Evidence for carbohydrate recognition and homotypic and heterotypic binding by the TIM family

Peter R. Wilker¹, John R. Sedy¹, Vadim Grigura², Theresa L. Murphy¹ and Kenneth M. Murphy¹,²

¹Department of Pathology and Immunology and ²Howard Hughes Medical Institute, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA

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Abstract

The T cell Ig domain and mucin domain (TIM) proteins form a conserved family of transmembrane cell-surface glycoproteins expressed by a variety of tissues. Each TIM protein contains a single V-type Ig domain, a glycosylated mucin-like domain, a transmembrane domain and a cytoplasmic domain. TIM proteins recognize a diverse array of ligands, including H-ferritin, galectin-9 as well as other TIM family members. In this study, we demonstrate that the Ig domains of murine TIM-1, -3 and -4 display calcium-dependent binding to ligands expressed by murine splenocytes and several non-murine cell lines, indicating non-species-specific ligand recognition. Further, the intrafamilial interaction of various TIM family Ig domains with surface-expressed TIM-1 and TIM-4 requires an intact TIM-1 and TIM-4 glycosylated mucin stalk. Importantly, we also uncovered the previously unrecognized potential for homotypic TIM interactions in forming ligand–receptor pairs. Using a glycan array screen, we identified the novel capacity of the TIM-3 Ig domain to recognize specific carbohydrate moieties, suggesting a role for carbohydrate modification along with protein epitopes in TIM ligand recognition. Identification of the carbohydrate-binding capacity of TIM proteins helps explain the diversity of ligands recognized by this family and adds to our understanding of homotypic and heterotypic interactions between TIM family members.

Introduction

T cell Ig domain and mucin domain (TIM) proteins were first identified on liver and kidney cells and were more recently observed on immune cells, particularly T cells and dendritic cells (DCs) (1–6). The first TIM family member was identified in the rat as kidney injury molecule 1 (7), and its homolog on human and African green monkey kidney cells was later recognized as a hepatitis A virus receptor (8, 9). The murine TIM family was recognized for its genetic linkage with the T cell and airway phenotype regulator locus (2). The TIM family now includes three human and eight murine genes (10). TIM proteins are differentially expressed on subsets of immune cells. TIM-1 and TIM-2 are preferentially expressed by T₉,2 cells, TIM-3 by T₈,1 cells (1, 4, 6, 11) and TIM-4 by macrophages and DCs (3, 12). Although their precise role is still somewhat obscure, TIM proteins exert in vivo regulatory effects, since blockade of TIM-3 or TIM-3 deficiency augment autoimmunity and block induction of peripheral tolerance (4–6).

TIM proteins have been described to bind a diverse set of ligands belonging to different gene families, in contrast to the CD28/B7 Ig superfamily whose ligand interactions are all restricted to the same gene family (13). For example, TIM-1 was reported to be the ligand for TIM-4, interpreted as a specific heterotypic pairing between TIM family members (3). TIM-2 binds H-ferritin, an iron transport protein that is expressed intracellularly and is secreted from liver and lymphoid cells (14, 15) and TIM-3 reportedly recognizes galectin-9, a member of a family of lectins that bind β-galactosides (16). Further, these studies have not claimed that these ligands represent the only TIM-interacting proteins, and there are some indications of the existence of additional TIM ligands (16).

In this study, we wished to determine the basis for ligand recognition by TIM proteins. Unexpectedly, we found that the Ig domains of several TIM proteins exhibit characteristics reminiscent of C-type lectins. First, in contrast to strict species-specific interactions exhibited by the Ig domains of the CD28/B7 family (13), we find that the Ig domains of murine TIM-1, -3 and -4 bind to epitopes expressed by cells from species as diverse as mammalian and insect cells. The
binding of TIM Ig domains to cells of all species is calcium sensitive, similar to the calcium-sensitive carbohydrate binding by C-type lectins (17), and is reduced in cells with defective O-linked and N-linked carbohydrate synthesis. Further, we find that interactions between TIM family members are not unique to the interaction between TIM-1 and TIM-4, as the Ig domains of TIM-1, -3 and -4 each bind to TIM-1 and TIM-4 expressed on cells in a manner that requires the intact mucin stalks of TIM-1 and TIM-4. Importantly, these results indicated an unreported potential for homotypic interactions by TIM family proteins. Finally, using a glycan array screen, we identified several specific purified carbohydrate moieties that interact with the isolated TIM-3 Ig domain. In summary, the TIM proteins appear to be a family with lectin-like activity that recognizes various non-species-specific carbohydrate epitopes. These results have implications for TIM protein function in cellular adhesion and immune regulation, and suggest broader interactions between TIM proteins and glycosylated receptors than previously appreciated.

**Methods**

**Reagents**

Anti-B220–Allophycocyanin (APC, 53-6.7), and streptavidin–PE were obtained from BD PharMingen (Franklin Lakes, NJ, USA). Anti-CD4–FITC (CT-CD4) was purchased from Caltag Laboratories (Carlsbad, CA, USA). D-(+)-galactose (Gal), N-acetyl-D-galactosamine and ethylene-bis(oxyethylenenitriol)tetraacetic acid (EGTA) were obtained from Sigma (St Louis, MO, USA). Anti-TIM-1 (RMT1-4) and anti-TIM-3 (RMT3-23) were purchased from eBioscience (San Diego, CA, USA). The PE–anti-human IgG (H&L) secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Human FGF R1β (IICl)–Fc chimera from R&D Systems (Minneapolis, MN, USA) was a gift from Lijuan Zhang (Washington University, St Louis, MO, USA).

**Cell lines**

BJAB cells were from A. Chan (Washington University); CHO-K1 and CHO-pgsA-745 cells were from L. Zhang (Washington University); IdID CHO cells were purchased from American Type Culture Collection (Manassas, VA, USA) after obtaining permission from M. Krieger (Massachusetts Institute of Technology, Cambridge, MA, USA); 293T cells were from R. Schreiber (Washington University) and High Five and Sf9 insect cells were from D. Fremont (Washington University).

**Plasmid construction**

A detailed description of the procedures and reagents used to generate each of the plasmids described below can be found in the Supplementary Methods (available at *International Immunology* Online).

**TIM protein production in Escherichia coli**

Recombinant protein preparations of the TIM-3 and TIM-4 Ig domains were produced in BL-21 (DE3) Codon Plus RIL competent cells (Stratagene, La Jolla, CA, USA) using the TIM3Ig-pET28a and TIM4Ig-pET28a vectors. The recombinant protein was purified from insoluble inclusion bodies and refolded essentially as previously described (18). The TIM3Ig-pET28a and TIM4Ig-pET28a vectors use a modified version of pET28a that fuses a biotinylation sequence to the C-terminus of the Ig domain (gift from Daved Fremont, Washington University), enabling *in vitro* biotinylation using BirA biotin–protein ligase (Avidity, Denver, CO, USA). The refolded, biotinylated TIM3Ig and TIM4Ig was purified by size exclusion chromatography.

**TIM protein production in insect cells**

Protein preparations of the extracellular domains of TIM-1, TIM-2, TIM-3 and TIM-4 were produced using a baculovirus system in insect cells. Recombinant bacmid DNA was generated by transformation of the TIM1-pFastBac1, TIM2-pFastBac1, TIM3-pFastBac1 and TIM4-pFastBac1 plasmids into DH10Bac *Escherichia coli* from Invitrogen (Carlsbad, CA, USA). Purified recombinant bacmid was transfected into Sf9 insect cells to generate recombinant baculovirus. TIM-1, TIM-2, TIM-3 and TIM-4 protein was produced in High Five insect cells by infection with high-titer recombinant baculovirus. The pFastBac1 vector used was modified to place a biotinylation sequence and histidine tag in frame with the C-terminus of the cloned TIM sequence (gift from Daved Fremont, Washington University), enabling purification over a nickel column following buffer exchange into 50 mM NaH2PO4, 50 mM sodium citrate, 300 mM NaCl and 0.01% NaN3, pH 6.40. Purified protein was biotinylated *in vitro* using the BirA biotin–protein ligase purchased from Avidity and the biotinylated protein purified by size exclusion chromatography.

All TIM protein tetramers were produced by adding biotinylated TIM protein to PE-conjugated streptavidin in a 4:1 molar ratio.

**TIM protein-expressing CHO cells**

Retrovirus was produced by transfecting 293T cells with the pCGP and pYITG plasmids for retrovirus packaging (gifts from W. Sha, University of California, Berkeley, CA, USA) and a retroviral vector containing the coding sequence of the protein to be surface expressed and an internal ribosome entry site (IRES) driving the expression of green fluorescent protein (GFP). The pCGP plasmid encodes the Moloney murine leukemia virus gag and pol genes and the pYITG plasmid encodes the vesicular stomatitis virus G glycoprotein. Bicistronic retroviral constructs used include TIM1FL-IRES-GFP, TIM3FL-IRES-GFP and TIM4FL-IRES-GFP to express the indicated full-length (FL) TIM protein; TIM1Ig-only-IRES-GFP and TIM4Ig-only-IRES-GFP to express Ig domain-only versions of TIM-1 and TIM-4 in which the mucin stalk region was deleted and BTLAIg–TIM-1 mucin-IRES-GFP to surface express a fusion protein consisting of the BTLA Ig domain fused to the TIM-1 mucin domain. Cell lines were generated by transducing CHO cells with retrovirus in the presence of 1 µg/ml polybrene (Sigma). Stably integrated cells were identified based on GFP expression and were sorted to >90% purity.
Consortium for Functional Glycomics screening for TIM-3 carbohydrate ligands

Purified TIM-3 Ig protein was screened for binding to a panel of natural and synthetic carbohydrate moieties as previously described by the Consortium for Functional Glycomics (19). Briefly, biotinylated TIM-3 Ig protein produced in E. coli as described above was screened at 30 μg ml⁻¹ in binding buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.05% Tween 20 and 1% BSA) for binding to glycosides plated in replicates of four (Glycan Array V3.8). Microtiter plates were washed and incubated with 5 μg ml⁻¹ Alexa 488–streptavidin. Plates were again washed and bound TIM-3 was detected using a Victor 2™ 1420 Multilabel Counter from PerkinElmer Life Sciences (Wellesley, MA, USA).

Results

TIM proteins recognize non-species-specific ligands in a calcium-dependent manner

To characterize the interactions of TIM proteins with their ligands, we generated high-avidity tetramers for several TIM proteins using two approaches. First, we used a prokaryotic expression system to express the Ig domains of TIM-1, -2, -3 and -4. In this system, each Ig domain is refolded in vitro to a native, but non-glycosylated, conformation, and is then biotinylated to enable tetramerization on a fluorochrome-labeled streptavidin support for staining. Properly refolded TIM-3 Ig and TIM-4 Ig tetramers were produced by this approach, but the TIM-1 Ig was toxic to E. coli and TIM-2 Ig could not be refolded in vitro. Second, we used a baculovirus expression system to produce the complete extracellular region of TIM-1, -2, -3 and -4 in insect cells. In this system, each TIM protein is natively folded and glycosylated, and is then biotinylated to enable tetramerization on a fluorochrome-labeled streptavidin for staining. The TIM-2 tetramer showed no binding to any cells tested and is not discussed further (data not shown). The TIM-3 tetramers produced by E. coli and baculovirus showed identical staining properties, and results for both will be shown for comparison. The TIM-4 tetramers produced by E. coli and baculovirus also showed identical staining properties, and hereafter only results for the TIM-4 Ig tetramer produced by E. coli will be shown.

The recombinant TIM proteins generated were purified and characterized by size exclusion chromatography, where each protein migrated as a single predominant species at the predicted molecular weight of monomeric TIM protein. Additionally, the purified recombinant proteins used in this study to generate tetramers migrated at appropriate molecular weights by SDS–PAGE, and were detectable by western blot using a streptavidin–HRP probe reflecting successful biotinylation (data not shown). Additionally, to allay concerns that the use of tetramers might detect low-affinity interactions that might not be biologically relevant, we also carried out all staining using monomeric TIM proteins in the place of each tetramer. In each experiment that follows, staining with monomeric TIM proteins showed the same pattern of binding as revealed by the tetramers (data not shown), demonstrating that TIM tetramer binding accurately reflects the behavior of monomeric protein and is not an artifact arising from the detection of irrelevant low-affinity interactions.

We first evaluated the binding of this panel of TIM tetramers to subsets of murine splenocytes (Fig. 1). As a control, we compared the binding of the TIM tetramers with a B and T lymphocyte associated (BTLA) tetramer, which binds herpesvirus entry mediator (HVEM) expressed by resting CD4⁺ and CD8⁺ T cells and CD11c⁺ DCs (20). First, each TIM tetramer showed a similar pattern of binding to the major splenocyte populations. The highest binding for TIM-1, TIM-3 and TIM-4 tetramers was to CD11c⁺ splenocytes (Fig. 1). Lower binding for each tetramer was found on B220⁺ B cells. CD4⁺ and CD8⁺ T cells bound the lowest level of each TIM tetramer.

We next characterized the binding parameters of each TIM tetramer, including the effects of pH, temperature and divalent cation dependence. We noted a strong effect of divalent cations on TIM tetramer binding to cells. Binding of TIM-3 tetramers derived from both E. coli and insect cells to all murine splenocyte subsets was completely ablated by 2 mM EGTA (Fig. 1). Binding of TIM-1 tetramers to CD4⁺ and CD8⁺ cells was reduced to background in the presence of EGTA, while there was a significant, but incomplete, reduction in binding to B220⁺ and CD11c⁺ cells (Fig. 1). The binding of TIM-4 tetramers was only modestly reduced on each splenocyte subset in the presence of EGTA (Fig. 1). As expected, BTLA tetramer staining was unaffected by the presence of 2 mM EGTA (Fig. 1).

As a control, we examined binding of TIM tetramers to cells of other species, including CHO cells, the insect cell line Sf9 and the human B cell line BJAB (Fig. 2). Unexpectedly, each TIM tetramer showed significant binding to each of these cell lines. Importantly, each tetramer displayed the same pattern of divalent cation dependence to the cell lines as was found for murine splenocytes. This divalent cation-dependent binding was due to a requirement for calcium, as the addition of calcium, but not magnesium, restored the binding of TIM tetramers to BJAB cells (Fig. 2C). In summary, TIM-1, TIM-3 and TIM-4 tetramers bind to ligands expressed by cells from several species with the same pattern of calcium dependence as seen with murine splenocytes. Calcium-dependent binding and non-species-specific interactions are two properties that are also exhibited by C-type lectins (21).

TIM proteins show both homotypic and heterotypic interactions that involve the Ig domain and mucin stalk regions

A recent report claimed that TIM-1 is a ligand for TIM-4 (3) based on the following findings: a TIM-1–Fc fusion protein displayed increased binding to cells that were transfected with TIM-4, but not with TIM-1 or TIM-3. Likewise, a TIM-4–Fc fusion protein displayed increased binding to cells transfected with TIM-1, but not with TIM-3 or TIM-4. Because the non-species-specific, calcium-dependent binding of the TIM tetramers suggested a potential role for carbohydrates in TIM tetramer binding, we wondered if binding of TIM-1 to TIM-4 reflected an interaction between the Ig domain of one TIM protein and the glycosylated mucin stalk of the other TIM protein. To test this, we generated a panel of cell lines that expressed either full length TIM proteins or truncated versions that contained the Ig domain but lacked the mucin
stalk and measured the binding of each TIM tetramer to this panel of cells (Fig. 3). This analysis confirmed the previously described interaction between TIM-1 and TIM-4 (3), since we found that our TIM-1 tetramer displayed increased binding to cells expressing full length TIM-4 and the TIM-4 tetramer bound more highly to cells expressing full length TIM-1 (Fig. 3). In contrast to the previous report, we found that the TIM-1 and TIM-4 tetramers also displayed homotypic interactions with surface expressed TIM-1 and TIM-4, respectively (3) (Fig. 3). Notably, in each case, the increased binding was lost when cells expressed the TIM-1 Ig domain only or the TIM-4 Ig domain only, indicating a requirement for the heavily glycosylated TIM-1 and -4 mucin stalks in TIM tetramer binding. Similarly, both forms of our TIM-3 tetramer displayed increased binding to cells expressing full length TIM-1 and TIM-4, but not to cells expressing TIM-1 and TIM-4 without their mucin stalk (Fig. 3). Finally, none of the TIM tetramers showed substantial increases in binding to cells expressing FL TIM-3, perhaps because the mucin stalk region of TIM-3 is substantially smaller than that present in TIM-1 and TIM-4. In summary, these results differ substantially from the previous interpretation (3) that TIM-1 and TIM-4

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**Fig. 1.** TIM-1, TIM-3 and TIM-4 display calcium-sensitive binding to ligands preferentially expressed on B220+ B cells and CD11c+ DCs. Splenocytes from BALB/c mice were stained with the indicated TIM protein tetramer–PE (open histogram) or streptavidin–PE alone (shaded histograms). Cell subsets were identified by staining with anti-CD4–FITC and anti-B220–APC or anti-CD11c–FITC and anti-CD8–APC. Cells were stained with the indicated reagents in untreated tissue culture media or in media containing 2 mM EGTA to sequester calcium ions. The BTLA-Ig tetramer–PE, which binds HVEM expressed by resting CD4+ and CD8+ T cells and CD11c+ cells, is included for comparison. TIM-1- and TIM-3-staining reagents consisting of the Ig and mucin extracellular domains were produced in insect cells. TIM-3Ig, TIM-4Ig, and BTLA-Ig-staining reagents consisting of the Ig domain only were generated in *Escherichia coli.*
are a specific receptor–ligand pair, and instead demonstrate that TIM-1, TIM-3 and TIM-4 are capable of more promiscuous intrafamilial interactions in a manner that requires an intact heavily glycosylated mucin stalk.

We next asked if the mucin stalk was sufficient for TIM tetramer binding. We evaluated TIM tetramer binding to CHO cells expressing a fusion protein consisting of the TIM-1 mucin stalk and the BTLA Ig domain. Surface expression of the
fusion protein was confirmed using a BTLA-specific antibody (Fig. 3), but fusion protein expression did not result in increased binding of TIM-1, TIM-3 and TIM-4 tetramers to these cells, indicating that the mucin stalk alone is not sufficient for TIM binding. This result could suggest either that TIM tetramers recognize a combinatorial epitope requiring the TIM-1 Ig domain and the mucin stalk or that the replacement of the TIM-1 Ig domain by the BTLA Ig domain alters carbohydrate or protein epitopes within the TIM-1 mucin domain such that it is no longer recognized by TIM-1, TIM-3 or TIM-4 tetramer.

The TIM-3 Ig domain can interact directly with carbohydrate moieties

The results above suggested that TIM protein binding may involve recognition of carbohydrates from several species and carbohydrates contained in the mucin stalks of other TIM proteins. To test directly for carbohydrate binding by TIM family proteins, we analyzed the capacity of the isolated TIM-3 Ig domain to bind a variety of purified carbohydrate structures using a glycan array screen (Fig. 4). Importantly, the TIM-3 protein used in this screen contains only the TIM-3 Ig domain, was produced in E. coli, and is not itself glycosylated. The glycan array screen contains >200 individual glycans, includes a series of negative controls for binding specificity, and uses internal positive controls consisting of known lectins and their corresponding carbohydrate ligand (see Supplementary Table 1, available at International Immunology Online). To analyze the results of the array, each glycan was ranked according to its signal-to-noise ratio (S/N) by dividing the mean relative fluorescence.
by the mean background generated in control wells that lacked glycosides. This value was compared with the average S/N for all wells in the array. This TIM-3 protein demonstrated highly specific interactions with four glycan moieties (Fig. 4). The highest interactions of the TIM-3 Ig domain were with Man$_{a}$$^{1-3}$(Xyl$_{b}$$^{1-2}$(Man$_{a}$$^{1-6}$Man$_{b}$$^{1-4}$GlcNAc$_{b}$$^{1-2}$Man$_{a}$$^{1-3}$Gal$_{b}$$^{1-4}$(Fuc$_{a}$$^{1-6}$GlcNAc$_{b}$$^{1-4}$(S/N of 22), NeuAc$_{a}$$^{2-8}$NeuAc$_{a}$$^{2-(3,6)}$Gal$_{b}$$^{1-4}$(S/N of 16) and Gal$_{b}$$^{1-4}$(Fuc$_{a}$$^{1-6}$GlcNAc$_{b}$$^{1-4}$Man$_{a}$$^{1-6}$Gal$_{b}$$^{1-4}$(Fuc$_{a}$$^{1-6}$GlcNAc$_{b}$$^{1-4}$(S/N of 13) (Fig. 4). Also, the Le$^{c}$ antigen, Gal$_{b}$$^{1-3}$GlcNAc$_{b}$, showed very high specific interactions with the TIM-3 Ig domain (S/N of 7). These results directly demonstrate the capacity of the TIM-3 Ig domain to bind carbohydrate epitopes, and are the first to show that any TIM family Ig domain can directly recognize carbohydrate moieties. The results of the TIM-3 Ig glycan array screen and, for comparison, numerous additional screens performed by the Consortium for Functional Glycomics can be viewed at http://www.functionalglycomics.org/glycomics/publicdata/primaryscreen.jsp.

Since Ig domains can interact with glycosaminoglycans (22–24), we next tested whether the TIM tetramers bind heparan sulfate and chondroitin sulfate. For this, we compared the binding of each TIM tetramer to CHO-K1 cells, that express heparan and chondroitin sulfate, and to CHO-pgsA-745 cells, which lack heparan and chondroitin sulfate due to xylosyltransferase deficiency (25) (Fig. 5). As a positive control, we showed that the soluble human FGF R1β (IICc–Fc chimera, which requires heparan sulfate for binding (24), bound to CHO-K1 cells, but not CHO-pgsA-745 cells, as expected (Fig. 5, compare panels a and f). Next, we tested the binding of each TIM tetramer to both of these cell lines. In each case, the TIM tetramers bound equally to CHO-K1 and CHO-pgsA-745 cells (Fig. 5). Thus, heparan sulfate and chondroitin sulfate are not exclusive ligands for TIM-1, TIM-3 or TIM-4.

However, we did find direct evidence that binding of TIM tetramers to cells requires glycosylation of cell-surface proteins. For this, we examined TIM tetramer binding to the IdID CHO cell line, which is defective in UDP-galactose/UDP-N-acetylgalactosamine 4-epimerase activity (26) (Fig. 6). Because of this deficiency, IdID CHO cells lack the ability to synthesize complete N-linked, O-linked and lipid-linked glycoconjugates de novo. However, IdID CHO cells can generate these glycoconjugates if grown in a media with a source of galactose (Gal) and N-acetylgalactosamine (GalNAc) to bypass the 4-epimerase defect. Specifically, IdID CHO cells grown with 10% FCS can compensate for the 4-epimerase defect through salvage pathways that provide Gal and GalNAc from serum-derived glycoproteins (26). We found that binding of each TIM tetramer to IdID CHO cells was dependent upon provision of either serum or Gal and GalNAc from serum-derived glycoproteins (26). We found that binding of each TIM tetramer to IdID CHO cells was significant reduced in conditions of 1% serum (Fig. 6, middle row). Importantly, TIM tetramer binding to IdID CHO cells was restored when 1% serum was supplemented with Gal and GalNAc (Fig. 6, lower row). Thus, binding of TIM-1, TIM-3 and TIM-4 tetramers to cells requires N- and O-linked carbohydrate addition to cell-surface proteins.
Discussion

This study suggests a role for carbohydrates and the potential for homotypic and heterotypic interactions in ligand recognition by TIM family proteins. We were initially led to examine these issues because of our unexpected finding that several TIM protein tetramers displayed binding to cells from non-murine species. This result suggested to us that the ligands for each TIM protein might be conserved, non-protein-encoded epitopes, such as carbohydrates or lipids. These interactions were non-species restricted and calcium dependent, which are two features that are also exhibited by C-type lectins (27). Further, we showed that isolated TIM Ig domains interact with other TIM proteins in a manner dependent upon the glycosylated mucin stalk of the TIM protein. We directly demonstrated carbohydrate-binding activity by identifying specific interactions between the TIM-3 Ig domain and several purified glycan structures. Finally, each TIM protein binds to cells in a manner requiring cell-surface protein glycosylation. These results are the first to suggest a carbohydrate-binding activity and the first to recognize homotypic interactions among the TIM family.

Our results describing TIM protein binding to splenocytes are in partial agreement with earlier reports. In agreement with previous work, we find that TIM-1 binds strongly to CD11c+ cells and that TIM-4 interacts strongly with B220+...
TIM family ligand-binding properties

TIM-2 was also previously identified as a Sema4A-binding partner by expression cloning from an EL4 cell cDNA library (29). This paper examined the ligand-binding properties of a Sema4A–Fc fusion protein and demonstrated an interaction with cells transfected with TIM-2. However, there was no data specifically addressing the binding properties of TIM-2 to splenocytes or cell lines. The TIM-2 tetramer we generated did not show detectable binding to any cells tested. We did not directly test whether our TIM-2 tetramer bound Sema4A, but Sema4A is reportedly expressed highly on CD11c+ DCs which did not detectably bind our TIM-2 tetramer (29). It is possible that our TIM-2 tetramer produced in insect cells is not natively folded or processed, accounting for the apparent discrepancy in TIM-2-binding characteristics. However, a recent report identifying H-ferritin as an intracellular ligand for TIM-2 was unable to reproduce the interaction between Sema4A and TIM-2, indicating that further confirmation of an interaction between Sema4A and TIM-2 is needed (14).

Several properties of TIM tetramer binding are also exhibited by lectins. First, TIM-1 and TIM-3 exhibit strongly calcium-dependent interactions with all cell types, whereas TIM-4 exhibits weak calcium dependence to most cell types. C-type lectin-like receptors such as the macrophage mannose receptor, DC-SIGN and lymphocyte adhesion molecules of the selectin family display calcium-dependent interactions with carbohydrate ligands (17, 30, 31). The interaction of C-type lectins with carbohydrate ligands is mediated by a conserved carbohydrate recognition domain (CRD). The TIM family Ig domains appear unrelated to the CRD of C-type lectins based on amino acid sequence comparisons, but are related to I-type lectins, called siglecs, in that each contains an extracellular Ig domain (32). Each siglec family member contains a V-set Ig domain that binds specific types of sialic acid attached to the terminal sugars of oligoosaccharide chains by specific glycosidic linkages (32). Thus, siglec family Ig domains provide a clear precedent for carbohydrate recognition by TIM family Ig domains.

These results have several potential implications. First, we have demonstrated the potential for homotypic and heterotypic interactions between TIM proteins that might underlie adhesion between various cell types, potentially influencing cellular activation, co-stimulation and migration. Second, carbohydrate-binding proteins are involved in cellular trafficking, pathogen recognition, antigen presentation, cellular adhesion, antigen uptake and regulation of immune activation (30). L-selectin is expressed by naive lymphocytes and regulates their migration through high endothelial venules into lymph nodes through recognition of a sialyl-Lewis X (sLeX) moiety (31). DC-SIGN recognizes pathogens via mannosenrich carbohydrate additions on the HIV-1 envelope protein gp120 (33). Notably, TIM-1 was already known as a hepatitis A virus receptor well before its linkage with asthma (2, 8, 9).

In summary, we present two new aspects of TIM family binding. We find broader specificity for TIM protein interactions than previously suggested, in that we show the potential for homotypic and heterotypic interactions between TIM
proteins, and show that surface-expressed TIM-1 and TIM-4 can serve as ligands for the TIM-3 Ig domain. Also, we find that TIM-1, -3 and -4 exhibit calcium-sensitive and non-host-specific binding properties and the TIM-3 Ig domain can interact with certain carbohydrate residues. The current challenge will be to determine the precise nature and specificity of carbohydrate binding by TIM family members and to identify proteins that contain these carbohydrate decorations.

Note added in proof
Following the submission of this manuscript, two studies were published that address TIM family protein crystal structures in relation to their ligand binding properties.


Supplementary data
Supplementary Methods and Table 1 are available at International Immunology Online.

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Abbreviations

- APC: Allophycocyanin
- BTLA: B and T lymphocyte associated
- CRD: carbohydrate recognition domain
- DC: dendritic cell
- EGTA: ethylene-bis(oxyethylenenitrilo)tetraacetic acid
- FL: full length
- Gal: galactose
- GaINAc: N-acetylgalactosamine
- GFP: green fluorescent protein
- HVEM: herpesvirus entry mediator
- IRES: internal ribosome entry site
- S/N: signal-to-noise ratio
- TIM: T cell Ig domain and mucin domain
- PO1 AI031238).

References

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