TIM-1 and TIM-3 enhancement of Th2 cytokine production by mast cells

Short title: Role of TIM-1 and TIM-3 in mast cell function

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Abstract

Members of the T cell immunoglobulin- and mucin-domain-containing molecule (TIM) family have roles in T cell-mediated immune responses. TIM-1 and TIM-2 are predominantly expressed on Th2 cells, whereas TIM-3 is preferentially expressed on Th1 and Th17 cells. We found that TIM-1 and TIM-3, but neither TIM-2 nor TIM-4, were constitutively expressed on mouse peritoneal mast cells and bone-marrow-derived cultured mast cells (BMCMCs). After IgE+Ag stimulation, TIM-1 expression was downregulated on BMCMCs, whereas TIM-3 expression was upregulated. We also found that recombinant mouse TIM-4 (rmTIM-4), which is a ligand for TIM-1, as well as an anti-TIM-3 polyclonal Ab, can promote IL-4, IL-6 and IL-13 production without enhancing degranulation in BMCMCs stimulated with IgE+Ag. Moreover, the anti-TIM-3 Ab, but neither anti-TIM-1 Ab nor rmTIM-4, suppressed mast cell apoptosis. These observations suggest that TIM-1 and TIM-3 may be able to influence T cell-mediated immune responses in part through effects on mast cells.
Introduction

Molecules of the TIM family are thought to contribute to the development of autoimmune and allergic diseases by modulating T cell function\textsuperscript{1-4}, and genetic polymorphism affecting these molecules may also play a role in such disorders\textsuperscript{1-4}.

TIM-1 enhances and TIM-2 suppresses Th2 cell activation, and TIM-3 down regulates Th1 cell function\textsuperscript{1-3}. Such effects are likely to be important. Thus, mice treated with an anti-TIM-1 Ab or a TIM-1 extracellular domain protein exhibited attenuated development of antigen-induced airway inflammation\textsuperscript{5} and of contact or delayed-type hypersensitivity responses\textsuperscript{6}; administration of TIM-2-Ig to mice ameliorated development of experimental autoimmune encephalomyelitis\textsuperscript{7} while TIM-2-deficient mice exhibited exacerbated airway inflammation\textsuperscript{8}; and blockade of the TIM-3 pathway exacerbated experimental autoimmune encephalomyelitis\textsuperscript{9}, diabetes (in nonobese diabetic mice)\textsuperscript{10} and acute graft-vs-host disease\textsuperscript{11}.

Mast cells are important effector cells in host defense, and also contribute to the development of autoimmune and allergic diseases\textsuperscript{12}. In this study, we examined mouse mast cells for expression of TIM family members and investigated whether such TIM expression by mast cells is functionally significant.
Study Design

Animal protocols were approved by the Stanford Administrative Panels on Laboratory Animal Care.

Mice and BMCMCs.

C57BL/6J and BALB/cJ mice, and C57BL/6Ka and BALB/cKa mice, were from the Jackson Laboratories and the Stanford Animal Facility, respectively. BMCMCs were obtained by culturing mouse bone marrow cells in WEHI-3 conditioned medium (containing IL-3) for 4-6 weeks, at which time >98% of the cells were identified as mast cells by flow cytometry for c-Kit and FcεRI.

Reagents and Abs.

rmIL-3, rmTIM-1, rmTIM-4, mouse mAbs for TIM-1 (222414) and TIM-3 (215015) and polyclonal Abs (pAbs) for TIM-1, TIM-3 and TIM-4 were from R&D systems. Anti-mouse TIM-3 mAb (8B.2C12), mTIM-1-Ig and mTIM-3-Ig were from eBioscience. Anti-mouse TIM-1 (RMT1-4 and RMT1-17), TIM-2 (RMT2-1 and RMT2-14) (Fig. S1) and TIM-3 (RMT3-23)\textsuperscript{11} mAbs (currently available from eBioscience or BioLegend) were from H. A. Anti-mouse TIM-1 (3B3)\textsuperscript{13} and TIM-4 (21H12) (Fig. S2) were from D. T. U. and R. H. D. Either mAbs or pAbs for TIM-1 or TIM-3 from R&D systems revealed bands (70-80 kDa) in Western blots of whole lysates from naïve BMCMCs; by contrast, we found that RMT1-4, RMT1-17, RMT3-23 and 3B3 were not appropriate for Western blot analyses (Fig. S3).
Flow cytometry, β-hexosaminidase release assay and cytokine ELISAs.

BMCMCs were sensitized with anti-DNP IgE (H1-e-26, at 10 µg/ml) at 37°C overnight and then washed. Naïve or IgE-sensitized BMCMCs were cultured with 20 µg/ml anti-TIM Abs or 20 µg/ml rmTIM-4 ± 20 ng/ml DNP-human serum albumin (DNP-HSA [Ag]: SIGMA) at 37°C for 1 h (β-hexosaminidase release assay), 6 h (for FACS and ELISAs) or for 0, 1, 2, 4 or 6 d (for apoptosis). FACS analysis and β-hexosaminidase release assay were performed as described. We used the Apoptosis Detection Kit and Bcl-2 Set (BD PharMingen) for annexin V staining and Bcl-2 expression. Cytokine levels in supernatants were measured by Mouse IL-3, IL-4 and IL-6 BD OptEIA™ ELISA sets (BD PharMingen) and Mouse IL-13 DuoSets (R&D systems).

Statistics.

The unpaired Student's t-test, 2-tailed, was used for statistical evaluation of the results; unless otherwise specified, results are shown as mean + SD (n = 3/group).

Results and Discussion

Expression of TIM family members on mouse mast cells.

TIM molecules exhibit splicing variants in several mouse strains. We therefore evaluated the expression of TIM family members using different mAbs for TIMs. Naïve BMCMCs from C57BL/6J mice constitutively expressed TIM-1, as detected by anti-TIM-1 mAbs (3B3, RMT1-4 or RMT1-17) (Figure 1A). TIM-1 expression appeared to be slightly decreased 6 h after IgE+Ag
stimulation (Figure 1A). Little or no TIM-2 or TIM-4 expression was detectable on naïve or IgE+Ag-stimulated C57BL/6J BMCMCs (Figure 1A), whereas TIM-2 expression was observed on Th2 cells (data not shown). TIM-1 or TIM-3 ligands were not detectable using TIM-1-Ig and TIM-3-Ig fusion proteins, either on naïve or IgE+Ag-stimulated C57BL/6J BMCMCs (Figure 1A). Similar results were obtained with C57BL/6Ka, BALB/cJ or BALB/cKa BMCMCs (data not shown).

Frisancho-Kiss et al. reported that BALB/cJ mouse PMCs constitutively express TIM-3, based on staining with the 8B.2C12 anti-TIM-3 mAb. Using the RMT3-23 anti-TIM-3 mAb, we detected constitutive expression of TIM-3 on C57BL/6J, C57BL/6Ka, BALB/cJ or BALB/cKa BMCMCs (Figure 1A and data not shown). By contrast, when we used the 8B.2C12 anti-TIM-3 mAb, we detected TIM-3 expression on C57BL/6Ka, BALB/cJ and BALB/cKa BMCMCs, but not on C57BL/6J BMCMCs (Figure 1A and data not shown), since the 8B.2C12 antibody does not recognize the TIM-3 allele expressed in the C57BL/6J background.

Expression of TIM-3 on C57BL/6J, C57BL/6Ka, BALB/cJ or BALB/cKa BMCMCs was increased slightly 6 h after IgE+Ag stimulation (Figure 1A and data not shown). Constitutive expression of TIM-1 and TIM-3, but not TIM-2 and TIM-4, also was observed on peritoneal mast cells (PMCs) from C57BL/6J mice (Figure 1B) and from C57BL/6Ka, BALB/cJ and BALB/cKa mice (data not shown).

**TIM-1 and TIM-3 can enhance mast cell-Th2 cytokine production.**

Interactions between TIM-1 on Th2 cells and TIM-4 on DCs can enhance Th2 cell function.
whereas administration of anti-TIM-1 Ab attenuated a Th2-associated mouse model of allergic airway inflammation. We searched for effects of TIMs on degranulation and/or Th2-associated cytokine production in naïve, IgE-sensitized or IgE-sensitized and Ag-stimulated BMCMCs.

Neither anti-TIM-1 pAbs or mAbs (RMT1-4, RMT1-17, 3B3 or 222414), rmTIM4, nor anti-TIM-3 pAbs or mAbs (RMT3-23, 8B.2C11 or 215015) detectably influenced degranulation or phosphorylation of ERK1/2, JNK or MAPK after IgE/Ag-stimulation in any of the C57BL/6J BMCMCs tested (Figure 2A, Figure S4 and data not shown). By contrast, rmTIM-4 slightly, but significantly, enhanced production of IL-4, IL-6 and IL-13, but didn’t detectably influence IL-17 or IFN-γ production (data not shown), by IgE+Ag-stimulated C57BL/6J BMCMCs, but not by naïve or IgE-sensitized cells (Figure 2B and C). The effects of rmTIM-4 were enhanced by treatment with anti-TIM-4 pAb (Figure 2D) and inhibited by rmTIM-1 (Figure 2E). Anti-TIM-1 mAbs (3B3, RMT1-4 and RMT1-17) can enhance Th2 cell activation or modulate B cell function (H. A., unpublished observations), but not mast cell activation (our results). These findings support the possibility that mAbs for TIM-1 may have distinct effects on different cell types. Our results suggest that interfering with TIM-1-TIM-4 interactions on mast cells, as well as blocking effects of TIM-4 on Th2 cells, may have therapeutic benefit in Th2-type disorders.

TIM-3 is expressed on Th9 and Th17 cells and either TIM-3 or galectin-9, a ligand for TIM-3, can negatively regulate Th1 cell function by inducing apoptosis. We found that anti-TIM-3 pAb, but not anti-TIM-3 mAbs (RMT3-23, 8B.2C12 or 215015), significantly enhanced IL-4, IL-6 and IL-13 production by IgE+Ag-stimulated BMCMCs (Figure 2B). The anti-TIM-3 pAb also slightly promoted IL-13, but not IL-6 and IL-4, production by naïve and
IgE-sensitized C57BL/6J BMCMCs in the absence of IgE +/- Ag (Figure 2B). These effects of anti-TIM-3 pAb could not be blocked by pre-incubation of BMCMCs with any of the anti-TIM-3 mAbs (data not shown).

The anti-TIM-3 pAb, but not other TIM-1 or TIM-3 Abs, also significantly inhibited IL-3-withdrawal-induced apoptosis of IgE-sensitized BMCMCs with or without exposure to Ag, as assessed by annexin V staining (Figure 2F) or trypan blue exclusion (data not shown). Anti-TIM-3 pAb also promoted IL-3 production, but not Bcl-2 expression, in BMCMCs (Figure 2G, I and H).

Results similar to those in Figure 2 were also obtained in BALB/cJ BMCMCs (data not shown). Our results are consistent with results of in vivo studies, in which anti-TIM-3 mAbs (RMT3-23 or 8B.2C12) functioned as blocking Abs whereas anti-TIM-3 pAb had agonist effects.

Our data demonstrate that mouse mast cells can constitutively express TIM-1 and TIM-3, but do not detectably express TIM-2 or TIM-4. Moreover, in contrast to evidence that TIM-3 can negatively regulate Th1 cells, our findings indicate that, in mast cells, TIM-3 can enhance IgE+Ag-dependent cytokine secretion and survival. These observations raise the possibility that TIM-1 and/or TIM-3 can influence the development of autoimmune and allergic disorders at least in part through effects on mast cells.

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References


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Figure 1. Expression of TIM family members on mouse mast cells

A. Expression of TIM-1, TIM-2, TIM-3, and TIM-4, and TIM-1 and TIM-3 ligands, on naïve or IgE+Ag-stimulated c-Kit⁺ FcεRIα⁺ BMCMCs derived from C57BL/6J or (for TIM-3) C57BL/6Ka mice.

B. Expression of TIM-1, TIM-2, TIM-3 and TIM-4 on peritoneal c-Kit⁺ FcεRIα⁺ mast cells from C57BL/6J mice.

Results in A and B are representative of similar results that were obtained in three independent experiments.
Figure 2. Effect of anti-TIM-1, anti-TIM-3 or rmTIM-4 on mast cell function

C57BL/6J BMCMCs were sensitized with anti-DNP IgE (H1-ε-26) at 37°C overnight and then washed. Naïve or IgE-sensitized C57BL/6J BMCMCs were cultured in the presence or absence of Ag (DNP-HSA, 20 ng/ml) together with:

(A) 20 µg/ml anti-TIM Abs or control IgG or 20 µg/ml rmTIM-4 at 37°C for 1 h for β-hexosaminidase (β-Hex) release assay (P + I = PMA [0.1 µg/ml] + Ionomycin [1.0 µg/ml]);

(B) 20 µg/ml anti-TIM Abs or control IgG or 20 µg/ml rmTIM-4 at 37°C for 6 h for cytokine production;

(C) 0-100 µg/ml rmTIM-4 at 37°C for 6 h for cytokine production in IgE/Ag-stimulated cells;

(D) 40 µg/ml rmTIM-4 and 80 µg/ml anti-TIM-4 pAb or control IgG (goat IgG) at 37°C for 6 h for cytokine production in IgE sensitized cells ± Ag;

(E) 40 µg/ml rmTIM-4 and 0-40 µg/ml rmTIM-1 at 37°C for 6 h for cytokine production in IgE/Ag-stimulated cells;

(F) 20 µg/ml anti-TIM Abs or control IgG or 20 µg/ml rmTIM-4 at 37°C for 0, 1, 2, 4 or 6 d in the absence of IL-3 for apoptosis (the same data for culture with IL-3, rat IgG and goat IgG, or without any reagents [medium] are shown in the upper and lower panels for each group of BMCMCs);

(G) 20 µg/ml anti-TIM Abs or control IgG at 37°C for 2 d for IL-3 production;

(H and I) 20 µg/ml anti-TIM Abs or control IgG at 37°C for 2 d in the absence of IL-3 for Bcl-2 expression (the FACS data shown are representative of those obtained in 3 experiments using 3 different batches of BMCMCs). Upper numbers = MFI for anti-Bcl-2 mAb staining, lower numbers = MFI for control IgG staining. “IL-3” = results for BMCMCs maintained in IL-3-containing WEHI-3 cell-conditioned medium.

(A-G and I) * p < 0.05 versus corresponding values for cells cultured with control IgG (B, D, F, G), or with no rmTIM-4 or rmTIM-1 (0 µg/ml) (C, E). Data shown in A-G and I are the average ± SEM (n = 3) of duplicate values for each condition obtained from an experiment done using three different batches of BMCMCs; such experiments were done 2-4 times each, and each of these gave similar results.
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