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Antigen-Independent Induction of Tim-3 Expression on Human T Cells by the Common γ-Chain Cytokines IL-2, IL-7, IL-15, and IL-21 Is Associated with Proliferation and Is Dependent on the Phosphoinositide 3-Kinase Pathway

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T cell Ig mucin domain-containing molecule 3 (Tim-3) is a glycoprotein found on the surface of a subset of CD8+ and Th1 CD4+ T cells. Elevated expression of Tim-3 on virus-specific T cells during chronic viral infections, such as HIV-1, hepatitis B virus, and hepatitis C virus, positively correlates with viral load. Tim-3−cytotoxic T cells are dysfunctional and are unable to secrete effector cytokines, such as IFN-γ and TNF-α. In this study, we examined potential inducers of Tim-3 on primary human T cells. Direct HIV-1 infection of CD4+ T cells, or LPS, found to be elevated in HIV-1 infection, did not induce Tim-3 on T cells. Tim-3 was induced by the common γ-chain (γc) cytokines IL-2, IL-7, IL-15, and IL-21 but not IL-4, in an Ag-independent manner and was upregulated on primary T cells in response to TCR/CD28 costimulation, as well as γc cytokine stimulation with successive divisions. γc cytokine-induced Tim-3 was found on naive, effector, and memory subsets of T cells. Tim-3− primary T cells were more prone to apoptosis, particularly upon treatment with galectin-9, a Tim-3 ligand, after cytokine withdrawal. The upregulation of Tim-3 could be blocked by the addition of a PI3K inhibitor, LY 294002. Thus, Tim-3 can be induced via TCR/CD28 costimulation and/or γc cytokines, likely through the PI3K pathway.

Chronic infections are associated with an inability of T cells to clear the pathogen, despite high levels of immune activation. Mechanisms of immune evasion include viral escape via epitope mutations, as well as the induction, directly or indirectly, of negative immune regulatory proteins, such as programmed death 1 (PD-1), CTLA-4, and T cell Ig mucin domain-containing molecule 3 (Tim-3), on the surface of Ag-specific T cells (1–3). Overexpression of these molecules on effector T cells dampens immunological control against infected cells, manifested by the failure of T cells to proliferate in response to Ag and by an inability to secrete effector cytokines, such as IFN-γ and TNF-α, in some cases leading to apoptosis.

Tim-3, an Ig superfamily member that was initially identified as a specific cell surface marker of murine Th1 CD4+ T cells (4). A putative ligand for Tim-3 in mice is galectin-9, a β-galactoside–binding lectin (5). Interaction of murine Tim-3 with its ligand, galectin-9, regulates Th1 responses by promoting the death of IFN-γ–producing Th1 cells (5). Tim-3 was identified as a negative regulator of T cells and evaluated in models of autoimmunity. In mice, blocking the interaction of Tim-3 with its ligand(s) abrogated experimental autoimmune encephalomyelitis (5). Subsequent work in human autoimmune diseases, such as rheumatoid arthritis (6) and multiple sclerosis (7), showed associations between a decreased frequency of Tim-3+ T cells and unchecked expansion of autoreactive T cells. Increased frequency of Tim-3+ T cells is reported in tuberculosis (8). We and other investigators subsequently showed that the chronic virus infections HIV-1 (9) and hepatitis C virus (10, 11) are also associated with upregulation of Tim-3 on T cells. In addition, elevated expression of Tim-3 on virus-specific T cells correlated with increased viral load and disease progression (8–11). Subsequent studies showed that Tim-3 expression is also upregulated on tumor-infiltrating T cells, thus providing an additional mechanism of tumor escape from the immune response (12). Thus, Tim-3 is a critical negative regulator of T cell activation.

In addition to its expression on Th1 T cells, Tim-3 can be found on CD8+, Th17 CD4+, and NKT cells, as well as monocytes, splenic dendritic cells, and activated macrophages (4, 13, 14). TCR engagement via CD3 and CD28 costimulation in vitro was demonstrated to induce Tim-3 expression on human CD4+ T cells, suggesting that antigenic contact with the TCR is an essential component of Tim-3 upregulation (14). However, although expression of Tim-3 is particularly elevated on HIV-1–specific T cells, these make up only a small minority of circulating T cells in the periphery: ~6.3% of total CD8+ and 0.7% of total CD4+ T cells (15). Hence, the observation that >50% of total CD8+ and >30% of total CD4+ T cells in the peripheral blood of HIV-1–infected subjects can express Tim-3 (9) cannot be accounted for by the direct stimulation of HIV-1–specific T cells by viral Ags. The mechanism behind this global upregulation of Tim-3 on bulk CD4+ and CD8+ T cells during chronic viral infection is poorly understood. To elucidate the pathway behind Tim-3 upregulation...
in chronic HIV-1 infection, we first postulated that a viral gene product could be driving Tim-3 expression. Indeed, HIV-1 protein Nef was shown to directly induce the expression of another exhaustion marker, PD-1, on the surface of HIV-1–infected CD4+ T cells (16). We hypothesized that viral factors, such as HIV-1 gp120, found in the serum of infected individuals, could upregulate Tim-3 by binding to certain receptors on the surface of T cells, such as the CD4 receptor, or that soluble HIV-1 Tat diffusing into T cells to drive HIV-1 replication could also be indirectly responsible (17). We also posited that the soluble bacterial endotoxin LPS, which is elevated in the plasma of acute and chronically HIV-1–infected individuals as a result of microbial translocation (18), could drive Tim-3 upregulation on the majority of CD8+ and CD4+ T cells. Lastly, we hypothesized that some other soluble inflammatory factor could be behind the Tim-3 expression witnessed during immune activation and tested the common γ-chain (γc) cytokines on T cells from individuals not infected with HIV-1. Indeed, IL-7, IL-15, and IL-21 were reported to be elevated in blood plasma during the course of chronic infections (19–23) and, thus, are likely candidates behind global Tim-3 upregulation. Finally, to investigate the signaling pathways involved in the Tim-3 expression pathway, we treated T cells with drug inhibitors to PI3K, MAPK-activated protein kinase 2 (MAPKAPK2), and MEK1 under strong Tim-3–inducing conditions.

Materials and Methods

Study participants
Healthy HIV-1–seronegative human volunteers were recruited for blood specimens. HIV-1–infected individuals’ blood was collected from patients of the Maple Leaf Medical Clinic. PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden). Informed consent was obtained in accordance with the guidelines for conduct of clinical research at the University of Toronto and St. Michael’s Hospital institutional ethics boards.

HIV-1 infection
CD4+ T cells from PBMC were isolated by negative selection (StemCell Technologies, Vancouver, BC, Canada) and plated at 2 × 10^6/ml with 20 U/ml IL-2 for 2 d. Then cells were washed and infected with HIV-1 NL4-3 (multiplicity of infection 0.2) (National Institutes of Health, Bethesda, MD) derived from transfection of 293T cells. Virus was magnetically labeled prior to infection with Viromag beads (OZ Biosciences, Marseille, France). Infection of CD4+ T cells was carried out per Soucha and Watkins (24). Cells were then stained at various time points following the infection with fluorochrome-labeled Abs for PD-1 (BioLegend, San Diego, CA), CD4 (BD Biosciences, San Diego, CA), Tim-3 (clone 344823; R&D Systems, Minneapolis, MN), and HIV-1 Gag (Kc57-RD1; Beckman Coulter, Miami, FL). Stained samples were run on the BD FACS Calibur flow cytometer, and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Cellular stimulation
PBMCs or negative selection–enriched CD4+ and CD8+ T cells (StemCell Technologies) were cultured at 1 × 10^6/ml in RPMI 1640 with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 20 mM L-glutamine (Wisent, Saint-Bruno, QC, Canada) final concentrations. Cells were stimulated with complete medium alone or with IL-2 (R&D Systems) at 0.04–125 ng/ml, IL-4 (R&D Systems) at 25 ng/ml, IL-10 at 25 ng/ml, IL-15 (R&D Systems) at 0.04–125 ng/ml, IL-21 (Invitrogen, Carlsbad, CA) at 25 ng/ml, staphylococcal enterotoxin B (SEB; Sigma-Aldrich, Germany) at 2 µg/ml, anti-CD3/CD28 (anti-CD3 clone OKT3, anti-CD28 clone CD28.2; eBioscience, San Diego, CA) at 1 µg/ml each (unless otherwise stated), or LPS (Sigma-Aldrich) at 4 µg/ml. All cells were cultured at 37°C with 5% CO₂.

γc receptor-neutrophil studies
An anti-human common γc receptor-neutrophilizing Ab (clone 633162; R&D Systems) was used at 10 µg/ml final concentration in cultures in the presence of Tim-3 inducers to assess Tim-3 induction’s dependence on the γc cytokines.

Phenotyping
Tim-3 was detected using the monoclonal anti-human Tim-3 Ab (clone 344823, R&D Systems). T cells were identified using anti-human CD4+ and CD8+ Abs (BD Biosciences). Rat IgG2a isotype control (clone R35-95; BD Biosciences) was used against the Tim-3 mAb. Memory phenotypes of T cells were identified using anti-human CD62L+ and CD45RA+ Abs (BD Biosciences). IFN-γ production was assessed by IFN-γ Ab (BD Biosciences). Allophycoerythrin-conjugated Annexin V (BD Biosciences) was used for apoptosis studies. All Abs were fluorochrome labeled. Stained samples were run on a BD FACS Calibur or BD FACS Canto II flow cytometer, and data were analyzed using FlowJo software.

Proliferation
PBMCs were stained with final 1 µM CellTrace CFSE (Invitrogen) and were plated at 1 × 10^6/ml in medium. Cells were stimulated individually with IL-2, IL-15, or anti-CD3/CD28 and cultured for 6 d. Samples were then stained with fluorescently labeled Abs to identify CD8+, CD4+, and Tim-3–expressing T cells to assess proliferation of T cells and their Tim-3 expression. Data were acquired on a BD FACS Calibur and analyzed using FlowJo software.

IFN-γ production of Tim-3+ T cells
PBMCs were stimulated with IL-2 or IL-15 at 25 ng/ml for 6 d. The cells were then washed and restimulated with 2 µg/ml SEB, and 1 µg/ml GolgiPlug/brefeldin A (BD Biosciences) was added for 12 h. Following this stimulation, samples were stained with fluorescent Abs to identify CD4+, CD8+ T cells, Tim-3, and IFN-γ. Data were acquired on a BD FACS Calibur and analyzed using FlowJo software.

Production and purification of human galectin-9
The medium isomorphism of human galectin-9 (UNIPROT ID: O00182-2) with a C-terminal 3XFLAG tag was codon optimized and subcloned into a pET15b vector. The resulting construct contained an N-terminal 6XHIS tag with a thrombin cleavage site and a C-terminal 3XFLAG tag. The plasmid was transformed into ArcticExpress (DE3) Competent cells (Agilent Technologies, Palo Alto, CA), which were grown per the manufacturer’s instructions to OD⁶⁰⁰nm of 0.3–0.4. The cells were induced with 0.5 mM isopropyl β-D-thiogalactoside and incubated at 12°C for 24 h. Cells were harvested and lysed using bacterial protein extraction reagent (Thermo Scientific, Rockford, IL) supplemented with lysozyme and DNase, as per the manufacturer’s instructions. The lysate was cleared via centrifugation at 25,000 × g for 30 min at 4°C. Functional galectin-9 was isolated using α-lactose agarose (Sigma-Aldrich) and eluted competitively using 200 mM α-lactose. Polishing purification was performed using a Superdex 75 (GE Healthcare) via AKTA HPLC. A purity > 90% was obtained, and endotoxin was <1 EU/µg protein.

Apoptosis studies
PBMCs were cultured in medium with or without 25 ng/ml IL-2, 25 ng/ml IL-15, or 1 µg/ml each anti-CD3/CD28 for 3 d at 1 × 10^6/ml. Samples were washed extensively (three times), and soluble galectin-9 was administered to the cultures at a final concentration of 62.5 nM. An equivalent volume of a “blank” buffer control for galectin-9 was added to a separate set to serve as a negative control. Samples were stained for CD8+, Annexin V, and Tim-3 8 h after galectin-9 treatment and run on a BD FACS Calibur, and data were analyzed on FlowJo software.

Drug-inhibition studies
PBMCs were cultured in medium with or without 25 ng/ml IL-2, 25 ng/ml IL-15, or 1 µg/ml anti-CD3/CD28 and were cultured at 1 × 10^6/ml for 6 d in the presence of either DMSO control (0.94% final) or one of the three inhibitory drugs, which were dissolved in DMSO (25): SB 203580 (Sigma-Aldrich), a p38 inhibitor (10⁻⁶ M); PD 98059 (Sigma-Aldrich), an ERK inhibitor (10⁻⁷ M); or LY 294002 (Sigma-Aldrich), a PI3K inhibitor (10⁻⁶ M). Following the 6-d culture period, samples were stained with CD8+, CD4+, and Tim-3 fluorochrome-conjugated Abs, and data were acquired on a BD FACS Calibur and analyzed using FlowJo software.

Statistical analyses
Data were analyzed with the two-tailed paired Student t test using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA).
Results

HIV-1 does not directly induce Tim-3 expression

We hypothesized that soluble HIV-1 viral products could induce Tim-3 expression on CD4+ T cells in an Ag-independent manner. Elevated Tim-3 levels on T cells could potentially negatively impact the immune system’s response toward all pathogens. Indeed, another exhaustion marker, PD-1, which exhibits characteristics on T cells similar to those that express Tim-3 in the acute and chronic phases of HIV-1 infection (3, 26–28) was reported to be upregulated by the HIV-1 protein Nef (16). Thus, we determined the effect of in vitro HIV-1 infection on CD4+ T cells’ PD-1 or Tim-3 expression up to 72 h postinfection (Fig. 1). Tim-3 and PD-1 levels of HIV-1–infected CD4+ T cells were compared with uninfected and bystander cells (HIV-1 Gag–cells within the same culture) at various time points. PD-1, but not Tim-3, was modestly upregulated on HIV-1 NL4-3–infected CD4+ T cells compared with bystander cells in the same culture (at 72 h postinfection, *p = 0.0384 for PD-1, *p = 0.4514 for Tim-3). Thus, Tim-3 expression in HIV-1–infected individuals is likely an indirect result of HIV-1 infection.

LPS does not induce Tim-3 expression

Bacterial endotoxin, LPS, levels were described to be significantly elevated following HIV-1 infection in the gastrointestinal tract and are associated with the systemic immune activation observed in gut tissue during acute and chronic stages of HIV-1 infection as a result of microbial translocation (18). We asked whether LPS could elevate Tim-3 levels on T cells between various time points. PD-1, but not Tim-3, was modestly upregulated on HIV-1–infected CD4+ T cells compared with bystander cells within the same culture (Fig. 1). Thus, the trend was similar for CD8+ T cells (6.1 ± 0.7% versus 7.0 ± 0.6%; *p = 0.3598). Thus, there was no significant upregulation of Tim-3 in response to LPS treatment (Fig. 2A).

The common γc cytokines IL-2, IL-7, IL-15, and IL-21 drive Tim-3 expression on CD4+ and CD8+ T cells in vitro

We then postulated that some other soluble factors, found to be elevated in the blood plasma of chronic HIV-1–infected subjects, could drive Tim-3 expression on T cells during systemic immune activation. The common γc cytokines, including IL-7, IL-15, and IL-21, were reported to be above normal levels in chronic HIV-1 infection (19–23) and, thus, were potential candidates behind Tim-3 induction on T cells. To further understand Tim-3 upregulation in the context of immune responses, PBMCs from healthy donors were stimulated with various cytokines, including members of the common γc family, and Tim-3 expression was analyzed on CD4+ and CD8+ T cells following a 6-d period of stimulation compared with cells cultured in plain medium alone (CD4+, 5.6 ± 2.9%; CD8+, 6.1 ± 5.1%) (Fig. 2A). T cells stimulated with the γc cytokines IL-2 (CD4+, 33.2 ± 10.8%; *p = 0.0004; CD8+, 65.9 ± 10.1%; *p < 0.0001; compared with no treatment), IL-7 (CD4+, 26.0 ± 11.6%; *p = 0.0011; CD8+, 36.2 ± 20.6%; *p = 0.0011), IL-15 (CD4+, 42.6 ± 13.9%; *p = 0.0005; CD8+, 79.3 ± 8.5%; *p < 0.0001), and, to a lesser extent, IL-21 (CD4+, 19.8 ± 3.7%; *p = 0.0050; CD8+, 23.0 ± 5.9%; *p = 0.0040) exhibited significantly elevated frequencies of Tim-3+ T cells (Fig. 2A, 2B) along with TCR-stimulated T cells via CD3 and CD28 costimulation (CD4+, 67.4 ± 4.3%, *p < 0.001; CD8+, 83.5 ± 8.2%, *p < 0.001) as was reported on CD4+ T cells (14). The γc cytokine IL-4 did not affect Tim-3 expression (CD4+, 8.3 ± 2.1%, *p = 0.8584; CD8+, 7.4 ± 1.8%, *p = 0.1747), which is consistent with this cytokine’s role in the maintenance and polarization of Th2 cells and suppression of the Th1 response, as well as the observation that Tim-3 is preferentially expressed on Th1 cells (4). The induction of Tim-3 expression by cytokines was more marked on CD8+ T cells. Representative flow plots for PBMC stimulation and Tim-3 expression with isotype control are shown in Fig. 2B.

Similarly, enriched CD4+ and CD8+ T cells from six normal volunteers were stimulated with IL-2, IL-7, IL-15, and anti-CD3/CD28 as above, and an increase in the frequency of Tim-3–expressing cells and Tim-3 mean fluorescence intensity (MFI) was observed (Fig. 2C, 2D). Isolated T cells stimulated with the common γc cytokines IL-2 (CD4+, 11.5 ± 3.0%; *p = 0.0005; CD8+, 38.5 ± 11.8%; *p = 0.0007; compared with no treatment), IL-7 (CD4+, 14.9 ± 6.6%; *p = 0.0051; CD8+, 20.7 ± 2.7%; *p = 0.0004), and IL-15 (CD4+, 25.8 ± 12.7%; *p = 0.0077; CD8+, 63.5 ± 8.5%, *p < 0.0001) exhibited elevated frequencies of Tim-3+ T cells in vitro compared with the untreated cells. Similarly, TCR stimulation (CD4+, 44.2 ± 10.47%; *p = 0.0035; CD8+, 50.7 ± 15.9%, *p = 0.0417) yielded a greater proportion of Tim-3–expressing T cells compared with untreated cells. An increase in the level of Tim-3 expression, as shown by MFI, compared with medium-alone T cells was also observed on isolated CD4+ and CD8+ T cells following treatment with the γc cytokines IL-2 and IL-15, as well as anti-CD3/CD28. Again, Tim-3 expression was more pronounced on CD8+ T cells.

The γc cytokines IL-2 and IL-15 and anti-CD3/CD28 stimulation were the most potent inducers of Tim-3 on T cells and, thus, were studied further. We analyzed Tim-3 expression on T cells in a kinetics study over a 6-d period. The frequency of Tim-3+ cells gradually increased with time with cytokine stimulation alone, as observed (Fig. 2C, 2D). Isolated T cells stimulated with a suboptimal dose (0.01 μg/ml) of IL-2, IL-7, IL-15, and anti-CD3/CD28 each, alone or in the presence of either IL-2 or IL-15 each at 25 ng/ml, yielded a greater proportion of Tim-3–expressing T cells compared with untreated cells. An increase in the level of Tim-3 expression, as shown by MFI, compared with medium-alone T cells was also observed on isolated CD4+ and CD8+ T cells following treatment with the γc cytokines IL-2 and IL-15, as well as anti-CD3/CD28. Again, Tim-3 expression was more pronounced on CD8+ T cells.

The addition of anti-CD3/CD28 costimulation with the γc cytokines IL-2 or IL-15 resulted in a significantly increased frequency of Tim-3+ CD8+ T cells within PBMCs (Fig. 2G). PBMCs were cultured with a suboptimal dose (0.01 μg/ml) of anti-CD3 and anti-CD28 Abs each, alone or in the presence of either IL-2 or IL-15 each at 25 ng/ml. A suboptimal dose was preferred so that anti-CD3/CD28 Abs did not directly induce Tim-3 expression. Isolated CD4+ T cells were infected in vitro with HIV-1 strain NL4-3; Tim-3 and HIV-1 Gag levels were assessed at various time points by flow cytometry. Tim-3 (left panel) and PD-1 (right panel) expression was compared between bystander (HIV-1 Gag+) cells and infected cells (HIV-1 Gag+) within the same population, as well as infected versus uninfected cells. HIV-1 infection data are not shown. Mean and SD from three independent experiments are shown. *p < 0.05.
FIGURE 2. Cytokine-mediated induction of Tim-3 expression on T cells contained in PBMCs and on purified T cells. (A) Total PBMCs were treated with 25 ng/ml IL-2, 25 ng/ml IL-4, 25 ng/ml IL-7, 25 ng/ml IL-10, 25 ng/ml IL-15, 25 ng/ml IL-21, or 1 μg/ml each anti-CD3/CD28 for 6 d and Tim-3 expression was assessed on CD4+ and CD8+ T cells. Data sets represent the mean and SD from nine independent experiments for the cytokines IL-2, IL-7, and IL-15, as well as for anti-CD3/CD28 stimulation; four independent experiments were performed for IL-4, IL-21, and LPS; and two experiments were performed for the cytokine IL-10. The p values refer to comparison with untreated cells. (B) Representative flow plots of cytokines that induced significant upregulation of Tim-3 in (A) on CD8+ T cells within PBMCs are shown with isotype control on anti-CD3/CD28–stimulated cells. CD8+ T cells within lymphocytes were gated with flow cytometry analysis, and their Tim-3 expression was assessed with each of the treatments. (C and D) The common γc cytokines IL-2, IL-7, and IL-15 directly induced Tim-3 expression on purified T cells. Purified T cells were stimulated with 25 ng/ml of all cytokines that resulted in significant Tim-3 expression from (A) and anti-CD3/CD28. Tim-3+ cell frequency (C) and MFI (D) in CD4+ and CD8+ subsets in purified T cells are shown. Data sets represent the mean and SD from six independent experiments. The p values refer to comparison with untreated cells. (E) Kinetics of cytokines IL-2 and IL-15 (each at 25 ng/ml) and anti-CD3/CD28–induced Tim-3 expression on T cells within PBMCs (CD8+ (Figure legend continues)}
CD3/CD28 stimulation alone would not induce Tim-3 expression to 100% of the CD8+ T cells within PBMCs. Tim-3 levels were assessed 4 d after stimulation. Compared with cells that received anti-CD3/CD28 stimulation alone, cells that received IL-2 displayed a greater frequency of Tim-3+ CD8+ T cells (49.7 ± 10.2% and 61.3 ± 7.4%, respectively; \( p = 0.015 \)). A similar observation was made with IL-15 and cells stimulated with 0.01 µg/ml anti-CD3/CD28; Tim-3+ frequency increased to 67.0 ± 8.4% (\( p = 0.007 \)). Thus, cells that received both anti-CD3/CD28 co-stimulation and γc cytokine exhibited an increased frequency of CD8+ Tim-3+ T cells.

**Common γc cytokine-mediated Tim-3 induction is abrogated by a γc cytokine receptor-neutralizing Ab**

We were able to diminish cytokine-stimulated induction of Tim-3 expression on CD8+ T cells within PBMCs in the presence of an anti-human common γc receptor Ab at 10 µg/ml while culturing with the γc cytokines IL-2, IL-7, and IL-15 (Fig. 2H). Compared with the PBMCs that did not receive this neutralizing Ab, CD8+ T cells cultured for 6 d with the γc cytokines at 25 ng/ml displayed a reduced frequency of Tim-3+ cells with each cytokine treatment. The reduction in Tim-3 expression was most clearly evident in the IL-2 treatments, in which Tim-3 expression was reduced from 64.8% without neutralizing Ab to 26.5% (\( p = 0.004 \)) with it. Similar trends were observed for IL-7 (30.0 to 25.4%, \( p = 0.021 \)) and IL-15 (83.2 to 63.1%, \( p = 0.043 \)). TCR-stimulated cells via anti-CD3/CD28 treatment were unaffected with regard to Tim-3 frequency, despite the addition of the γc-neutralizing Ab (\( p = 0.238 \)). This suggests that γc cytokine stimulation is sufficient for Tim-3 upregulation on T cells and that TCR-dependent signals upregulate Tim-3 expression, irrespective of γc cytokine signaling.

**Tim-3 can be upregulated on naive, effector, and memory subsets of T cells**

To define the particular subsets of T cells that upregulate Tim-3 in response to IL-2, IL-15, or anti-CD3/CD28 treatments, CD4+ and CD8+ T cells were further characterized into naïve (CD45RA+CD62L+), effector (CD45RA+CD62L−), central memory (CD45RA−CD62L+), and effector memory (CD45RA−CD62L−) subsets by flow cytometry, and the levels of Tim-3 were analyzed for each fraction. Differential Tim-3 expression of Tim-3 on either of the subsets would provide insight into the stage of T cell maturation. Tim-3 would act on a cell to negatively regulate their effector functions (e.g. late-stage effector T cells versus naive T cells). Interestingly, we observed significantly elevated levels of Tim-3 on each subset of the cells stimulated with cytokine for 6 d compared with cells in medium alone (Fig. 3), suggesting that Tim-3 can potentially function as an immune regulator at all stages of T cell maturation. Following TCR stimulation, we were unable to detect any naive or effector T cells as the result of downregulation of CD45RA.

**Tim-3 expression increases on proliferated T cells**

We hypothesize that Tim-3 expression is tied to exhaustion on T cells after multiple rounds of proliferation and failure to suppress infection in vivo. To examine this correlation between proliferation status and Tim-3 expression, PBMCs from healthy donors were stained with CFSE and stimulated with the cytokines IL-2 or IL-15 or anti-CD3/CD28. Tim-3 expression was analyzed on non-proliferating, CFSEhigh, and proliferating cells, as demonstrated by CFSE dilution in the CFSEmid and CFSELow populations (Fig. 4). CFSEmid T cells have undergone two rounds of proliferation, whereas the CFSELow population represents T cells that underwent at least three rounds of proliferation. Tim-3 was dramatically upregulated on proliferated cells (CFSEmid and CFSELow) compared with the nonproliferating cells (CFSEhigh) within the same population with each of our treatments, and this comparison was used to determine statistical significance. The \( p \) values were compared between CFSEhigh and CFSEmid fractions, as well as between CFSEmid and CFSELow fractions, for each treatment. Tim-3 expression was greatest in the CFSELow fraction, and its frequency was significantly elevated compared with the CFSEmid population. The CFSEmid population exhibited a significantly greater frequency of Tim-3+ T cells compared with the CFSEhigh subset. This result suggests that Tim-3 is upregulated successively on T cells undergoing multiple rounds of replication. Culture of cells in plain medium alone did not stimulate T cell proliferation. Therefore, Tim-3 expression is induced on cells undergoing multiple rounds of proliferation.

**γc cytokine-driven Tim-3+ T cells are weakly responsive to SEB stimulation**

Tim-3 expression on T cells, as described previously (9, 10), marks exhausted cells that are unable to produce IFN-γ upon antigenic stimulation. We note similar characteristics whereby cytokine-driven Tim-3+ T cells failed to produce IFN-γ upon SEB stimulation (Fig. 5A) in vitro. Briefly, PBMCs from healthy donors were stimulated with 25 ng/ml IL-15 for a 6-d period, treated with SEB and brefeldin A for 12 h, and analyzed for IFN-γ production by flow cytometry. The majority of CD8+ T cells that produced IFN-γ in response to SEB was found in the Tim-3+ fraction. Previous work showed that short-term treatment of ex vivo T cells from HIV-1–infected individuals with IL-2 and IL-15 could enhance IFN-γ production of these cells (29). As described before in chronic untreated HIV-1 infection, ex vivo T cells exhibit an increased frequency of Tim-3–expressing T cells compared with uninfected individuals (9). We asked whether short-term treatment of ex vivo T cells from untreated chronically infected individuals with IL-2 or IL-15 could reduce Tim-3 expression or increase cytokine expression on Tim-3–expressing cells. We stimulated PBMCs directly with IL-2 (25 or 100 ng/ml) or IL-15 (25 ng/ml) for 2 h and then stimulated cells for 6 h with 2 µg/ml SEB, in the presence of 1 µg/ml brefeldin A, to induce IFN-γ production. We observed that, although total CD8+ T cells increased IFN-γ production (Fig. 5C), the majority of cytokine expression was still in the Tim-3− fraction (as previously reported (9) compared with Tim-3+ cells (Fig. 5B); ~90% of IFN-γ production came from Tim-3+ cells (Fig. 5B). Also, short-term, 2-h cytokine treatment did not affect the expression level of Tim-3. Thus, prior short-term treatment with the γc cytokines did not reverse the exhausted...
phenotype of Tim-3+ CD8+ T cells seen in ex vivo samples, as measured by their ability to produce IFN-γ. Tim-3+ T cells are more susceptible to apoptosis via galectin-9 ligation
Tim-3 ligation on Th1 cells by its ligand, galectin-9, has been associated with apoptosis induction in Tim-3–expressing cell lines, but this has not been tested in primary cells (5). We tested whether the ligation of Tim-3 with galectin-9 on primary T cells would result in enhanced apoptosis of cells with γc cytokine-driven Tim-3 expression (Fig. 6). Briefly, cells from normal human volunteers were given various stimuli to induce Tim-3 expression for a 3-d period, washed thrice to remove cytokines, and then treated for 8 h with either 62.5 nM recombinant human galectin-9 or equivalent volume of a “blank” buffer negative control. A period of 3 d was selected so that the frequencies of Tim-3+ and Tim-3− cells were comparable, given that longer cytokine stimulation would induce Tim-3 expression on the majority of T cells in culture as per this study. These cells were then analyzed for phosphatidylserine (PS) levels via Annexin V staining on Tim-3+ and Tim-3− CD8+ T cells in both groups (Fig. 6). Cells that received galectin-9 displayed PS at significantly elevated levels within the Tim-3+ fraction compared with the Tim-3− population, indicating greater apoptotic activity upon ligation. However, Tim-3+ cells, also exhibited a greater tendency toward apoptosis versus the Tim-3− cells, irrespective of the addition of exogenous galectin-9. We did not measure endogenous galectin-9 in these experiments.

To better understand the signaling pathways involved in Tim-3 upregulation, we cultured PBMC with inhibitory drugs specific to MAPKAPK2 (SB 203580), MEK1 (PD 98059), and PI3K (LY 294002)-signaling pathways while driving Tim-3 expression on T cells, as described earlier, via the γc cytokines IL-2 and IL-15 or anti-CD3/CD28. We found that LY 294002, the PI3K inhibitor, was most efficient at blocking Tim-3 expression upon stimulation (Fig. 7A). DMSO control-treated T cells were compared with each inhibitory drug in their corresponding conditions. We observed the greatest reductions in Tim-3 expression on CD4+ and CD8+ T cells in the anti-CD3/CD28 TCR-stimulated cultures between DMSO control (CD4+, 46.1 ± 13.4%; CD8+, 71.3 ± 3.8%) and the PI3K inhibitor LY 294002 (CD4+, 7.6 ± 5.4%, p = 0.0094 compared with DMSO control; CD8+, 13.2 ± 7.7%, p < 0.001). The two other drugs, MAPKAPK2 inhibitor SB 203580 (CD4+, 42.1 ± 8.8%, p = 0.1829; CD8+, 68.6 ± 0.7%, p = 0.3223) and MEK1 inhibitor PD 98059 (CD4+, 39.3 ± 7.0%, p = 0.1272; CD8+, 63.2 ± 8.7%, p = 0.2845) were not statistically significant in dampening TCR-mediated Tim-3 induction. Similar findings were observed with IL-2 and IL-15 treatments, with greatest reductions in Tim-3 expression using the PI3K inhibitor LY 294002.
PI3K activation has been shown after CD28 signaling (30). We stimulated isolated CD8+ T cells from healthy individuals’ PBMCs with anti-CD3, anti-CD28, or anti-CD3/CD28. We observed that anti-CD28 treatment alone on isolated CD8+ T cells minimally induced Tim-3 expression to a level over the back-
ground untreated T cells. Anti-CD3 treatment alone induced Tim-3 expression; however, combined anti-CD3/CD28 stimulation had a synergistic effect on Tim-3 expression (Fig. 7B).

**Discussion**

Our study provides further insight into the mechanism of the regulation of the T cell exhaustion marker, Tim-3. We find that Tim-3 can be upregulated through stimulation of the TCR and is linked to proliferation, with increasing levels observed on cells that have undergone multiple rounds of expansions. This is consistent with the notion that chronic antigenic stimulation leading to multiple rounds of proliferation would successively upregulate Tim-3 on Ag-specific T cells. We find that Tim-3 can also be upregulated independently of TCR or antigenic stimulation if cells are provided an inflammatory environment rich in cytokines, specifically IL-2, IL-7, IL-15, and IL-21. In fact, Tim-3 could be upregulated on T cells of any differentiation state in vitro, including naive cells, suggesting that stimulation through IL-2Rα/IL-15Rα/β/γ may be sufficient to upregulate Tim-3. This suggests that in inflammatory states, where IL-2 and IL-15 are being produced, Tim-3 is upregulated, possibly to dampen the inflammatory process. Tim-3 was originally described as a Th1-related molecule and was recently shown to be upregulated on cells that have turned on the Th1 transcriptional protein T-bet (31). We find that Tim-3 is more easily induced on CD8+ T cells compared with CD4+ T cells in human samples, consistent with previous ex vivo studies (9). We also report that expression of Tim-3 via γc cytokines was markedly higher in T cells within PBMCs as opposed to isolated T cells. This might have to do with cytokine signaling in the presence of other cell types. For example, effective signaling by IL-15 requires the cytokine to be trans-presented to a T cell by another accessory cell (32). Furthermore, we were able to diminish γc-induced Tim-3 expression on T cells in the presence of a γc cytokine receptor-neutralizing Ab, proving a direct role in these cytokines’ ability to upregulate Tim-3 expression on T cells. T cells stimulated with submaximal doses of anti-CD3/CD28 plus γc cytokines had greater Tim-3 induction compared with stimulation through TCR or γc alone, suggesting that, although these two pathways individually are sufficient for Tim-3 induction, they can also function in an additive manner.

Our observations that IL-2 and IL-15 were the most potent inducers of Tim-3 expression on T cells are consistent with the role...
of these cytokines in Th1 immunity, as well as the importance of Tim-3 in regulating Th1 responses. The upregulation of Tim-3 in response to prolonged IL-15 or IL-2 stimulation indicates an important negative regulatory role of Tim-3 in cells responding to these cytokines. Although Tim-3 was shown to mark T cells that have lost the ability to produce cytokines, we now show that γc cytokines, such as IL-2 and IL-15, are capable of inducing Tim-3 to dampen the effector functions of the cell. This is observed in the case of IFN-γ secretion and Tim-3 expression, and our findings are consistent with the previously proposed roles of Tim-3 expression on T cells (33). In our study, we observed increased Tim-3 expression on T cells belonging to the CFSEHigh or undivided cells fraction, especially in response to TCR stimulation and marginally to the γc cytokine-stimulated cells, suggesting that proliferation alone was not responsible for the elevated levels of Tim-3 observed in our cultures. Multiple signaling pathways are induced in T cells during stimulation through the TCR and γc chain cytokines (38). We found that we could most potently block cytokine- or TCR-induced Tim-3 expression by blocking the PI3K pathway. For antigenic (TCR)
stimulation, costimulation through CD28 was shown to activate PI3K, which results in the phosphorylation of Akt. Our findings suggested that CD28 signaling may be important in Tim-3 upregulation during Ag stimulation. We observed that CD28 signaling induced a synergistic effect on Tim-3 upregulation compared with CD3 stimulation alone. Many signaling pathways induce PI3K activation, although the level of PI3K activation and the downstream effector molecules vary depending on the signaling pathway. TCR cross-linking/ligation can activate PI3K, in part through Lck (39) and Gab2 adapter proteins (40). CD28 can directly activate PI3K through recruitment of p85 subunit (41). Also, it was shown that PI3K activation is greatest with CD3/CD28 costimulation (42), indicating that there is likely a threshold at which Tim-3 is upregulated. These findings suggest that, although CD28 costimulation is generally believed to enhance function and proliferation of T cells, counter-regulatory pathways that induce Tim-3 can also be triggered.

IL-2 and IL-15 signaling results in STAT5 and STAT3 phosphorylation, whereas IL-4, which was shown not to upregulate Tim-3, activates STAT6 and STAT3 but not STAT5 (32, 43). Thus, our findings suggest that γc cytokine signaling-mediated STAT5 phosphorylation plays a role in Tim-3 upregulation. Recently, it was shown that p-STAT5 can activate Akt through PI3K at the cell surface, suggesting a direct effect of STAT5 on PI3K induction (44).

FIGURE 6. Tim-3+ cells are prone to apoptosis upon treatment with galectin-9. PBMCs were treated with medium, the common γc cytokines IL-2 or IL-15 (25 ng/ml), or anti-CD3/CD28 (1 μg/ml each) for 3 d. Then PBMCs were washed thrice in PBS and treated with either a final concentration of 62.5 nM human galectin-9 or with a blank buffer negative control of equivalent composition as the galectin-9 for 8 h. CD8+ T cells within the PBMCs were analyzed for PS levels via Annexin V staining and compared between the Tim-3+ and the Tim-3− fractions. (A) PS MFI s on CD8+ T cells given various stimuli on three healthy individuals’ (not HIV-1–infected) PBMCs: OM620, OM630, and OM672. (B) Representative flow plots of OM672 CD8+ T cells within PBMCs treated with blank buffer (left panels) or galectin-9 (right panels) in Tim-3+ (bottom panels) and Tim-3− (upper panels) fractions that were stimulated with anti-CD3/CD28.
In addition, p-STAT5 has putative protein binding sites with the DNA binding cofactors p100 and p300 (44), the latter of which also have putative binding sites in the promoter region of Tim-3 (45). IL-2, IL-15, and IL-21 were reported to increase T-bet mRNA in T cells (46–48). For IL-2, this mechanism involves an increase in the levels of p-STAT5, which, in turn, induces expression of T-bet (48). Tim-3 expression is regulated directly by T-bet (31). Although IL-15 and IL-21 also increase IFN-γ production (46), it is interesting to note that, through the induction of Tim-3 via T-bet, these cytokines can play a counter-regulatory role as well. PI3K was also shown to interact with the adaptor protein GAB2, which is phosphorylated by Jak3 (49). Jak3 is critical for common γc cytokine signaling (50) and, therefore, serves as another pathway for IL-2Rγmediated Tim-3 expression. In another possible role for PI3K-associated Tim-3 expression, active Akt was shown to restore T-bet expression and Th1 differentiation in rictor (an essential subunit of mTOR complex 2)-deficient mice (51), suggesting that PI3K signaling induces Tim-3 expression via the expression of T-bet. Future studies will examine the interactions among STAT5, T-bet, and GAB2 signaling on Tim-3 expression via the PI3K pathway.

Chronic HIV-1 infection is characterized by systemic immune activation. TCR-mediated antigenic contact as a result of viral replication is partially responsible for the increased expression of Tim-3 on virus-specific T cells; however, other factors are potentially responsible for elevated Tim-3 expression on nonvirus-specific T cells. LPS translocation through the gut is also implicated in immune activation during chronic HIV-1 infection (18); however, LPS stimulation of PBMCs did not directly upregulate Tim-3 in our assays. The production of γc cytokines, such as IL-15 or IL-7, during chronic immune activation in HIV-1 infection (19, 22) may provide another stimulus to induce Tim-3 on bulk T cells in the periphery of infected individuals. In this regard, serum IL-15 and IL-7 levels are increased during chronic untreated HIV-1 infection compared with healthy volunteers (19, 22). Thus, an inflammatory milieu that contains IL-15 or IL-7 in HIV-1 infection could maintain Tim-3 expression on T cells.

Recent in vivo models of the administration of IL-7 to lymphocytic choriomeningitis virus (LCMV) clone 13-infected mice, a mouse model of T cell exhaustion, showed different effects on the exhaustion marker, PD-1, depending on when it is measured in association with IL-7 administration. One study reported that IL-7 injection reversed virus-specific T cell exhaustion, as measured by PD-1 expression (52), whereas another recent study reported that administration of IL-7 to LCMV clone 13-infected mice improved

FIGURE 7. Tim-3 expression is associated with the PI3K pathway. The frequency of Tim-3+ T cells is shown following treatment with drugs that block various signaling pathways. (A) PBMCs were treated with medium, the common γc cytokines IL-2 or IL-15 (25 ng/ml), or anti-CD3/CD28 (1 μg/ml each) in the presence of each individual drug (LY 294002, SB 203580, and PD 98059), or with the maximal concentration of DMSO in which the drugs were dissolved (0.94%) as a control, for 6 d. Following the 6-d stimulation, cells were analyzed for Tim-3 expression on CD8+ and CD4+ T cells via flow cytometry. All p values are compared with the corresponding DMSO Tim-3+ frequency. Mean and SD of six independent experiments are shown. (B) CD28 signaling alone does not induce Tim-3 expression via the PI3K pathway. CD8+ T cells were isolated from six healthy (not HIV infected) individuals’ PBMCs and stimulated with anti-CD3, anti-CD28, or anti-CD3/CD28 (all treatments at 1 μg/ml each); Tim-3 expression was analyzed after 6 d following treatment. **p < 0.01, ***p < 0.001, compared with negative control, paired Student t test.
CTL function without altering PD-1 (53). The level of Tim-3 on T cells was not analyzed in either of these studies; thus, it is unknown what effect IL-7 treatment would have on Tim-3 expression on T cells in this model. Of note, however, was the fact that the absolute number of PD-1+ T cells increased markedly during IL-7 treatment in LCMV-infected mice (see figure 3B in Ref. 52), which then decreased at the end of IL-7 therapy, at which point the virus was also undetectable. Furthermore, in both of these studies, PD-1 was primarily measured after viral and Ag clearance and not during IL-7 administration. It is clear from our current and previous studies that the chronicity of infection and repeated antigenic contact of T cells play a significant role in the overexpression of Tim-3 in diseases such as HIV-1 (9). Thus, loss of Ag would mitigate ongoing TCR stimulation and cytokine induction, possibly explaining a lack of PD-1 upregulation in a model involving viral clearance.

Several γc cytokines are being tested in various therapies to boost T cell numbers and effector functionality in patients with various diseases, such as HIV-1. IL-2 and IL-7 administered to HIV-1-infected individuals was shown to increase the frequency of CD4+ T cells (29, 54–56). In SIV-infected macaques, IL-15 overexpression of Tim-3 is concomitantly expressed with inflammatory factors that negatively regulate T helper type 1 immunity. Nature 415: 536–541. It is clear from our studies, PD-1 was primarily measured after viral and Ag clearance and not during IL-7 administration. It is clear from our current and previous studies that the chronicity of infection and repeated antigenic contact of T cells play a significant role in the overexpression of Tim-3 in diseases such as HIV-1 (9). Thus, loss of Ag would mitigate ongoing TCR stimulation and cytokine induction, possibly explaining a lack of PD-1 upregulation in a model involving viral clearance.

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References


