian immunodeficiency virus–infected nonhuman primates. We hypothesized that CD8⁺ T cells could exert a proatherosclerotic effect via paracrine actions on monocytes. We found that T-cell receptor–stimulated CD8⁺ T cells induce monocytes to express tissue factor, a potent activator of coagulation. Tumor necrosis factor was both necessary and sufficient for this effect.

Keywords. CD8⁺ T cells; monocytes; tissue factor; TNF; HIV; cardiovascular disease.

Human immunodeficiency virus (HIV)–infected individuals sustain elevated levels of inflammation that are linked to increased risk of cardiovascular disease despite virus suppression by antiretroviral therapy [1]. Many factors promote this persistent inflammatory state, including low-level virus replication, infection with copathogens such as cytomegalovirus (CMV), translocation of microbial products from the gut to the bloodstream, and abundance of proinflammatory lipids such as oxidized low-density lipoprotein cholesterol [1]. Although each of these stimuli can activate innate immune defenses and alter endothelial cell and immune cell functions, the mechanisms linking these proinflammatory pathways to comorbid cardiovascular disease are not well understood.

HIV infection is associated with a highly prothrombotic immune phenotype, with demonstrated increases of tissue factor

(TF)–expressing platelets, platelet microparticles, monocytes, and platelet-monocyte aggregates [2, 3]. These changes are due in part to an expansion of inflammatory (intermediate) and patrolling (nonclassical) monocytes, which are enriched for TF expression [4] and platelet aggregation [5]. Recently, it has been shown that TF expression on monocytes is associated with coagulopathy in simian immunodeficiency virus (SIV)–infected pigtail macaques [6], and earlier we reported that the prothrombotic monocyte phenotype in HIV infection is similar to that seen in patients with active atherothrombosis and acute coronary syndrome [4]. In contrast to other (classical) monocytes, inflammatory and patrolling monocytes express the chemokine receptor CX3CR1, a vascular endothelium-homing chemokine receptor, also expressed by platelets, that tethers immune cells to activated endothelium via its membrane-bound ligand, fractalkine (CX3CL1).

In HIV-uninfected persons, acute coronary syndrome is associated with a relative expansion of effector memory CD8⁺ T cells [7], and there is a profound expansion of CD8⁺ T cells with an effector phenotype in HIV-infected persons who are CMV coinfecting [8]. This latter expansion is linked to cardiovascular morbidity, although the mechanisms driving this association are poorly understood [9]. Importantly, the expanded CD8⁺ T cells are enriched for expression of CX3CR1, the thrombin-responsive protease-activated receptor 1 (PAR-1), and P-selectin glycoprotein ligand 1, which binds activated platelets [10]. Conjugates of CD8⁺ T cells and activated platelets are increased in the circulation of HIV-infected individuals [11]. CX3CL1 levels are increased in plasma during HIV infection [12] and on the endothelium during HIV infection and atherosclerosis [13], suggesting that CX3CR1⁺CD8⁺ cells may be recruited to prothrombotic, inflamed endothelium. Indeed, we and others have recently identified accumulation and colocalization of macrophages and CD8⁺ T cells in the aortic endothelium of SIV-infected macaques at sites of endothelial cell dysfunction [6, 14].

Inflammatory cytokines have previously been shown to exert a “priming” influence on macrophage/monocytes, leading to exaggerated responsiveness, but may also lead to tolerance, immune paralysis, and cross-tolerance [15]. Using a coculture system, we sought to test the hypothesis that activated CD8⁺ T cells might contribute to the induction of a prothrombotic monocyte phenotype.

METHODS

Cell Harvest, Culture, and Stimulation

CD8⁺ T cells and monocytes were purified by negative selection, using RosetteSep isolation kits (Stemcell Technologies), from

Received 24 October 2018; editorial decision 23 January 2019; accepted 28 January 2019; published online January 29, 2019.

Presented in part: 16th International Congress of Immunology, Melbourne, Australia, 21–26 August 2016.

Correspondence: M. L. Freeman, PhD, Center for AIDS Research, Division of Infectious Diseases and HIV Medicine, Department of Medicine, Case Western Reserve University, School of Medicine, 2109 Adelbert Rd, BRB 1048B, Cleveland, OH 44106 (MLF62@case.edu).

The Journal of Infectious Diseases® 2019;220:73–7

© The Author(s) 2019. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com. DOI: 10.1093/infdis/jiz051

peripheral blood specimens collected with informed consent from 11 HIV-uninfected volunteers. CD8⁺ T cells were stimulated via the T-cell receptor (TCR), using plate-bound anti-CD3 (5 µg/mL; BD Biosciences) and soluble anti-CD28 (5 µg/mL; BD Biosciences), or were left unstimulated. After 3 hours, the CD8⁺ T cells were harvested, washed, added to purified monocytes, and cocultured for an additional 3 hours. In some assays, antibodies to interferon γ (IFN-γ; 10 µg/mL), tumor necrosis factor (TNF; 10 µg/mL), or immunoglobulin G1 isotype control (all from BD Biosciences) were added during coculture to neutralize the effect of stimulated CD8⁺ T cells on monocytes. In parallel assays, monocytes were cultured for 3 hours in the presence or absence of exogenous lipopolysaccharide (20 ng/mL; Sigma), recombinant IFN-γ (250 ng/mL; R&D Systems), or recombinant TNF (250 ng/mL; R&D Systems). At the end of the cultures, cells were harvested, stained with antibodies to CD14 (BD Biosciences) and TF (Biomedica Diagnostics), and analyzed by flow cytometry on a MACSQuant (Miltenyi) cytometer. All experiments were approved by the Institutional Review Board of University Hospitals Cleveland Medical Center (protocol 01-98-55) in accordance with the Declaration of Helsinki.

Intracellular Cytokine Staining

For detection of intracellular cytokines, peripheral blood mononuclear cell preparations from 6 HIV-uninfected volunteers were stimulated for 6 hours with plate-bound anti-CD3 (5 µg/mL; BD Biosciences) and soluble anti-CD28 (5 µg/mL; BD Biosciences) or medium control in the presence of brefeldin A (GolgiPlug, BD Biosciences). After stimulation, cells were harvested; stained with LiveDead Aqua viability dye (ThermoFisher) and fluorescently labeled antibodies to surface antigens CD3 (BD Biosciences), CD8 (BD Biosciences), and CX3CR1 (BioLegend); fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) per the manufacturer's instructions; stained with fluorescently labeled antibodies to IFN-γ and TNF (both from BD Biosciences); and analyzed by flow cytometry a LSRFortessa (BD Biosciences) cytometer.

Stimulation of Human Aortic Endothelial Cells

Primary human aortic endothelial cells (PromoCell) were cultured per the manufacturer's instructions. Cells were stimulated for 7 days in the presence of IFN-γ (20 ng/mL), TNF (20 ng/mL), or medium control. Culture supernatants were then harvested, and CX3CL1 levels were quantified by enzyme-linked immunosorbent assay (Quantikine; R&D Systems) per the manufacturer's instructions.

Statistical Analyses

We compared continuous variables by using the Mann-Whitney *U* test for unpaired analyses, the Wilcoxon signed rank test for paired analyses, the Friedman test with the Dunn multiple comparison correction, or 1-way analysis of variance with the Holm-Sidak multiple comparison correction. *P* values

< .05 were considered significant. All statistic analyses were performed using Microsoft Excel or GraphPad Prism software.

RESULTS

In a recent study, we found CD8⁺ T cells and CD68⁺ macrophage/monocytes in close proximity in the aortas of SIV/simian-human immunodeficiency virus (SHIV)-infected rhesus macaques, which is consistent with both CD8⁺ T cells and monocytes expressing the vascular endothelium-homing receptor CX3CR1. Given that monocytes have increased expression of the coagulation element TF in both HIV disease and acute coronary syndromes, we sought to identify whether CD8⁺ T cells could be a factor driving increased TF expression on monocytes, a potential mechanistic link between HIV infection and increased cardiovascular disease risk. To examine whether TCR-stimulated CD8⁺ T cells could induce expression of TF on monocytes, we set up a short-term coculture assay (Figure 1A). At the end of the culture, cells were harvested, and surface TF expression was measured on the monocytes by flow cytometry. We found that TCR-stimulated CD8⁺ T cells but not unstimulated CD8⁺ T cells induced an increase of surface TF expression on monocytes (Figure 1B and 1C). Lipopolysaccharide-stimulated monocytes were used as a positive control for monocyte TF expression (Figure 1A and 1B).

CD8⁺ T cells activated via the TCR release cytokines and chemokines that may affect surrounding cells. In particular, 2 CD8⁺ T-cell-derived effector cytokines—IFN-γ and TNF—have monocyte-activating properties [15]. We stimulated peripheral blood mononuclear cells through the TCR and examined the intracellular accumulation of IFN-γ and TNF in CX3CR1⁺CD8⁺ T cells and CX3CR1[−]CD8⁺ T cells by flow cytometry (Figure 2A). A greater proportion of CX3CR1⁺CD8⁺ T cells than CX3CR1[−]CD8⁺ T cells made IFN-γ and TNF in response to TCR stimulation, consistent with CX3CR1⁺CD8⁺ T cells being enriched in terminally differentiated cells with proinflammatory potential [10]. To determine whether IFN-γ and/or TNF were necessary for the CD8⁺ T-cell-induced upregulation of TF on monocytes, we added neutralizing antibodies to IFN-γ (10 µg/mL) and TNF (10 µg/mL) during the coculture. Blocking TNF but not IFN-γ reduced the TCR-stimulated CD8⁺ T-cell enhancement of surface TF expression on the monocytes (Figure 2B), indicating that TNF is necessary for the TCR-stimulated CD8⁺ T-cell-mediated effect. To determine whether TNF or IFN-γ was sufficient to induce TF upregulation, we treated monocytes with recombinant TNF or IFN-γ. TNF treatment alone, in the absence of activated T cells, induced TF expression on the monocytes (Figure 2C). Importantly, both IFN-γ and TNF induced cultured endothelial cells to release fractalkine (CX3CL1), the chemokine ligand for CX3CR1 (Supplementary Figure 1). Thus, CX3CR1⁺CD8⁺ T-cell-derived cytokines drive both monocyte expression of TF and aortic endothelial cell expression of CX3CL1, which may

Figure 1. Activated CD8⁺ T cells promote tissue factor (TF) expression on human monocytes. *A*, Schematic of CD8⁺ T-cell and monocyte coculture assay. *B*, TF expression on monocytes in indicated conditions. *C*, Summary of coculture experiments. Boxes represent median values and interquartile ranges, and error bars extend to minimum and maximum values. Statistical significance was determined by the Wilcoxon signed rank test. LPS, lipopolysaccharide; MFI, mean fluorescence intensity; TCR, T-cell receptor.

promote the accumulation of CD8⁺ T cells and monocytes in proximity at sites of vascular endothelial cell activation.

DISCUSSION

Expression of the prothrombotic element TF on peripheral blood cells is elevated in both treated HIV infection and in HIV-uninfected patients with active atherothrombosis and acute coronary syndrome [4]. Monocyte TF expression in particular is associated with coagulopathy in SIV-infected pigtail macaques [6]. In this study, we demonstrated that activated CD8⁺ T cells promote the expression of TF on monocytes through TNF production—a possible mechanism driving elevated cardiovascular disease in treated HIV infection.

CX3CR1 binds fractalkine (CX3CL1), a chemokine and adhesion molecule expressed by vascular endothelial cells. CX3CR1 is expressed by platelets, monocytes, and some T cells, and CX3CR1⁺CD8⁺ T cells are increased in the circulation of people living with treated HIV infection [10]. We show here that the CX3CR1⁺CD8⁺ T-cell-derived cytokines IFN- γ and TNF promote CX3CL1 secretion by aortic endothelial cells. Thus, CX3CR1⁺CD8⁺ T cells are potentially key players in the

recruitment, retention, and accumulation of other CX3CR1-expressing cells at sites of endothelial activation, a phenomenon we have observed in the aortas of SIV- and SHIV-infected rhesus macaques [14].

Notably, monocytes can also make TNF [15], although it is unclear whether monocyte TNF induces autocrine expression of TF, and determining the relative roles of T-cell-derived TNF versus monocyte-derived TNF in monocyte TF expression in vivo is an important future direction. Other TNF-producing cells, such as TCR-activated CX3CR1⁺CD4⁺ T cells (Supplementary Figure 2A), could also potentially induce TF expression on monocytes, although CX3CR1⁺CD4⁺ T cells are far less frequent than CX3CR1⁺CD8⁺ T cells (Supplementary Figure 2B). Systemic levels of TNF are increased in antiretroviral therapy recipients living with HIV infection, particularly in those coinfecting with CMV [8], consistent with the role of CMV as a risk factor for cardiovascular disease [9], and it will be of great interest to determine whether in vivo TNF blockade in HIV-infected individuals is associated with a decrease in monocyte TF expression. We also note that activation by TNF is not the only method for TF upregulation on monocytes. In the

Figure 2. Tumor necrosis factor (TNF) is required for activated CD8⁺ T cells to induce tissue factor (TF) expression on human monocytes. *A*, Interferon γ (IFN- γ) and TNF expression by CX3CR1⁻CD8⁺ and CX3CR1⁺CD8⁺ T cells (*left*). Summary of T-cell receptor (TCR) stimulations (*right*). Statistical significance was determined by the Wilcoxon signed rank test. Boxes represent median values and interquartile ranges, and error bars extend to minimum and maximum values. *B*, Blocking TNF prevents the activated CD8⁺ T-cell-mediated upregulation of TF on monocytes. Boxes represent median values and interquartile ranges, and error bars extend to minimum and maximum values. Statistical significance was determined by the Friedman test with Dunn multiple comparison correction. *C*, Recombinant TNF enhances monocyte TF expression. Boxes represent median values and interquartile ranges, and error bars extend to minimum and maximum values. Statistical significance was determined by the Wilcoxon signed rank test. *D*, Proposed model in which activated CD8⁺ T cells expressing CX3CR1 contribute to atherothrombotic risk via TNF-mediated induction of TF on monocytes at sites of endothelial activation. CVD, cardiovascular disease; MFI, mean fluorescence intensity; r, recombinant.

absence of activated T cells, lipopolysaccharide stimulation can induce monocyte TF expression in a TNF-independent manner (Supplementary Figure 3).

CX3CR1⁺CD8⁺ T cells and monocytes express the thrombin receptor PAR-1, and direct thrombin exposure through PAR-1 promotes TCR stimulation-induced cytokine release by CD8⁺ T cells [10]. Thus, thrombin activation may be another component of the prothrombotic cross-talk between CX3CR1⁺CD8⁺ T cells and monocytes. Platelets are likely to be another player in the development of cardiovascular disease in HIV infection. Platelets express CX3CR1, TF, and PAR-1 and have been

found aggregating with both blood CD8⁺ T cells and monocytes during HIV infection [2, 11].

In conclusion, our findings indicate that TNF is both necessary and sufficient for activated CD8⁺ T cells to induce surface expression of the potent procoagulant protein TF on blood monocytes. These data support a model in which activated CD8⁺ T cells expressing CX3CR1 contribute to atherothrombotic risk via TNF-mediated induction of TF on monocytes at sites of endothelial activation (Figure 2D), thereby contributing mechanistically to the cardiovascular disease risk observed in persons living with HIV.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. M. L. F. conceived the study design, performed experiments, and wrote the manuscript; S. P. conceived the study design, performed experiments, and made editorial revisions; B. C. performed experiments and made editorial revisions; S. J. performed experiments and made editorial revisions; S. F. S. conceived the study design and made editorial revisions; M. M. L. conceived the study design and made editorial revisions; N. T. F. conceived the study design and made editorial revisions; D. A. Z. conceived the study design and contributed to drafting the manuscript; and all authors approved the final version of the manuscript.

Financial support. This work was supported by National Institutes of Health (grants HL134544 [to N. T. F.], AI076174, and AI069501), the Richard J. Fasnmyer Foundation (EIN 34-1627457 and BRIDGE number 2094523224 [award to M. M. L.]), the CWRU Center for AIDS Research (catalytic award AI036219 to M. L. F. and D. A. Z.), and the Clinical and Translational Science Collaborative of Cleveland (KL2TR000440 to D. A. Z.).

Potential conflicts of interest. N. T. F. serves as a consultant for Gilead. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Lederman MM, Funderburg NT, Sekaly RP, Klatt NR, Hunt PW. Residual immune dysregulation syndrome in treated HIV infection. *Adv Immunol* **2013**; 119:51–83.
2. Liang H, Duan Z, Li D, et al. Higher levels of circulating monocyte-platelet aggregates are correlated with viremia and increased sCD163 levels in HIV-1 infection. *Cell Mol Immunol* **2015**; 12:435–43.
3. Mayne E, Funderburg NT, Sieg SF, et al. Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression. *J Acquir Immune Defic Syndr* **2012**; 59:340–6.
4. Funderburg NT, Zidar DA, Shive C, et al. Shared monocyte subset phenotypes in HIV-1 infection and in uninfected subjects with acute coronary syndrome. *Blood* **2012**; 120:4599–608.
5. Singh MV, Davidson DC, Jackson JW, et al. Characterization of platelet-monocyte complexes in HIV-1-infected individuals: possible role in HIV-associated neuroinflammation. *J Immunol* **2014**; 192:4674–84.
6. Schechter ME, Andrade BB, He T, et al. Inflammatory monocytes expressing tissue factor drive SIV and HIV coagulopathy. *Sci Transl Med* **2017**; 9:eaam5441.
7. Zidar DA, Mudd JC, Juchnowski S, et al. Altered maturation status and possible immune exhaustion of CD8 T lymphocytes in the peripheral blood of patients presenting with acute coronary syndromes. *Arterioscler Thromb Vasc Biol* **2016**; 36:389–97.
8. Freeman ML, Mudd JC, Shive CL, et al. CD8 T-cell expansion and inflammation linked to CMV coinfection in ART-treated HIV infection. *Clin Infect Dis* **2016**; 62:392–6.
9. Freeman ML, Lederman MM, Gianella S. Partners in crime: the role of CMV in immune dysregulation and clinical outcome during HIV infection. *Curr HIV/AIDS Rep* **2016**; 13:10–9.
10. Mudd JC, Panigrahi S, Kyi B, et al. Inflammatory function of CX3CR1+ CD8+ T cells in treated HIV infection is modulated by platelet interactions. *J Infect Dis* **2016**; 214:1808–16.
11. Green SA, Smith M, Hasley RB, et al. Activated platelet-T-cell conjugates in peripheral blood of patients with HIV infection: coupling coagulation/inflammation and T cells. *AIDS* **2015**; 29:1297–308.
12. Kulkarni M, Bowman E, Gabriel J, et al. Altered monocyte and endothelial cell adhesion molecule expression is linked to vascular inflammation in human immunodeficiency virus infection. *Open Forum Infect Dis* **2016**; 3:ofw224.
13. Liu W, Jiang L, Bian C, et al. Role of CX3CL1 in diseases. *Arch Immunol Ther Exp (Warsz)* **2016**; 64:371–83.
14. Panigrahi S, Freeman ML, Funderburg NT, et al. SIV/SHIV infection triggers vascular inflammation, diminished expression of krüppel-like factor 2 and endothelial dysfunction. *J Infect Dis* **2016**; 213:1419–27.
15. Decano JL, Mattson PC, Aikawa M. Macrophages in vascular inflammation: origins and functions. *Curr Atheroscler Rep* **2016**; 18:34.