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Soluble T Cell Immunoglobulin Mucin Domain 3 Is Shed from CD8$^+$ T Cells by the Sheddase ADAM10, Is Increased in Plasma during Untreated HIV Infection, and Correlates with HIV Disease Progression

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ABSTRACT

Chronic HIV infection results in a loss of HIV-specific CD8$^+$ T cell effector function, termed "exhaustion," which is mediated, in part, by the membrane coinhibitory receptor T cell immunoglobulin mucin domain-3 (Tim-3). Like many other receptors, a soluble form of this protein has been described in human blood plasma. However, soluble Tim-3 (sTim-3) is poorly characterized, and its role in HIV disease is unknown. Here, we show that Tim-3 is shed from the surface of responding CD8$^+$ T cells by the matrix metalloproteinase ADAM10, producing a soluble form of the coinhibitory receptor. Despite previous reports in the mouse model, no alternatively spliced, soluble form of Tim-3 was observed in humans. Shed sTim-3 was found in human plasma and was significantly elevated during early and chronic untreated HIV infection, but it was not found differentially modulated in highly active antiretroviral therapy (HAART)-treated HIV-infected subjects or in elite controllers compared to HIV-uninfected subjects. Plasma sTim-3 levels were positively correlated with HIV load and negatively correlated with CD4 counts. Thus, plasma sTim-3 shedding correlated with HIV disease progression. Despite these correlations, we found that shedding Tim-3 did not improve the function of CD8$^+$ T cells in terms of gamma interferon production or prevent their apoptosis through galectin-9. Further characterization studies of sTim-3 function are needed to understand the contribution of sTim-3 in HIV disease pathogenesis, with implications for novel therapeutic interventions.

IMPORTANCE

Despite the overall success of HAART in slowing the progression to AIDS in HIV-infected subjects, chronic immune activation and T cell exhaustion contribute to the eventual deterioration of the immune system. Understanding these processes will aid in the development of interventions and therapeutics to be used in combination with HAART to slow or reverse this deterioration. Here, we show that a soluble form of T cell exhaustion associated coinhibitory molecule 3, sTim-3, is shed from the surface of T cells. Furthermore, sTim-3 is elevated in the plasma of treatment-naive subjects with acute or chronic HIV infection and is associated with markers of disease progression. This is the first study to characterize sTim-3 in human plasma, its source, and mechanism of production. While it is still unclear whether sTim-3 contributes to HIV pathogenesis, sTim-3 may represent a new correlate of HIV disease progression.

Despite significant advances in the development of highly active antiretroviral therapy (HAART) to reduce viral replication in subjects chronically infected with human immunodeficiency virus type 1 (HIV), the immune system is incapable of completely eliminating the virus. The resulting persistent antigen levels drive a process called "T cell exhaustion," whereby responding T cells undergo hierarchical loss of their effector functions, including their ability to proliferate, their cytotoxic potential, and their ability to produce cytokines (1). Coinhibitory molecules, including programmed death receptor 1 (PD-1) (2–6), lymphocyte activation gene-3 (LAG-3) (5, 7, 8), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (9–12), and T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) (4, 12–14) contribute to T cell exhaustion, reducing potentially harmful, persistent T cell activation. However, this also results in suboptimal HIV-specific responses and ultimately poor control of the virus. Understanding the mechanisms of regulation beyond receptor/ligand expression is important, as these mechanisms affect whether these processes play a role in pathogenesis

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and how the specific coinhibitory pathway would respond to therapeutic intervention.

Coinhibitory pathways can be regulated by the production of endogenous soluble coinhibitory receptors. Soluble receptors have been reported for multiple coinhibitory molecules, including LAG-3 and CEACAM1 (9, 15, 16). The mechanisms for soluble receptor production are different for LAG-3 and CEACAM1. While LAG-3 is "shed" from the surface of the T cell by the matrix metalloproteinases ADAM10 and ADAM17 (15), soluble CEACAM1 (sCEACAM1) production is independent of matrix metalloproteinase activity, suggesting the presence of an alternatively spliced isoform (16). Interestingly, while LAG-3 shedding enhances proliferation of the parent cell due to loss of surface inhibitory receptor (intrinsic effect), the shed protein exhibits no apparent effect on other cells of the immune system (extrinsic effect). This has been attributed to poor binding of the soluble receptor to its ligand (15). In contrast, binding of sCEACAM1 to the membrane-bound form results in inhibition of this negative regulatory pathway in NK cells (9, 16). However, multiple studies have also shown that the use of sCEACAM1 enhances inhibitory signaling through the membrane-bound CEACAM1 pathway, resulting in T cell inhibition (9, 10). Thus, the type of construct (surface shed or alternatively spliced) and the interaction with other receptors and ligands can dictate the overall regulation of these pathways.

Human Tim-3 is a type I transmembrane protein with extracellular IgV-like and mucin domains with 2 N-linked and 10 O-linked glycosylation sites (17, 18). Tim-3 is expressed on multiple immune cells, including activated/exhausted T cells and monocytes (13, 19–26). Known ligands for Tim-3 include phosphatidylserine (27), galectin-9 (28), CEACAM1 (12), and HMGB1 (29). A soluble form of Tim-3 (sTim-3) was first described in mice as an alternatively spliced product (30). While the full-length Tim-3 transcript encodes a signal peptide, IgV-like domain, mucin domain, transmembrane region, and cytoplasmic tail, the alternatively spliced variant in mice lacks both the mucin domain and transmembrane region. Further characterization of this protein suggested a role in inhibiting T cell responses to tumor antigen *in vitro* and *in vivo* (31). Using mass spectrometry, Hansen et al. first discovered a Tim-3 construct in human plasma which was found to be associated with graft-versus-host disease (32). However, this plasma Tim-3 construct is poorly characterized, and it is unclear whether this soluble Tim-3 is elevated in chronic infections such as HIV. Interestingly, Möller-Hackbarth et al. described a mechanism whereby full-length membrane Tim-3 can be cleaved from the cell surface by matrix metalloproteinases ADAM10 and ADAM17, yielding a soluble Tim-3 ectodomain (IgV-like domain and mucin domain) (33). Thus, it is unclear which construct, the alternatively spliced or the membrane-cleaved sTim-3, represents the sTim-3 form found in human plasma. Furthermore, while sTim-3 has been correlated with graft-versus-host disease (32), it is unknown whether it can modulate immune pathways. Given Tim-3's role in HIV-associated T cell dysfunction (12–14), sTim-3 might play a role in modulating T cell responses during HIV infection. However, it is unclear what role sTim-3 may play, and further, the functional properties of soluble receptors may be dependent on the type of construct produced. For example, surface shedding may enhance T cell responses, similar to LAG-3, while production of an alternatively spliced form of sTim-3 might inhibit T cell responses, as was shown in mice (31). To obtain an in-depth understanding of human sTim-3, here we characterized human soluble Tim-3 produced by T cells and assessed its role in HIV infection.

MATERIALS AND METHODS

Ethics statement. Informed consent was obtained in accordance with the guidelines for conduct of clinical research at the University of Toronto and Maple Leaf Clinic institutional ethics boards. Written informed consent was provided for this study, which was reviewed by research ethics board of the University of Toronto, Canada, and of St. Michael's Hospital, Toronto, Canada. The study was also approved by the institutional review board of the University of California, San Francisco and the committee of human subjects at the University of Hawaii. All individuals gave written informed consent, and investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Patient groups. We studied individuals from three cohorts of prospectively observed HIV-infected subjects: the Toronto-based cohort (Maple Leaf Clinic and St. Michael's Hospital, Toronto, Canada), the San Francisco-based SCOPE (Study of the Consequences of the Protease Inhibitor Era, San Francisco, U.S.) cohort, and the San Francisco-based Blood Systems cohort. Samples were obtained from subjects comprising the following patient groups: (1) treatment-naive acute HIV infection (infected <6 months, with detectable viral load, HIV Western blot serology negative); (2) treatment-naive early HIV infection (infected <6 months, with detectable viral load, HIV Western blot serology positive); (3) treatment-naive chronic HIV infection (infected >1 year, with detectable viral load); (4) HAART-treated chronic HIV infection (treated for >6 months, undetectable viral load); (5) treatment-naive chronic HIV infection with elite viral control (elite controllers; untreated, HIV infected with undetectable viral load, as previously described [34]); (6) demographically matched HIV-seronegative subjects. The Toronto-based cohort consisted of samples from groups 2 ($n = 15$), 3 ($n = 22$), 4 ($n = 5$), and 6 ($n = 13$). The SCOPE cohort consisted of samples from groups 3 ($n = 16$), 4 ($n = 19$), 5 ($n = 18$), and 6 ($n = 16$), as previously described (34). The Blood Systems cohort consisted of plasma samples from groups 1 ($n = 19$) and 2 ($n = 13$) as previously described (35). Whole blood was collected in anticoagulant-treated tubes and spun at 2,000 × *g* for 10 min to collect plasma, which was stored at −80°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated from the remaining blood material by using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) and stored at −150°C until use.

Stimulation assays and flow cytometry. Isolated monocytes and isolated, activated $CD4^+$ and $CD8^+$ T cells were stained with fluorescently conjugated anti-Tim-3 monoclonal antibody (MAb; clone 344823; R&D Systems, Minneapolis, MN) with anti-ADAM10 MAb (Biolegend, San Diego, CA) or isotype control MAbs (Biolegend and R&D Systems) and fixed in 2% formalin–phosphate-buffered saline (PBS). For peptide stimulation assays, HIV-infected chronic naive PBMCs were thawed and stimulated with dimethyl sulfoxide (DMSO) control, 1 μg/ml of overlapping HIV-1 clade B Gag peptide pool (National Institutes of Health AIDS Reagent Program, Bethesda, MD), or 1 μg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich, Germany) in the presence of brefeldin A (Sigma), with or without 9 μM (optimized concentration [36]) ADAM10 metalloproteinase inhibitor GI 254023X (R&D Systems) for 6 h. Following stimulation, the cells were stained with anti-Tim-3 MAb (clone 344823; R&D Systems) or an isotype control (R&D Systems) and antibodies against CD3 and CD8 (Biolegend). Following fixation and permeabilization, intracellular staining was performed using anti-gamma interferon (anti-IFN-γ; Biolegend) and final fixing in 2% formalin–PBS.

For apoptosis assays, HIV-infected chronic naive PBMCs were thawed and stimulated with DMSO control, 1 μg/ml of overlapping HIV-1 clade B Gag peptide pool (National Institutes of Health AIDS Reagent Program), or 1 μg/ml SEB (Sigma-Aldrich) in the presence of brefeldin A (Sigma), with or without 9 μM ADAM10 metalloproteinase inhibitor GI 254023X (R&D Systems) for 6 h. During the last hour of stimulation, 125 nM recombinant galectin-9 (produced as described in reference 37) was

added to the cultures. Following stimulation, the cells were stained with anti-Tim-3 MAb (clone 344823; R&D Systems) or isotype control (R&D Systems), and antibodies against CD8 (Biolegend), followed by Annexin-V staining as per the manufacturer's instruction (BD Biosciences, San Jose, CA). Following fixation and permeabilization, intracellular staining was performed using anti-IFN-γ (Biolegend) and final fixing in 2% formalin–PBS. All data were acquired on a BD FACSCalibur apparatus (BD Biosciences) and analyzed using FlowJo software.

Soluble Tim-3 ELISA. To measure levels of sTim-3 in human plasma and *in vitro* culture supernatant samples, Immulon 2HB 96-well plates (Thermo Scientific, Rockford, IL) were coated overnight at 4°C with an unconjugated anti-Tim-3 MAb (clone 344823; R&D Systems) at 2.1 μg/ml in PBS. The next day, frozen patient plasma was thawed on ice and spun at 10,000 × *g* to remove any aggregated material and residual cells. *In vitro* culture supernatants were concentrated approximately 10× by using Amicon Ultra-4 10-kDa molecular mass cutoff centrifugal filter units (Millipore, Billerica, MA). Each sample was diluted 1-in-2 with blocking buffer (3% bovine serum albumin [BSA] in PBS-Tween 20 [PBST]). Standards for the enzyme-linked immunosorbent assay (ELISA) consisted of recombinant sTim-3, which was produced from HEK293T cells and purified as previously described (13). sTim-3 was diluted in blocking buffer to create a 2.5-fold dilution series ranging from 1 ng/ml to 97.6 ng/ml. The plate was washed, blocked with blocking buffer, and incubated with duplicates of the sTim-3 standards and plasma samples. Following washing, the wells were incubated with anti-Tim-3 polyclonal antibody (pAb; R&D Systems), followed by anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. The plate was developed, and absorbance was measured at 405 nm.

Cell isolations, *in vitro* culture, and activation. PBMCs from HIV-uninfected individuals were plated at 2×10^6 cells in 2.5 ml complete R-10 medium (RPMI 1640, 10% fetal bovine serum, 1 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine; Wisent, Saint-Bruno, QC, Canada) in a 12-well plate. Cells were stimulated with medium alone, 1 μg/ml LEAF-purified anti-CD3 MAb (clone OKT3; BioLegend), and 1 μg/ml anti-CD28 MAb (clone 28.8; BioLegend), 1% phytohemagglutinin M form (PHA; Invitrogen, Carlsbad, CA), or 1, 10, 100, or 1,000 pg/ml of lipopolysaccharide (LPS; Sigma-Aldrich). PBMCs were harvested 4 days after stimulation and frozen at −80°C for future RNA isolation. Cellular supernatants were also collected from PBMC cultures and stored for future use. For 1-hour shedding experiments, monocytes or $CD4^+$ and $CD8^+$ T cells were isolated from HIV-uninfected PBMCs via negative enrichment (EasySep; StemCell Technologies, Vancouver, BC, Canada). T cells were activated in culture for 6 days with 1 μg/ml anti-CD3 MAb (clone OKT3; BioLegend), 1 μg/ml anti-CD28 MAb (clone 28.8; BioLegend), and 50 U/ml of recombinant interleukin-2 (IL-2; National Institutes of Health, Bethesda, MD) to upregulate Tim-3. Isolated cell populations were washed and then received medium, 1 μg/ml SEB (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), or 5 μg/ml LPS (Sigma-Aldrich) for 1 h followed by supernatant collection. For assays using the ADAM10 metalloproteinase inhibitor GI 254023X (R&D Systems), cells were cultured with 9 μM inhibitor for 30 min at 37°C prior to stimulation.

Analysis of gene expression by reverse transcription-PCR. Frozen PBMCs, $CD4^+$ and $CD8^+$ T cells, or monocytes were thawed and lysed, and the RNA was purified using an RNeasy Plus minikit (Qiagen, Venlo, Limburg, Netherlands). Total RNA was reverse transcribed with the SuperScript III first-strand synthesis system (Invitrogen) using oligo$(dT)_{18}$ primers, and RNA complementary to the cDNA was removed using *Escherichia coli* RNase H (Invitrogen). For the amplification of Tim-3 transcripts, primers were designed against the untranslated regions (UTRs): 5′-UTR (5′-GAGAGTTAAAACTGTGCCTAACAG-3′) and 3′-UTR (5′-CTCCAAAACCAGTCAGGTGACACA-3′) as previously described (30). Cycling conditions were as follows: denaturation at 95°C for 60 s, annealing at 55°C for 30 s, extension at 72°C for 3 min for 35 cycles, and a final extension at 72°C for 10 min. Human Tim-3 parent vector containing the full mRNA transcript was used as a positive control for all PCR amplifications. Products were visualized using ethidium bromide on a 1% agarose gel.

Immunoprecipitation and Western blot analysis. Frozen patient plasma was thawed on ice and spun at 10,000 × *g* to remove any aggregated material and residual cells. One milliliter of cleared plasma was incubated with 5 μg of isotype or anti-Tim-3 pAb (catalog number AF2365; R&D Systems)-coupled protein G Dynabeads (Invitrogen) overnight at 4°C. Beads were washed 5 times with 0.2 ml of ice-cold washing buffer (PBS plus 0.2% Tween 20), and bound protein was eluted in lithium dodecyl sulfate (LDS) sample buffer with 50 mM dithiothreitol (DTT) as per the manufacturer's instruction (Invitrogen). Samples were prepared in LDS sample buffer and 50 mM DTT as per the manufacturer's instruction and analyzed via 4-to-12% bis-Tris SDS-PAGE with subsequent Western blotting (WB). Membranes were probed using anti-Tim-3 pAb (R&D Systems) followed by the appropriate light-chain-specific secondary antibody (Jackson ImmunoResearch). Detection was performed using chemiluminescence (Thermo Scientific).

Soluble CD14 ELISA. Plasma samples from subjects used for the sTim-3 ELISA were diluted 1:400. The sCD14 ELISA (R&D Systems) was performed as per the manufacturer's instructions.

Statistical analysis. Prism version 6.0 software (GraphPad Software, La Jolla, CA) was used to determine statistical significance between experimental conditions using a paired Student's *t* test. Statistical analysis for plasma sTim-3 levels was performed using the Mann-Whitney test. For regression analysis, data sets were first subjected to normality tests. For data sets that showed normal distributions, Pearson's correlation coefficients were calculated. For data sets that did not show normal distributions, Spearman's correlation coefficients were calculated.

RESULTS

Tim-3 is shed from the surface of T cells. As previously described by Moller-Hackbarth et al. (33), human Tim-3 was shown to be shed from the surfaces of monocytes by the matrix metalloproteinases ADAM10 and ADAM17, in response to the stimuli PMA and ionomycin. Tim-3 is constitutively expressed on monocytes normally and is weakly expressed on T cells, but it is upregulated on T cells in chronic viral infections (13, 38). We previously showed that Tim-3-expressing T cells respond poorly (i.e., IFN-γ production or proliferation) to cognate antigen (13, 14, 38). We thus initially determined if Tim-3 is shed from T cells *ex vivo* from individuals with chronic HIV infection. PMA/ionomycin are very strong stimuli, which bypass proximal signaling, mimicking second messengers and inducing calcium flux, respectively. To determine if physiological stimulation with antigen could induce Tim-3 shedding on the surface of $CD8^+$ T cells, we stimulated whole PBMCs taken *ex vivo* from treatment-naive HIV chronically infected subjects for 6 hours with a Gag peptide pool (to measure HIV-specific responses) or SEB superantigen (positive control) in the presence or absence of the ADAM10 inhibitor GI254023X, followed by intracellular staining for IFN-γ to assess effector function and Tim-3 coexpression. As shown in Fig. 1A (top panels), stimulation with Gag peptide or SEB in a 6-hour stimulation assay produced the typical functional profile observed for coinhibitory molecules, i.e., the $Tim\text{-}3^+$ and $IFN\text{-}\gamma^+$ populations are mutually exclusive, suggesting that Tim-3-expressing T cells are dysfunctional. However, inclusion of the ADAM10 inhibitor in the 6-hour stimulation assay resulted in Tim-3 retention on responding cells (Fig. 1A, lower panels, and B). This suggested that Tim-3 is shed from the surface of responding cells by ADAM10. This also suggests that some Tim-3-expressing cells are indeed

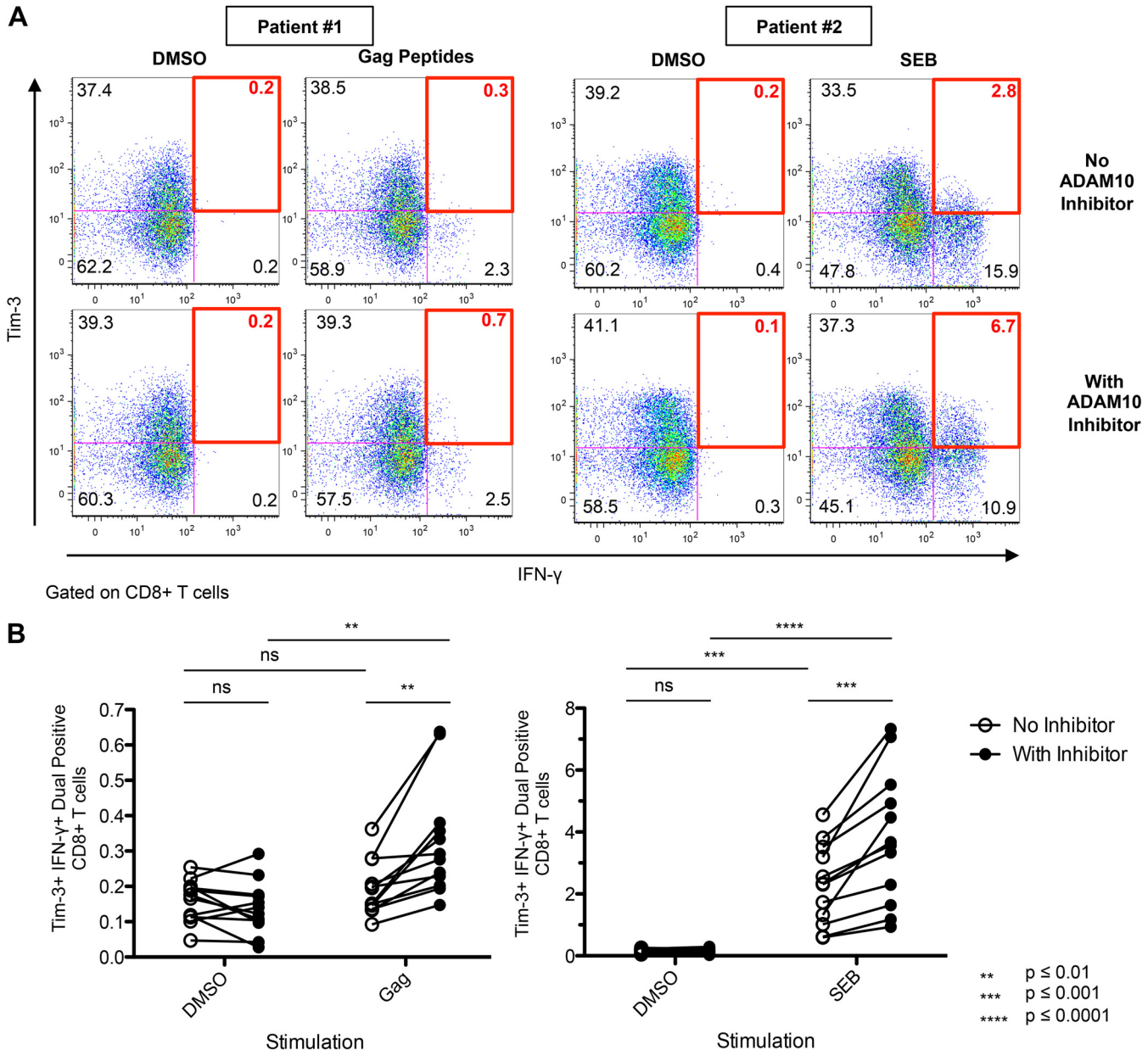


FIG 1 Tim-3 is shed from the surface of responding $CD8^+$ T cells by ADAM10. (A) Tim-3 expression on responding $CD8^+$ T cells. *Ex vivo* PBMCs from chronically HIV-infected viremic subjects were stimulated for 6 h with DMSO, Gag peptides, or SEB, with or without 9 μM ADAM10 inhibitor GI 254023X, followed by analysis of Tim-3 and IFN-γ expression on $CD8^+$ T cells via flow cytometry. Shown are representative dot plots of strong responses from two different subjects. (B) ADAM10 inhibition significantly increases Tim-3 expression on responding $CD8^+$ T cells. Results from 4 independent experiments are shown, comparing changes in frequency of $Tim\text{-}3^+$ $IFN\text{-}\gamma^+$ $CD8^+$ T cells stimulated with DMSO, Gag peptides, or SEB. Shown are matched results from 12 subjects. Statistics were performed using a paired Student *t* test.

functional in terms of IFN-γ production in response to antigen during chronic HIV infection.

To further confirm that Tim-3 is shed during HIV infection, we developed a Tim-3 sandwich ELISA (optimized for culture supernatants and human plasma) by using a combination of Tim-3 monoclonal and polyclonal antibodies and recombinant sTim-3 as a standard (see Fig. S1 in the supplemental material). *In vitro* activation of PBMCs with T cell stimuli (anti-CD3/anti-CD28 or PHA) shown to upregulate Tim-3 (39) or monocyte stimuli (LPS) shown to induce Tim-3 shedding on monocytes (33) resulted in significant increases in sTim-3 detected in culture supernatants (Fig. 2A). Further, we confirmed that Tim-3 and ADAM10 were coexpressed on monocyte and activated T cell populations (Fig. 2B), which could be responsible for sTim-3 production by PBMCs (Fig. 2A). To confirm that ADAM10-mediated cleavage of Tim-3 was responsible for the sTim-3 detected in cultures, we stimulated these isolated monocyte and activated T cell populations with medium (negative control), SEB (T cell receptor [TCR] stimuli), LPS (Toll-like receptor 4 [TLR4] stimuli), or ionomycin (positive control

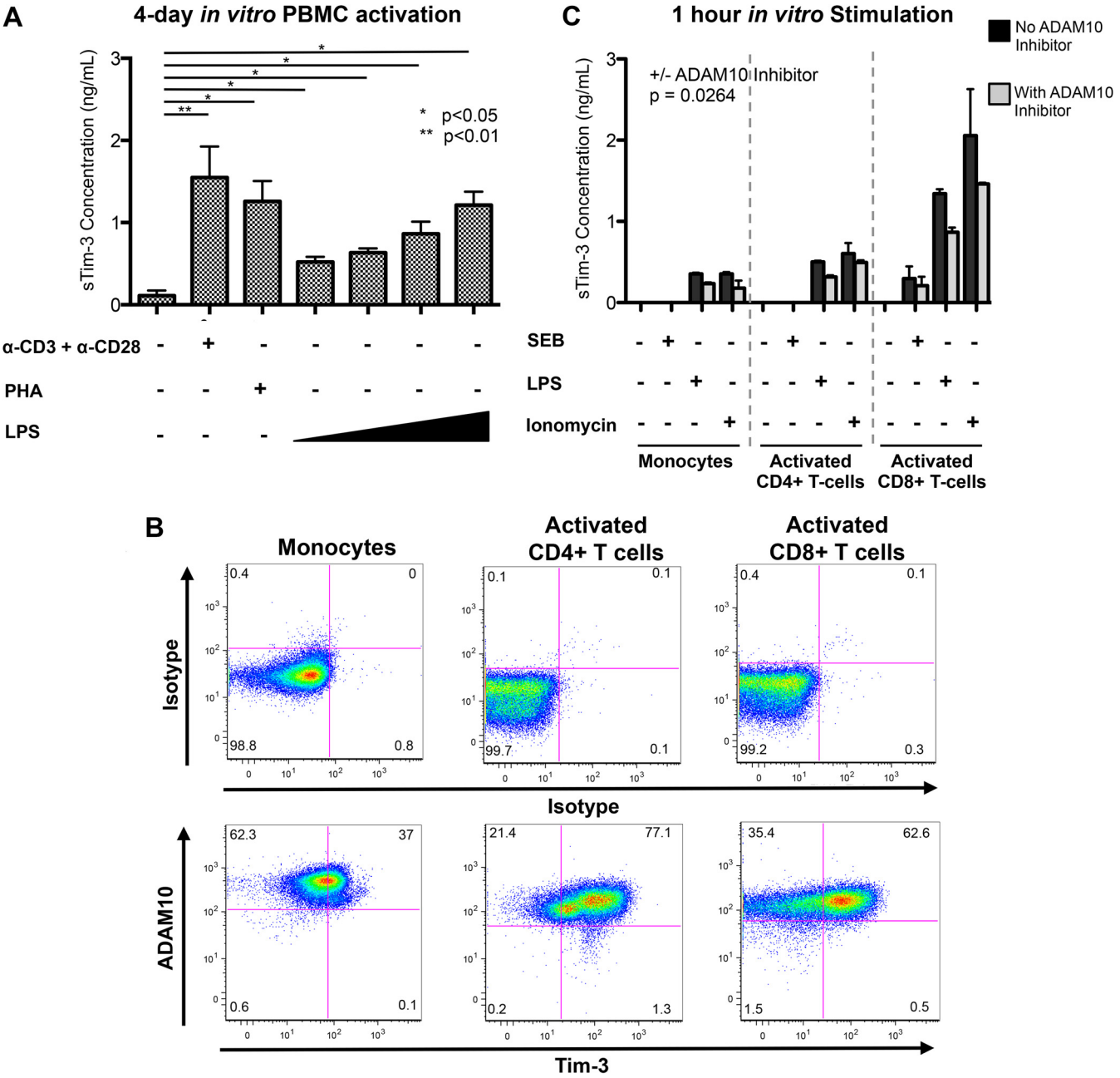
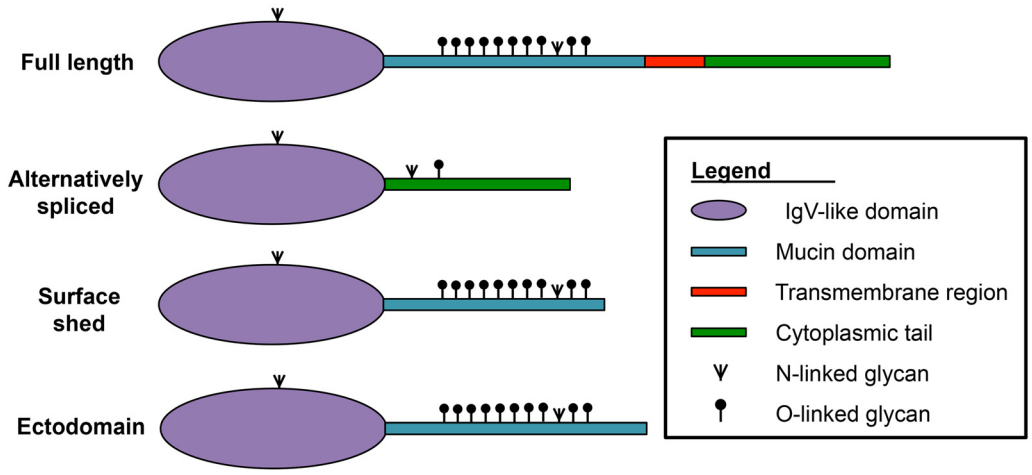


FIG 2 Detection of sTim-3 in culture. (A) Measurements of sTim-3 levels in culture. PBMCs from HIV-uninfected volunteers were activated *in vitro* using media, anti-CD3 and anti-CD28, PHA, or LPS (1, 10, 100, or 1,000 ng/ml) for 4 days. Following activation, the culture supernatants were collected and sTim-3 levels were measured via an sTim-3 ELISA. The bar graph shows the results from 4 independent experiments. Statistical analysis was performed using a paired Student *t* test. (B) Surface Tim-3 and ADAM10 expression on activated T cells and monocytes. *In vitro* activated, isolated $CD4^+$ or $CD8^+$ T cells and isolated *ex vivo* monocytes from an HIV-uninfected volunteer were stained for Tim-3 and ADAM10 and analyzed via flow cytometry. (C) Induction and inhibition of Tim-3 shedding. Activated $CD4^+$ or $CD8^+$ T cells and monocytes were stimulated with medium, SEB (TCR stimulus), LPS (TLR4 stimulus), or ionomycin (ADAM10 stimulus) in the presence of absence of 9 μM ADAM10 inhibitor GI 254023X for 1 h in serum-free medium. The supernatants were collected, and sTim-3 levels were measured via an ELISA. The bar graph shows the results from 3 independent experiments. Statistical analysis was performed using a paired Student *t* test for results with or without ADAM10 inhibitor.

[33]) for 1 h in the presence or absence of the ADAM10 inhibitor (Fig. 2C). Ionomycin treatment yielded the highest levels of sTim-3 in all cell types as expected. The SEB stimulus only yielded sTim-3 production in $CD8^+$ T cells, while the LPS stimulus yielded responses in all cell types. The inclusion of the ADAM10 inhibitor resulted in a significant reduction in the levels of sTim-3 produced across all populations ($P = 0.0264$); however, it did not completely prevent the production of sTim-3. While this could be a dosing issue with the inhibitor, ADAM17 may have contributed to Tim-3 shedding, as previously described (33). However, inhibitors specific for ADAM17 are not currently commercially available. An alternative explanation for the lack of complete shedding inhibition seen in Fig. 2C is that a portion of the sTim-3 detected is alternatively spliced.

A



B

Tim-3 Construct	Predicted Molecular Weight (-glycans)	Number of N-linked Glycans	Number of O-linked Glycan	Predicted Molecular Weight (+ glycan)
Full length	31kDa	2	10	55 – 67 kDa
Alternatively spliced	21kDa	2	1	27 – 30 kDa
Surface shed	20kDa	2	10	44 – 53 kDa
Ectodomain (recombinant sTim-3)	22kDa	2	10	45 – 56 kDa

FIG 3 Tim-3 constructs. (A) Schematic representation of four Tim-3 constructs described in the literature. Sequences of "full-length" human Tim-3 and the Tim-3 "ectodomain" sequences were obtained from Uniprot, the "alternatively spliced" human sequence was based on the mouse construct described by Sabatos et al., and the sequence of the "surface-shed" construct was inferred from Möller-Hackbarth et al. N-linked glycan predictions were made using the NetNGlyc 1.0 server (R. Gupta, E. Jung, S. Brunak, unpublished data), and O-linked glycan predictions were made using the NetOGlyc 4.0 server. (B) Summary of the constructs, the corresponding molecular weights predicted using ExPASy's Protparam tool, number of N-linked and O-linked glycans, and the predicted molecular masses of each glycoprotein, assuming glycans have an average molecular mass of 2 to 3 kDa.

Human sTim-3 is not an alternatively spliced soluble form. Previous work by Sabatos et al. described for mice an alternatively spliced Tim-3 construct lacking the mucin and transmembrane domains (30). However, this construct has never been described in humans. Thus, there exist three potential Tim-3 constructs: full-length, alternatively spliced, and shed Tim-3 (Fig. 3A and B). It is important to characterize which construct represents the sTim-3 produced from human cells in culture, as the alternatively spliced and surface-shed Tim-3 could potentially elicit different functions due to the differences in peptide composition and post-translational modifications C terminal to the IgV-like domain (Fig. 3A). In addition, unlike production of an alternatively spliced isoform, shedding a surface coinhibitory receptor could potentially elicit intrinsic effects on the parent cell, similar to what is seen with LAG-3 (15).

To assess the potential for production of an alternatively spliced sTim-3 construct, we amplified all potential Tim-3 constructs by using 5′ and 3′ UTR primers, equivalent to what was used for the initial discovery of murine sTim-3 (30). A full-length Tim-3 cDNA clone was used as a positive control. Since an alternatively spliced Tim-3 product has not yet been described in humans, we did not have a positive control for this construct. However, Sabatos et al. showed that these two murine constructs share the same 5′ and 3′ UTRs. As shown in Fig. 4A, we amplified a Tim-3 transcript of approximately ~1,000 bp (green arrow) from activated PBMCs, $CD4^+$ and $CD8^+$ T cells and monocytes (the same cells used to produce sTim-3 in culture [Fig. 2]). This represents the full-length Tim-3 construct. No smaller, alternatively spliced Tim-3 construct (predicted to be ~800 bp [30]; red arrow) was amplified from any of the cell populations, suggesting that human PBMCs do not produce an alternatively spliced Tim-3 construct. Further, as shown in Fig. 4B, using Western blotting we confirmed that the sTim-3 produced by activated $CD8^+$ T cells in culture (Fig. 2C) is smaller than full-length Tim-3 (obtained from $CD8^+$ T cell lysates) and is the approximate size of recombinant sTim-3 (Tim-3 ectodomain; green arrow). No smaller Tim-3 with the predicted molecular weight of alternatively spliced Tim-3 (red arrow) was observed. Following up on studies describing a soluble form of Tim-3 in plasma (32), we performed a Tim-3 immunoprecipitation on plasma from a treatment-naive chronically HIV-infected subject. As shown in Fig. 4C, a Tim-3 construct was observed at the approximate molecular weight of the Tim-3 ectodomain (green arrow), similar to the shed Tim-3 in Fig. 4B. Further, no construct was observed at the predicted molecular weight of alternatively spliced Tim-3 (red arrow). Thus, these results suggest that the sTim-3 observed in culture supernatants and in plasma is surface-shed Tim-3, not an alternatively spliced soluble form.

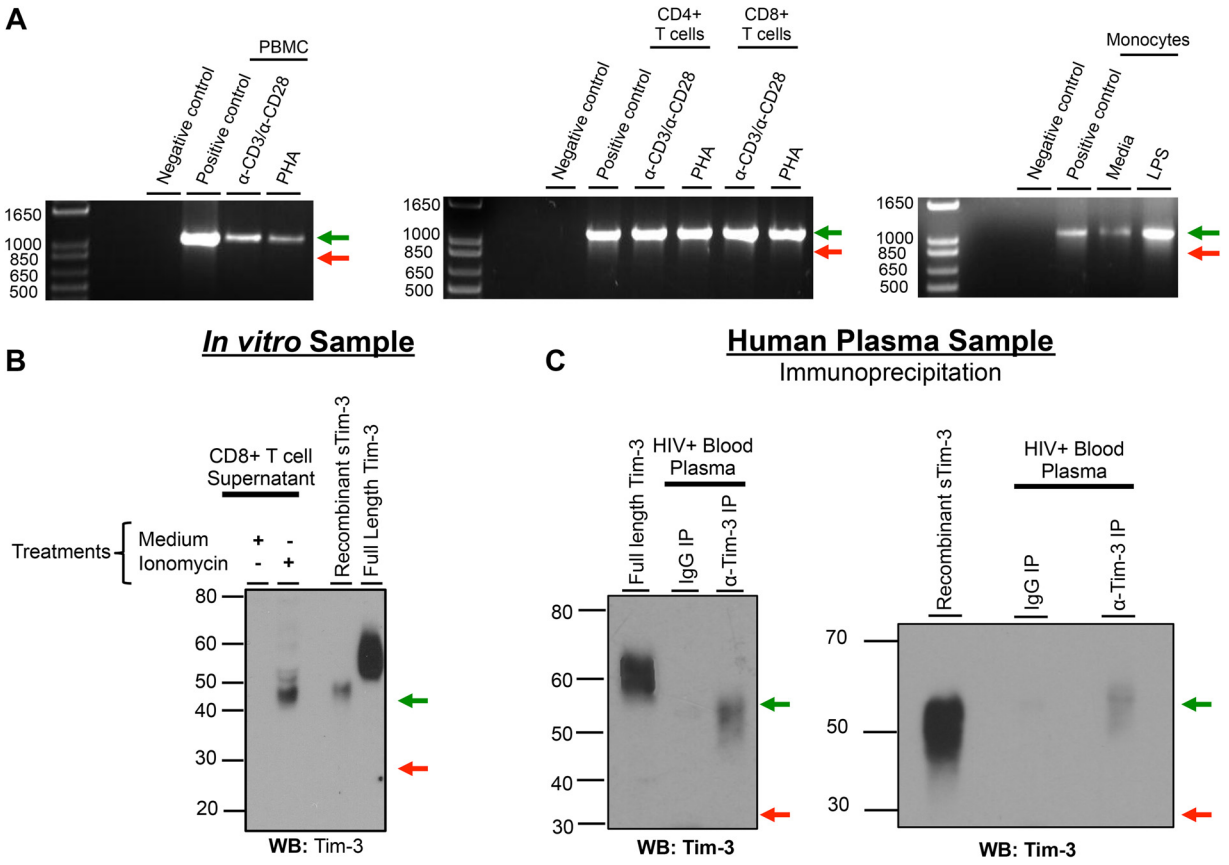


FIG 4 Characterization of sTim-3 in culture and in plasma. (A) Amplification of Tim-3 transcripts. PBMCs or isolated $CD4^+$ T cells, $CD8^+$ T cells, and monocytes (Fig. 2) were activated for 4 days with the appropriate stimulus to upregulate Tim-3. cDNA created from cellular mRNA was used to amplify Tim-3 transcripts using primers directed against the 5′ and 3′ UTRs. PCR products were separated on a 1% agarose gel, and DNA was visualized using ethidium bromide. The predicted full-length Tim-3 amplicon is approximately 1,000 bp (green arrow), while the predicted alternatively spliced Tim-3 amplicon is approximately 800 bp (red arrow). (B) Analysis of the shed sTim-3. Concentrated $CD8^+$ T cell supernatants used in the ELISA (shown in Fig. 2C) were analyzed via SDS-PAGE with subsequent Tim-3 Western blotting (WB). Tim-3^+ $CD8^+$ T cell lysate containing full-length Tim-3 and 50 ng/ml of recombinant sTim-3 were run for size comparisons. The green arrow denotes the predicted size of shed sTim-3 (the approximate size of recombinant sTim-3), while the red arrow denotes the predicted size of alternatively spliced sTim-3 (Fig. 3). The results shown are representative of 3 independent experiments. (C) Immunoprecipitation (IP) of sTim-3 from patient plasma. Immunoprecipitated Tim-3 protein from chronically HIV-infected subject plasma was analyzed via SDS-PAGE and Tim-3 WB. Tim-3^+ $CD8^+$ T cell lysate, containing full-length Tim-3, and 50 ng/ml of recombinant soluble Tim-3 were run for size comparisons (left blot and right blot, respectively). The green and red arrows denote predicted sizes of shed sTim-3 and alternatively spliced sTim-3, respectively, as in panel B. Results are representative of 3 independent experiments.

sTim-3 is elevated in HIV infection. While we confirmed that a shed, sTim-3 construct is found in human plasma, it remained to be determined if this sTim-3 plays a role in HIV disease pathogenesis. We obtained plasma samples from three different cohorts of HIV-infected and uninfected subjects covering 6 different clinical categories (Table 1), including treatment-naive acute HIV infection (acute), treatment-naive early HIV infection (early), treatment-naive chronic HIV infection (chronic naive), HAART-treated chronic HIV infection (chronic treated), treatment-naive chronic HIV infection with elite viral control (elite controller), and demographically matched HIV-seronegative subjects (HIV-uninfected; see Materials and Methods for more details on clinical classifications). Using the ELISA, we measured the plasma sTim-3 level in each subject and compiled results from the three cohorts (Fig. 5A). sTim-3 was significantly elevated in the acute (median, 10.2 ng/ml; interquartile range [IQR], 9.1 to 12.0 ng/ml), early (13.4 ng/ml; IQR, 9.0 to 24.8 ng/ml), and chronic naive (12.2 ng/ml; IQR, 7.8 to 19.6 ng/ml) patient groups compared to the HIV-uninfected control group (7.4 ng/ml; IQR, 5.6 to 9.0 ng/ml) (Fig. 5A). Chronic treated (8.3 ng/ml; IQR, 6.4 to 11.9 ng/ml) and elite controller (7.1 ng/ml; IQR, 6.4 to 13.9 ng/ml) groups were not statistically different from the HIV-uninfected control. Furthermore, sTim-3 levels from the chronic naive group were significantly higher than the chronic treated group ($P = 0.0165$) and there was trending toward significantly higher levels for the elite controller group ($P = 0.0770$). These results suggest that sTim-3 is involved in HIV disease pathogenesis or serves as a biomarker of disease progression.

sTim-3 is associated with parameters of HIV disease progression. With simple regression analysis, sTim-3 levels were positively correlated with HIV viral loads among all viremic patient groups ($P = 0.0200$) (Fig. 5B), suggesting that antigen could be driving sTim-3 production. Given our results in Fig. 2A that suggested that physiological levels (1 to 100 ng/ml) of LPS may contribute to production of sTim-3, we attempted to measure plasma LPS, a marker of bacterial translocation known to be elevated in

TABLE 1 Characteristics of study subjects

Patient classification parameter[a] and cohort	Value for HIV infection status group					
	Negative	Acute	Early	Chronic naive	Chronic treated	Elite controller
No. of samples						
Toronto cohort	13		15	22	5	
SCOPE cohort	16			16	19	18
Blood Systems cohort		19	13			
Median mos. infected (range)						
Toronto cohort	NA[b]		<6	53 (10–400)	26 (7–302)	
SCOPE cohort	NA			>12	>12	>12
Blood Systems cohort		<6	<6			
HAART treated?						
Toronto cohort	NA		No	No	Yes	
SCOPE cohort	NA			No	Yes	No
Blood Systems cohort		No	No			
Median (range) viral load (copies/ml)						
Toronto cohort	NA		25,947 (485–94,617)	62,628 (287–500,000)	39 (39–49)	
SCOPE cohort	NA			42,700 (13,633–500,000)	40 (40–75)	49 (40–75)
Blood Systems cohort		32,000 (54–2,800,000)	270,000 (2,500–1,600,000)			
Median (range) CD4 count (cells/μl)						
Toronto cohort	NA		720 (290–1,130)	405 (190–890)	600 (340–1,160)	
SCOPE cohort	NA			562 (368–1,088)	752 (433–1,693)	808 (518–1,850)
Blood Systems cohort		ND[c]	ND			

[a] Patient classifications are described in Materials and Methods.
[b] NA, not applicable.
[c] ND, not determined.

HIV-infected subjects (40–42). However, available assays to measure plasma LPS yielded inconsistent and variable results (data not shown), as previously reported (43). Thus, we measured plasma sCD14, a surrogate marker for plasma LPS (42). No correlation was found between plasma sTim-3 and sCD14 ($P = 0.7235$) (Fig. 5C), suggesting that even though physiological levels of LPS induced shedding *in vitro*, this mechanism may not contribute to sTim-3 production *in vivo*. Finally, sTim-3 was negatively correlated with $CD4^+$ T cell counts of chronic HIV-infected treatment-naive subjects ($P = 0.0294$) (Fig. 5D), suggesting that sTim-3 is correlated with disease progression.

Functional effects of Tim-3 shedding. Despite sTim-3's correlation with HIV disease progression, it is unclear if it actually contributes to pathogenesis. There are two possible functions Tim-3 shedding could play. First is the intrinsic effect of shedding, i.e., modulation of T cell signaling and function following shedding of Tim-3. As a coinhibitory molecule, one would predict that shedding Tim-3 would result in enhanced T cell function, as is seen with LAG-3 shedding (15). Second is the extrinsic effect of shedding, i.e., the potential for sTim-3 to exhibit a cytokine-like function, modulating function of other cells of the immune system, as is seen with sCEACAM1 (9, 16). While we have previously shown that recombinant sTim-3 is able to enhance HIV-specific T cell responses (13), it is unclear if this endogenous sTim-3 exhibits the same function.

Tim-3 was originally characterized as a coinhibitory molecule which modulated IFN-γ production (20), induced apoptosis when stimulated with its ligand galectin-9 (28), and inhibited proliferation (13). Thus, to characterize the intrinsic effects of Tim-3 shedding, we first assessed changes in total levels of IFN-γ with or without the ADAM10 inhibitor. Analyzing the same data described in Fig. 1, we found there was no significant difference in the amount of total IFN-γ produced in response to either Gag peptides or SEB (Fig. 6A and B). Second, we assessed the apoptotic potential of SEB-responding cells. Using Annexin-V to bind to plasma membrane outer leaflet phosphatidylserine (an early marker of apoptosis), we found that the $Tim\text{-}3^+$ and $Annexin\text{-}V^+$ populations were mutually exclusive (Fig. 7A), suggesting that $Tim\text{-}3^+$ cells do not succumb to spontaneous apoptosis. Since we showed that the addition of the ADAM10 inhibitor significantly increased the frequency of responding $Tim\text{-}3^+$ $CD8^+$ T cells (Fig. 1), we assessed the apoptotic potential of SEB-responding cells. We found that inhibiting Tim-3 shedding via an ADAM10 inhibitor did not significantly alter the "spontaneous" apoptotic potential of SEB-responding $CD8^+$ T cells (Fig. 7B and C). In fact, of the responding cells, the majority of the $Annexin\text{-}V^+$ cells were still $Tim\text{-}3^-$ (Fig. 7B, right, first and third panels). Hypothesizing that galectin-9 is needed to enhance Tim-3's putative apoptotic function, we included 125 nM galectin-9 in the last hour of stimulation. While this significantly enhanced apoptosis, there was still no difference between ADAM10-treated and untreated samples (Fig. 7B). Finally, we assessed differences in $CD8^+$ T cell proliferation. Following long-term stimulation with SEB or plate-bound anti-CD3/anti-CD28, we discovered that the ADAM10 inhibitor killed the T cells (data not shown). Thus, given current reagent limitations, we were not able to assess the effects of Tim-3 shedding on

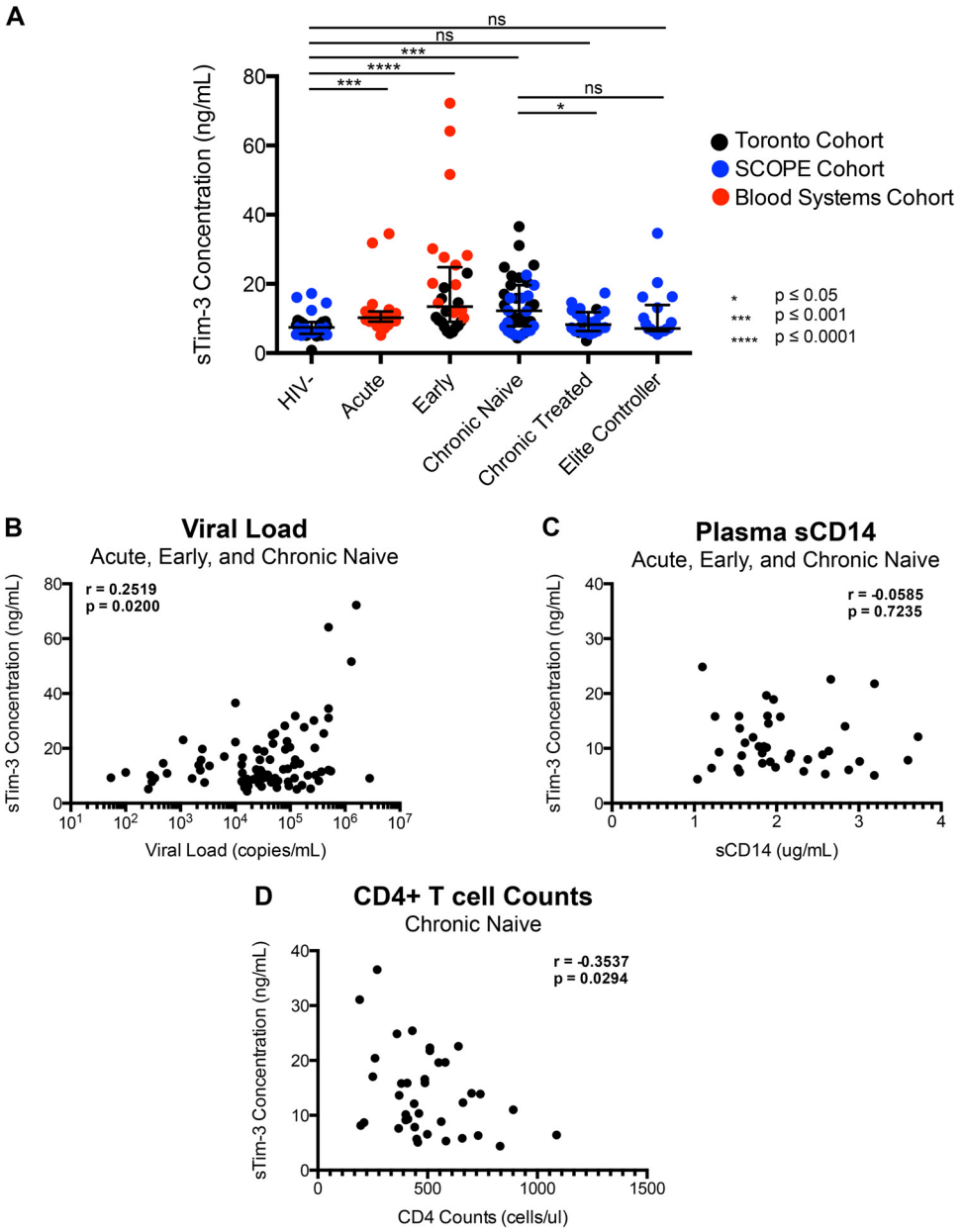


FIG 5 Quantification of sTim-3 in human plasma. (A) Measurement of sTim-3 levels in plasma from HIV-infected subjects. An sTim-3 ELISA was used to quantify the levels of sTim-3 in the plasma of HIV-infected acute, early, chronic-naive, chronic-treated, elite controller subjects and HIV-uninfected volunteers. Shown are the medians and the interquartile ranges. Statistics were performed using the Mann-Whitney test. (B) Correlation between sTim-3 levels and the viral load for HIV-infected acute, early, and chronic naive subjects. (C) Correlation between sTim-3 and sCD14 levels for HIV-infected acute, early, and chronic naive subjects. (D) Correlation between sTim-3 levels and $CD4^+$ T cell counts for HIV-infected chronic naive subjects. Statistical analyses for correlations were performed using the Spearman's correlation test for viral load (B); Pearson's correlation test was used for sCD14 (C) and $CD4^+$ T cell count (D) data.

proliferation, and it is still unclear what intrinsic effects Tim-3 shedding has on T cell function.

To assess the extrinsic effects of sTim-3, we attempted to isolate endogenous sTim-3 from large-scale *in vitro* culture as well as from human plasma to use in *in vitro* stimulation assays. Using Tim-3 monoclonal and polyclonal antibodies, we were able to immunoprecipitate Tim-3 from culture supernatants and plasma. However, despite our best efforts, we were unable to efficiently perform a native elution of the protein; only denaturing elution removed the sTim-3 from the column (data not shown). Thus, we were unable to determine what effect endogenous sTim-3 has on T cell responses. While we could infer that it acts similar to recombinant sTim-3, these proteins are not identical (Fig. 3). Further experiments assessing the effects of endogenous sTim-3 are warranted.

DISCUSSION

Tim-3 plays a functional role in T cell exhaustion during chronic HIV infection (13). Our study provides novel insight into the regulation of Tim-3 surface expression on stimulated $CD8^+$ T

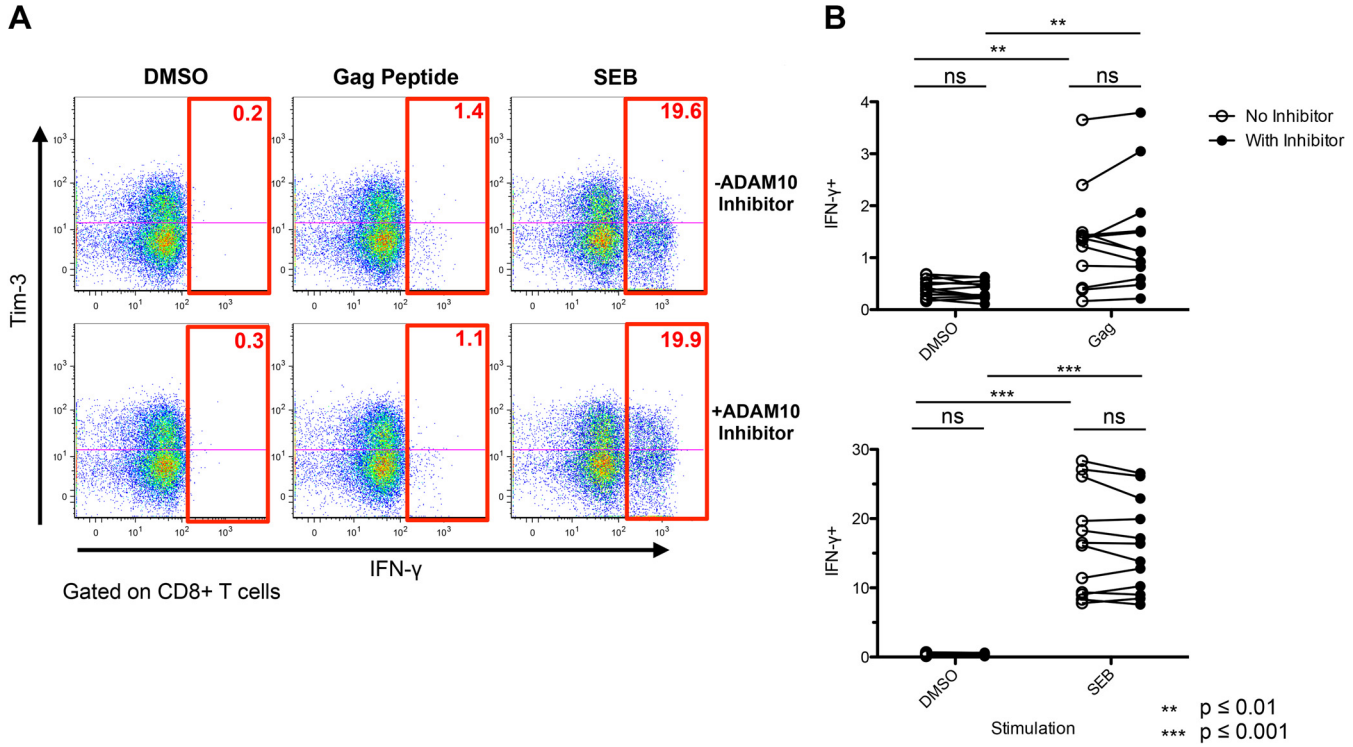


FIG 6 Intrinsic functional effects of Tim-3 shedding on IFN-γ production. (A) Total IFN-γ production. *Ex vivo* PBMCs from chronically HIV-infected viremic subjects were stimulated for 6 h with DMSO, Gag peptides, or SEB with or without 9 μM ADAM10 inhibitor GI 254023X, followed by analysis of total IFN-γ expression on $CD8^+$ T cells via flow cytometry. Shown are representative dot plots from 4 independent experiments. (B) ADAM10 inhibition does not affect IFN-γ production. Results from 4 independent experiments, comparing changes in the frequency of total IFN-γ^+ $CD8^+$ T cells stimulated with DMSO, Gag peptides, or SEB. Shown are matched results from 12 patients. Statistics were performed using a paired Student *t* test.

cells. We have provided evidence to show that Tim-3^+ cells are able to respond by shedding their Tim-3 from the surface via matrix metalloproteinase ADAM10. Thus, responding cells appear Tim-3 negative as a consequence of shedding. Further, via a novel sTim-3 ELISA, we confirmed the presence of a soluble Tim-3 construct in human plasma, which represents the by-product of Tim-3 shedding and not of an alternatively spliced isoform, as previously reported in mice (30). Plasma sTim-3 is significantly elevated during early and chronic untreated HIV infection and correlates with disease progression. This suggests that sTim-3 (or Tim-3 shedding) may play a role in HIV disease pathogenesis and could potentially be used as a novel marker of HIV disease progression. To address this, we assessed the intrinsic and extrinsic effects of shedding. Surprisingly, Tim-3 shedding did not modulate IFN-γ expression in response to stimuli, nor did it preferentially protect the cells from spontaneous or galectin-9-induced apoptosis. Further work is needed to completely delineate the intrinsic and extrinsic effects of Tim-3 and its role in disease pathogenesis.

One of the most surprising results from this study is that Tim-3 was shed from responding $CD8^+$ T cells (Fig. 1). ADAM10 is stimulated after successful T cell activation (44). This suggests that Tim-3^+ cells are able to undergo proximal TCR signaling, enough to induce effector function and activate ADAM10 to induce Tim-3 shedding. However, we also observed Tim-3^+ cells that remain unresponsive. This could be explained by lack of specificity for the peptide/superantigen or, alternatively, by a lack of overall proximal signal strength through the TCR resulting in a lack of cytokine production and ADAM10-mediated Tim-3 shedding. Whether Tim-3 (as a coinhibitory molecule) contributes to this TCR signal blockade is still unknown; however, if this were the case, then one would hypothesize that inhibiting Tim-3 shedding also inhibits cytokine production. This was not the case, as seen in Fig. 6. This does not exclude the possibility that Tim-3 may inhibit other pathways linked to other T cell activities, such as proliferation. Indeed, previous work by our lab showed that blocking the Tim-3 pathway enhanced T cell proliferation (13). While we did attempt to assess the effect of Tim-3 shedding on proliferation, we discovered that the ADAM10 inhibitor was toxic to the cells over a longer period of time (5 days) (data not shown). The use of an ADAM10 inhibitor is not ideal for these experiments, as ADAM10-mediated cleavage is not exclusive toward Tim-3; the matrix metalloproteinase is able to cleave multiple substrates (15, 36, 45–51). Ideal future experiments should employ Tim-3 knockout $CD8^+$ T cells, which ectopically express a wild-type or a cleavage-deficient mutant Tim-3 (33), in order to assess the effect of Tim-3 shedding on proliferation, similar to what has been performed with LAG-3 (15).

Initial characterization of Tim-3 and its ligand, galectin-9, showed that the interaction induced apoptosis of T cells (28). Hypothesizing that shedding of Tim-3 protects responding $CD8^+$ T cells from apoptosis, we added galectin-9 to the cells during the last hour of SEB stimulation. While we performed similar stimulations with Gag peptides in parallel, galectin-9 treatment decreased the sensitivity of the assay. Thus, only strong IFN-γ responses (elicited by SEB) could be detected in the presence of

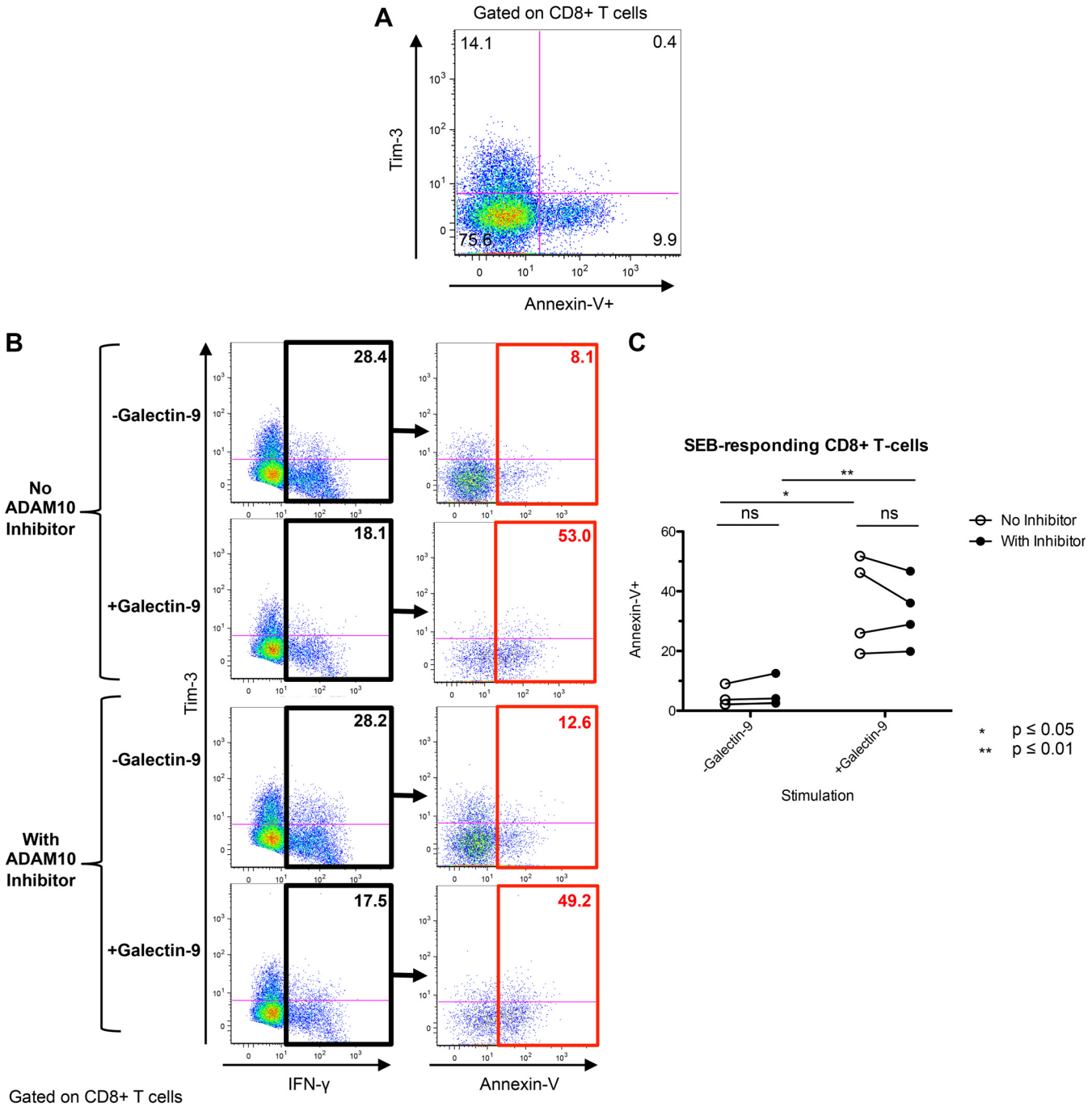


FIG 7 Intrinsic functional effects of Tim-3 shedding on galectin-9 mediated apoptosis. (A) Spontaneous apoptotic potential of Tim-3$^+$ CD8$^+$ T cells. *Ex vivo* PBMCs from a chronic naive HIV-infected subject were stimulated with SEB for 6 h, followed by assessment of Tim-3 expression and apoptotic potential via Annexin-V staining. (B) Apoptotic potential of responding cells. *Ex vivo* PBMCs from chronic naive HIV-infected subjects were stimulated for 6 h with DMSO or SEB with or without 9 μM ADAM10 inhibitor GI 254023X, with or without 125 nM recombinant galectin-9 for the last hour of stimulation. The SEB response was measured via IFN-γ expression on CD8$^+$ T cells, while apoptotic potential was measured via surface Annexin-V staining and subsequent flow cytometry. Shown are representative dot plots from 2 independent experiments. (C) ADAM10 inhibition does not affect total apoptotic potential. Results from 2 independent experiments comparing changes in frequency of Annexin-V on SEB-responding cells (gated on IFN-γ$^+$). Shown are matched results from 4 patients. Statistics were performed using a paired Student *t* test.

galectin-9. In the absence of galectin-9, the majority of SEB-responsive cells exhibiting "spontaneous" apoptosis were Tim-3 negative (Fig. 7A). This was in contrast to what is seen with PD-1 (52). Furthermore, inhibiting Tim-3 shedding did not enhance total apoptosis of the SEB-responding T cell population (Fig. 7B and C). Adding galectin-9 dramatically enhanced apoptosis; however, inhibiting Tim-3 shedding did not enhance galectin-9-mediated apoptosis. Initially, this was surprising, since blocking both

Tim-3 and galectin-9 with antagonistic antibodies has been shown to reduce galectin-9-mediated apoptosis (53). However, in our assays, we found that only the galectin-9 antibody (clone 9M1-3), not the Tim-3 antibody (clone 2E2), blocked apoptosis (unpublished observations). Indeed, galectin-9 is able to bind to multiple immune accessory molecules (37, 54, 55) and induce apoptosis in a Tim-3-independent manner (54). Discrepancies in these studies could be due to the *in vitro* culture systems used; here, we assessed responses in human primary *ex vivo* $CD8^+$ T cells. Thus, these results suggest that for primary human $CD8^+$ T cells, Tim-3^+ cells do not undergo spontaneous apoptosis. Further, Tim-3 shedding does not protect responding cells from spontaneous or galectin-9-induced apoptosis.

Extrinsic effects of sTim-3 greatly depend on the construct that is produced. Indeed, murine studies characterizing sTim-3 have shown that the alternatively spliced construct is able to extrinsically inhibit T cell responses (31); however, this construct has never been described in humans. More recently, Moller-Hackbarth et al. described a surface-shed Tim-3 ectodomain; however, the extrinsic function of this sTim-3 was not characterized (33). Further, while a soluble Tim-3 construct was detected via mass spectrometry in human plasma, it was unclear which construct, the alternatively spliced or surface-shed construct, was represented. We confirmed that sTim-3 produced from primary, human immune cells is not an alternatively spliced isoform but exclusively a by-product of Tim-3 surface shedding (Fig. 4). In addition, this construct represents the sTim-3 found in human plasma. Knowing the type of construct will help us delineate its extrinsic function. We attempted to isolate the construct from culture supernatants and plasma to assess its effects on HIV-specific immune responses; however, we were unable to natively purify the protein (data not shown). While isolation of native protein is ideal, future studies could focus on expression of N-terminally tagged Tim-3 in cell lines, followed by ionomycin-induced shedding and subsequent tag purification of the protein to use in culture assays.

In theory, sTim-3 could act as a decoy to block the Tim-3 pathway and enhance HIV-specific immune responses, similar to the results of functional studies using recombinant sTim-3 performed by Jones et al. (13). However, the presence of the soluble form of a receptor does not necessarily result in blockade of the receptor pathway. For example, ADAM10/ADAM17-induced shedding of LAG-3 has no extrinsic effect on the immune system (15). Li et al. showed that sLAG-3 was unable to bind to major histocompatibility complex class II antigen (MHC-II) and was therefore unable to block the full-length LAG-3 or CD4 from binding to MHC-II. In contrast to LAG-3, Tim-3 has multiple ligands, including phosphatidylserine, CEACAM1, galectin-9, and HMGB1, the latter two being soluble proteins. While it is unclear which ligand interaction signals through Tim-3 on T cells to induce negative signaling, sTim-3 has the potential to "mop up" these soluble proteins, or block phosphatidylserine or CEACAM1 interactions, reducing their bioavailability to Tim-3 as well as other receptors. Indeed, this may prove beneficial, as levels of plasma galectin-9 are significantly elevated in HIV infection and correlate with disease progression (34).

Given Tim-3's role in T cell exhaustion and HIV disease pathogenesis, we sought to delineate sTim-3's role in HIV infection. The findings that plasma sTim-3 was significantly increased during acute, early, and chronic untreated HIV infection, but not during chronic treated infection or in elite controllers, suggest that viral load may drive sTim-3 production. Indeed, viral load was significantly correlated with sTim-3 levels during these stages of HIV infection (Fig. 5B). This was in contrast to plasma sCD14, a surrogate marker for plasma LPS (42), which did not correlate with sTim-3 (Fig. 5C). This was surprising, as we showed that physiological levels of LPS were able to induce production of sTim-3 *in vitro* (Fig. 2A). However, sCD14 is only a surrogate marker for LPS; levels of the two are not perfectly correlated (42). Thus, in the future, when more-accurate LPS assays become available, it would be prudent to test the correlation of plasma sTim-3 with plasma LPS. The source of plasma sTim-3 from our studies suggests the source is primarily from T cell activation rather than LPS stimulation of monocytes/macrophages, but future studies are needed to confirm this. Further, we showed that sTim-3 levels were negatively correlated with $CD4^+$ T cell counts during untreated chronic HIV infection (Fig. 5D), suggesting that at the very least, sTim-3 could be a new marker for HIV disease progression.

The discovery of shed forms of coinhibitory receptors raises questions about the function of shedding in terms of T cell exhaustion. While ADAM10/ADAM17-mediated shedding requires signaling induced by TCR (44), it is unclear what TCR signaling threshold is required for shedding and whether exhausted T cells can shed coinhibitory receptors. Indeed, high expression levels of some coinhibitory receptors (LAG-3 and Tim-3) on the surface of exhausted cells may be a result of accumulation due to lack of shedding. While shedding may improve T cell responses, it may also contribute to chronic immune activation and progression to AIDS, particularly if there is viral epitope escape (44). While our results suggest that sTim-3 correlates with HIV disease progression, it is still unclear how Tim-3 shedding or sTim-3 itself affects immune responses. In terms of shedding, it would be prudent to first determine Tim-3 function. While multiple studies have shown that Tim-3 is coinhibitory, others have shown that Tim-3 can be costimulatory (56, 57). Indeed, our results suggest that a population of Tim-3^+ cells can signal and induce effector function (Fig. 1). Thus, it is possible that these $CD8^+$ T cells shed their costimulatory Tim-3, rendering them less functional. These findings highlight the complexities of the role of Tim-3 in immune responses in chronic infection and will require further investigation.

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