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Upregulation of Tim-3 on T cells during acute SIV infection and on antigen specific responders

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Abstract

Objective—Understanding the role of T cell Immunoglobulin and mucin domain-containing molecule 3 (Tim-3) on T cells and DCs during the course of SIV infection.

Design/methods—Sequentially collected PBMCs from uninfected and SIVmac239 infected rhesus macaques were evaluated for Tim-3 expression by flow cytometry and antigen specific responses.

Results—Blood Innate immune cells (DCs) and B cells showed high constitutive expression of Tim-3, while compared to humans, only a minority of macaque T cells did. However TIM-3 expression was transiently up-regulated on both CD4⁺ and CD8⁺ T-cells during acute SIV infection correlating with plasma viral loads, CD4 counts, and Ki67 expression up to 6 weeks post infection (pi) and returned to baseline values by 8 weeks pi. Upon antigen specific stimulation, most Tim-3⁺ T-cells produced various cytokines, suggesting that this marker is up-regulated on effector antigen specific T-cells and not associated with T cell exhaustion. Among mDCs, a clear separation was seen between blood mDCs expressing Tim-3 and those expressing PD-L2, a ligand for inhibitory receptor PD-1.

Conclusion—Rhesus macaques show constitutive expression of Tim-3 primarily on innate immune cells but markedly lower levels on T cells compared to humans. Nevertheless, Tim-3 expression on T cells is transiently up-regulated during acute, but not chronic SIV infection, and appears to be a marker of antigen specific effector cells. The exact role and contribution of Tim-3 to the modulation of antiviral responses in vivo will require additional investigation.

Keywords

Tim-3; PD-L2; SIV (Simian immunodeficiency virus); PD-1

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Introduction

During chronic viral infections with sustained antigen production, up to seven different inhibitory receptors have been associated with the down-modulation of T-cell responses extending to a state of “immune exhaustion” [1]. One such receptor is the T cell immunoglobulin and mucin protein 3 (Tim-3), initially identified as a marker of T helper1 (Th1) but not Th2 cells [2], and reasoned to be involved in the maintenance of peripheral tolerance and Treg function [3, 4] by interacting with Tim-3 ligands [3]. Previous studies suggested that Tim-3, similar to programmed death 1 (PD-1) might be an exhaustion marker with the progressive loss of cytokine production by Tim-3⁺ T-cells in tumors [5-7].

Tim-3 expression has also been reported to increase during chronic viral infections on antigen specific CD4⁺ and CD8⁺ T-cells in mice and humans [8-11]. In addition, Tim-3 expression has been associated with a state of T-cell exhaustion in terms of both cytokine production [9] and degranulating ability in cells from HIV and HCV infected patients [11-13]. However, there have been no reports on the expression and role of Tim-3 in nonhuman primate macaques in the context of simian immunodeficiency virus (SIV) infection until a recent report published during the redaction of the present article [14], which provided the rationale for the studies reported herein. Results from our previous studies showed that blocking the PD-1/PDL pathway using soluble recombinant PD-1 protein led to the restoration of T-cell function but resulted in limited clinical benefit [15]. The present study was initiated as a follow up to investigate whether there might be a rationale for blocking both the Tim-3 and PD-1 pathway simultaneously in the context of SIV infection to enhance antiviral responses.

Materials and Methods

All animals used in this study were born at Yerkes National Primate Research Center and were maintained at the Yerkes National Primate Research Center of Emory University in accordance with the regulations of the Guide on the Care and Use of Laboratory Animal Resources. The experiments were approved by the Emory Institutional Animal Care and Use Committee as well as Biosafety Committee. The animals were inoculated with 200 TCID₅₀ (50% tissue culture infective dose) of SIVmac239 intravenously and served as a source for blood at various time points post infection (pi). Human blood was obtained from healthy donors after providing written informed consent and the protocol was approved by the Institutional Review Board of Emory University.

Cryopreserved PBMCs were stained for live/Dead marker (Alexa 430 Invitrogen A10169) and following antibodies against CD3 (SP34-2), CD4 (L200), CD8 (RPA-T8), CD20 (L27), CD28 (CD28.2), CD95 (DX2), PD1 (EH12.2H7), Tim-3 (344823, PE-conjugate, R&D or isotype control rat IgG2A-PE), Mamu-A*001 APC- tetramer (loaded with peptide CTPYDINQM) and Ki67 (B56), CD14 (M5E2), CD16 (3G8), HLA-DR (TU36), CD123 (7G3), CD11c (3.9), PD-L2 (24F.10C12) and CD86 (IT2.2) were used. Stained cells were fixed in 1% paraformaldehyde (PFA), and acquired on a LSRII flow cytometer (BD Biosciences). Analysis of the acquired data was performed using FlowJo software (version

9.2; TreeStar, Ashland, OR). Statistical analyses were performed using GraphPad InStat version 3.00 and the graphs were generated in GraphPad Prism version 5.00.

SIVgag specific T cell responses from freshly isolated PBMCs of infected monkeys were performed as previously described [15].

Results

We evaluated the expression of Tim-3 on PBMCs from uninfected rhesus macaques and normal human donors. As shown in Figure 1A, there are significant differences in the Tim-3 expression profile of PBMCs from healthy human donors and SIV naïve RM. The frequencies of Tim-3 expressing cells were markedly lower for CD4⁺ (p=0.0043), CD8⁺ T cells (p=0.0043), CD3⁺/CD8α⁺/NKG2A⁺ NK cells (p<0.01), mDCs (p=0.02) and pDCs (p=0.0007) from RM compared to humans (Fig. 1A). In contrast, the frequencies of CD20⁺ B cells from RM that expressed Tim-3 were markedly higher than from humans (p=0.0043). The significant differences between these two species might suggest a potentially distinct role of Tim-3 in the context of a viral infection. We then investigated Tim-3 expression on PBMCs collected sequentially from rhesus macaques that were experimentally infected with SIVmac239. Four distinct subsets were identified for both CD8⁺ and CD4⁺ T-cells (PD-1⁺Tim-3⁻, PD1⁺Tim-3⁺, PD-1⁻Tim-3⁺ and PD-1⁻Tim-3⁻) but expression of Tim-3 was essentially restricted to PD1⁺T cells (Fig 1B). Further characterization of PD1⁺Tim-3⁺ T cells, using CD95 and CD28 indicated that these are primarily effector memory CD8⁺ and central memory CD4⁺ T cell subsets (data not shown). The longitudinal data from baseline (wk-8) to wk49 pi, showed a clear but only transient up-regulation of Tim-3 on both CD8⁺ and CD4⁺ T-cells (wk2 to 6-8, Fig. 1C, D). Of note, Tim-3 expression levels returned to baseline values by wk8 (CD8⁺ T-cells, Fig. 1C) or wk 12 (CD4⁺ T-cells, Fig. 1D). There was also a significant increase in the MFI of Tim-3 on both CD4 and CD8 T cells at wks 2 and 4, respectively and though the frequencies dropped back to baseline values after wk8, the MFI remained higher than baseline levels (supplementary Fig S1).

In contrast to T-cells, the frequencies of Tim-3⁺CD20⁺ B cells did not vary significantly as a function of time post SIV infection (Fig. 1E). Tim-3 expression by NKG2A⁺CD3⁻ cells before and after SIV infection showed a sustained increase as a function of time pi (Fig. 1F), confirming previous observations on human NK cells which showed that Tim-3 expression is up-regulated after activation [16] and that most mature NK cells (CD56^{dim}CD16⁺) express Tim-3 [17]. Investigating p11CM tetramer⁺ CD8⁺ T-cells following the acute phase of infection showed high levels of PD-1 (as previously known), but only low levels of Tim-3 expression, which interestingly did not markedly vary over the 16 wk follow-up period (Fig. 1G).

A correlation analysis was performed between Tim-3 expressing T cell frequencies and acute events of SIV infection. Tim-3 expression on CD8⁺ T-cells showed a modest positive correlation with plasma viral loads (p=0.05) (Fig. S2A), but a strong positive correlation by Tim-3⁺CD4⁺ T-cells (p=0.004, Fig. S2B). There was also a strong negative correlation between Tim-3 expression on CD8⁺ and CD4⁺ T-cells with absolute circulating CD4⁺ T-cell counts (Fig. S2C, D). A strong positive correlation was also observed for Tim-3 and Ki67

(activation/proliferation marker) expression by CD8⁺ and CD4⁺ T-cells during acute SIV infection (Fig. S2E, F). This strong association with early events of SIV infection supports Tim-3 as a marker of “acute” T-cell activation.

The CD14⁺CD16⁻ (classical), CD14^{dim}CD16⁺⁺ (non-classical) monocytes in PBMC did not show any detectable expression of Tim-3 irrespective of the SIV infection status (data not shown). This finding markedly differs from reports of Tim-3 expression by human monocytes [18-21]. However, CD14⁺CD16⁺ (Intermediate) monocytes showed modest Tim-3 expression with a mean value of 5.4% ± 6% SD at baseline and 3 ± 0.6% SD from wk1 to wk6 pi (data not shown). One-way Analysis of Variance (ANOVA) using the Tukey-Kramer Multiple Comparisons Test showed that the variation of these frequencies was not statistically significant (data not shown).

PD-L1 (B7-H1) and PD-L2 (B7-DC) are well-studied ligands for PD-1 and while they are structurally and genetically homologous, each have unique functional properties [22-25]. The expression of PD-L2 and Tim-3 was analyzed on DCs in PBMC. Surprisingly, myeloid DCs (mDCs), defined as lineage (CD3, CD20, CD8, CD14, CD16) negative but HLADR⁺/CD123⁻/CD11c⁺, showed a clear separation between Tim-3⁺ and PD-L2⁺ mDCs (Fig. 1H, upper panel). Monitoring Tim-3 and PD-L2 expression longitudinally showed a significant transient decrease in Tim-3 expressing mDCs at wk4 pi (P=0.02, Fig. 1I, solid line) followed by a restoration of Tim-3⁺ mDCs by wk6, but overall lower levels during chronic infection (wk12 to 49 pi, P<0.05). Interestingly, PD-L2 expressing mDCs showed an opposite pattern to Tim-3⁺ mDCs which increased significantly after SIV infection and reached a peak at wk 12 (P=0.002, Fig. 1I, broken line), suggesting a potential functional difference. There was a highly significant negative correlation between PD-L2 and Tim-3 expressing mDCs (Fig. 1J). Plasmacytoid DCs (pDCs) defined as lineage negative but HLADR⁺/CD11c⁻/CD123⁺ also express Tim-3 (Fig 1H, lower panel) but the levels were relatively low compared to mDCs. Increase in these frequencies was seen at wk2 but later samples were similar to baseline values before a new increase was noted at wk49 pi (Fig 1K, solid line)

PD-L2 expressing blood pDCs increased significantly after SIV infection peaked at wk 8 (P=0.0002) but then drastically dropped to baseline levels at wk 12 and remained low up to wk29, followed by modest increases on wk37 and beyond (Fig. 1K, broken line). However, unlike mDCs, the expression of Tim-3 and PD-L2 on pDCs showed no correlation (Fig. 1L).

We also examined the antigen specific responses in a 6 hr ICS assay with SIV gag peptides (2 µg/ml) using PBMCs collected at the acute (wk4 pi) and chronic (~1 year pi) phase of infection. Total CD4⁺ and CD8⁺ T-cells were divided into four different subsets based on PD-1 and Tim-3 expression. The responding cells among these 4 subpopulations were assessed for individual cytokine responses (Fig. 2A, B) and for polyfunctionality by using Boolean gates in FlowJo (Fig 2C). Values shown are net values adjusted by subtraction of responses obtained on an aliquot of the same cells without stimulation. As seen for both samples collected early and late pi, Tim-3⁺ T-cells were the major cytokine (IFN-γ, TNF-α, IL-2 and CD107a) producing populations compared to other subsets regardless of whether they expressed PD-1 or not. Dual (d) and triple (t) cytokine producer T-cells were predominantly Tim-3⁺ (Fig. 2C), suggesting that Tim-3 may be up-regulated primarily

during antigenic stimulation. Similar data have been reported for effector CD4⁺ and CD8⁺ T-cells from human patients with active TB infection [26].

Discussion

This report to our knowledge is the first analysis of the kinetics of Tim-3 expression on various cell lineages during SIV infection in rhesus macaques, though during submission of this manuscript, another report confirmed the low expression of TIM-3 on macaque T cells [14]. The transient up-regulation of Tim-3 levels on T-cells during acute but not chronic SIV infection makes this finding difficult to reconcile with the notion of Tim-3 being a marker of T-cell exhaustion in monkeys, as suggested by several studies on viral persistence and cancer [6, 7, 9, 11, 13, 27]. However, more profound was the finding of strong association with the early activation events during SIV infection strongly suggests its role as an “acute” activation marker due to the rapid mobilization of the immune response to the nascent infection and not one of exhaustion. The low levels of Tim-3 on p11CM tetramer specific cells compared to PD-1, shows that these two inhibitory receptors are differentially regulated. However, the findings that TIM-3⁺ T-cells are indeed antigen specific effector cells even during chronic SIV infection further argues against an association of Tim-3 expression on macaque T-cells and exhaustion, a fact that was corroborated in human CD4⁺ and CD8⁺ T-cells from patients with active TB infection [26], but contrasts a recent report of decreased antigen specific IFN- γ responses in T cells from SIV infected macaques [14]. The reasons for this discrepancy are unclear at present, though this report used a polyclonal instead of the monoclonal anti TIM-3 reagent used herein, and the antigen specific cytokine analyses were restricted to IFN- γ . Furthermore, the constitutive expression of Tim-3 on B cells and innate immune cells raises a note of caution regarding the use of Tim-3 blockade as a therapeutic strategy. Interestingly, RM also showed high Tim-3 expressing mDCs as reported for humans [28], and might have a similar role of secreting pro-inflammatory cytokines. The role of Tim-3 on DCs as impeding anti-tumor function within the tumor microenvironment was shown in mice [29], but its role in HIV/SIV still remains to be addressed. Our data also supports a previous study [28] reporting opposing roles of Tim-3 in the adaptive and innate immune systems, as shown by the increase in Tim-3⁺ T-cells versus the decrease in Tim-3⁺ mDCs after SIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A. The frequencies of Tim-3 expressing CD8⁺, CD4⁺ T cells, CD3⁺NKG2A⁺, CD20⁺ cells, mDCs and pDCs in normal humans (Hu, n=6, line at median) and naïve rhesus macaques (Rh, n=5, line at median). B. Representative FACS plots illustrating the profile of Tim-3 vs PD-1 expression on CD8⁺ and CD4⁺ T cells from SIV infected rhesus macaques at 2 wks post infection. C, D, E, F. Longitudinal analysis of Tim-3 expression on the gated population of PD1⁺CD8⁺ T cells, PD1⁺CD4⁺ T cells, CD20⁺ and CD3⁺NKG2A⁺ cells after SIV infection. Mann-Whitney test was used for the statistical analysis, error bars indicate SEM, *p<0.05, **p<0.01, ***p<0.001. G. Top panels, gating strategy utilized to analyze the expression of PD-1 and Tim-3 on p11CM⁺ tetramer⁺ CD8⁺ T cells in the blood from Mamu-A*001⁺ animals. Lower panels, comparative analysis of the expression levels of Tim-3 and PD-1 on p11CM⁺ tetramer⁺ CD8⁺ T cells from 6 weeks post SIV infection. H. Representative FACS plots showing the evaluation of Tim-3 and PD-L2 expression on mDCs and pDCs. I. Tim-3⁺PD-L2⁻ (solid line) and PD-L2⁺Tim-3⁻ (broken line) frequencies pre and post SIV infection on mDCs (n=8). J. Spearman's rank correlation test on Tim-3⁺PD-L2⁻ and PD-L2⁺Tim-3⁻ frequencies on mDCs. K. Tim-3⁺PD-L2⁻ (solid line) and PD-L2⁺Tim-3⁻ (broken line) frequencies pre and post SIV infection on pDCs (n=8). L. Spearman's rank correlation test on the Tim-3⁺PD-L2⁻ and PD-L2⁺Tim-3⁻ frequencies on pDCs. Results are the mean +/- SEM of data obtained on 8 RM and include pre and longitudinal data up to week 16 post infection.

Figure 2. Tim-3 expression on antigen specific T

A, B. SIVgag specific CD8⁺ and CD4⁺ T cell responses in aliquots of PBMC samples from rhesus macaques at 4 weeks pi (acute) and 1year pi (chronic) are illustrated. PBMCs were stimulated in vitro with a pool of SIVmac239 gag peptide pools (NIH AIDS Research & Reagent Repository Program) for 6 hr in presence of anti-CD28 (CD28.2), anti-CD49d (9F10), monensin (GolgiStop, BD), degranulation marker anti-CD107a-FITC (clone eBioH4A3) and brefeldin A (10 μ g/ml, added after initial 2hr of incubation). The cells were then stained for cytokines with anti-IFN- γ -v450 (B27), anti-TNF- α -Alexa700 (MAb11), anti-interleukin-2-APC (IL-2) (MQ1-17H12) for CD8⁺ (C) and CD4⁺ (D) T cells. The data is divided into 4 subsets based on PD-1 and Tim-3 expression. C. Boolean gating strategy was used on Flow Jo to identify dual (d) and triple (t) cytokine responders. The stimulation data was normalized using its own mock stimulated control. The notation of the monkeys and the number of samples analyzed from SIV infected animals are listed in the top panel.