TIM-4, a Receptor for Phosphatidylserine, Controls Adaptive Immunity by Regulating the Removal of Antigen-Specific T Cells


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TIM-4, a Receptor for Phosphatidylserine, Controls Adaptive Immunity by Regulating the Removal of Antigen-Specific T Cells

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Adaptive immunity is characterized by the expansion of an Ag-specific T cell population following Ag exposure. The precise mechanisms, however, that control the expansion and subsequent contraction in the number of Ag-specific T cells are not fully understood. We show that T cell/transmembrane, Ig, and mucin (TIM)-4, a receptor for phosphatidylserine, a marker of apoptotic cells, regulates adaptive immunity in part by mediating the removal of Ag-specific T cells during the contraction phase of the response. During Ag immunization or during infection with influenza A virus, blockade of TIM-4 on APCs increased the expansion of Ag-specific T cells, resulting in an increase in secondary immune responses. Conversely, overexpression of TIM-4 on APCs in transgenic mice reduced the number of Ag-specific T cells that remained after immunization, resulting in reduced secondary T cell responses. There was no change in the total number of cell divisions that T cells completed, no change in the per cell proliferative capacity of the remaining Ag-specific T cells, and no increase in the development of Ag-specific regulatory T cells in TIM-4 transgenic mice. Thus, TIM-4–expressing cells regulate adaptive immunity by mediating the removal of phosphatidylserine-expressing apoptotic, Ag-specific T cells, thereby controlling the number of Ag-specific T cells that remain after the clearance of Ag or infection. The Journal of Immunology, 2010, 185: 6839–6849.

During an immune response to infection, Ag-specific T cells proliferate and expand in number, mediating the development of immunological memory and adaptive immunity. Following clearance of the infection, a large fraction of Ag-specific T cells is removed to prevent accumulation of no-longer needed and potentially dangerous effector cells and to leave room for future expansions (1). However, a sizable number of Ag-specific T cells still remains and some become memory cells, although the precise mechanisms that regulate the specific number of remaining Ag-specific T cells are poorly understood. This process requires the induction of apoptosis in the Ag-specific T cells, as well as their subsequent engulfment and removal by phagocytic cells. Whereas the first step involves both extrinsic and intrinsic apoptotic pathways, removal of the apoptotic cell requires the expression by phagocytic cells of receptors that recognize phosphatidylserine (PtdSer), a specific marker of apoptosis (2). The expression of PtdSer on the external surface of the plasma membrane is a key signal for recognition of apoptotic cells by phagocytes, and T cells expressing PtdSer over a threshold level are marked for rapid removal by phagocytic cells (3).

Several PtdSer-binding molecules have been identified, including cell surface receptors such as T cell/transmembrane, Ig, and mucin (TIM)-4, BA11, and stabilin-2, as well as soluble PtdSer-binding molecules such as GAS6 and MFG-E8 that bind cell surface receptors. Of these, TIM-4 is the only one whose expression is limited to immune cells, suggesting an important role for TIM-4 in clearing apoptotic cells, including the 90% of Ag-specific T cells that die during the contraction phase of an immune response. TIM-4 is a member of the TIM gene family, identified by positional cloning using a congenic mouse model, in which genetic variants of TIM1 were associated with Th2-biased immune responses and the development of allergen-induced airway hyperreactivity (AHR) (4, 5). Whereas TIM-1, TIM-2, and TIM-3 have been shown to play important roles in T cell activation and tolerance induction (6–10), the function of TIM-4 in immune responses has not been fully understood.

TIM-4 is expressed primarily on APCs, including CD11c⁺ dendritic cells (DCs), macrophages (11–15), and CD169⁺ (MOMA-1⁺) marginal metallophilic macrophages (14), and also on peritoneal B-1 B cells (16). To understand and characterize the role of TIM-4 in adaptive immune responses, we generated TIM-4–specific mAbs as well as TIM-4 transgenic (Tg) mice, which over-
expressed TIM-4 on APCs by means of a MHC class II promoter. Using these reagents, we demonstrated that blockade of TIM-4 during immunization with Ag or infection with influenza A virus increased the number of Ag-specific CD4+ T cells present both at the peak and during the contraction phase of the immune response. Conversely, overexpression of TIM-4 on APCs in Tg mice reduced the number of Ag-specific T cells that remained after immunization, resulting in greatly reduced secondary T cell responses. These findings suggest, to our knowledge, a novel pathway for immune regulation in which TIM-4—expressing phagocytic cells efficiently engulf and clear PtdSer-expressing, Ag-specific T cells. Thus, TIM-4 regulates immunity by affecting lymphocyte fate and determining the proportion of Ag-specific T cells that are purified versus the number that proceed into the memory cell compartment.

Materials and Methods

Mice

BALB/cBy mice were purchased from The Jackson Laboratory (Bar Harbor, ME); OVA-specific TCR Tg DO11.10 Rag−/− mice were used as donors of OVA-specific CD4+ T cells. For some experiments, TIM-4 Tg mice were crossed to MD4 mice Tg for hen egg lysozyme (HEL)-specific IgG and IgD expressed in B cells (17, 18). The Animal Care and Use Committee, Children’s Hospital Boston, approved all animal protocols.

Generation of mAbs

Female Lewis strain rats (Harlan Sprague-Dawley, Hayward, CA) were immunized s.c. with mouse TIM-4−Ig (12) in CFA and were boosted multiple times with mouse TIM-4−Ig in PBS or IFA. At 1 d following the last boost, lymph node cells were depleted of T cells using MACS beads (Miltenyi Biotec), and were used to test serum neutralizing activity. mAbs were purified using protein G-agarose (Pierce Biotechnology, Rockford, IL), and coupled to PE or allophycocyanin by Reametrix (San Carlos, CA).

Generation of Tim-4 Tg mice

To generate TIM-4 Tg mice, a 1032-bp EcoRI fragment containing the complete open reading frame of BALB/c mouse TIM-4 was ligated into the EcoRI site of the pDOI-5 vector (19). The 4587-bp Bgl I fragment, which included the promoter of the MHC class II Eα, the TIM-4 cDNA, and the intron and polyadenylation site of rabbit β-globin gene, was excised from the resulting plasmid, microinjected into B6CBAF/J mouse eggs, and transferred to recipients. Tail biopsies from potential founders were screened by Southern blot analysis and PCR using forward primer 5′-TGTTACATAAGGGGTGGTG-3′ and reverse primer 5′-TGAAAAATTTCTTATTTGGA-3′. Injections were performed under the direction of Dr. Yannu Chen-Tsai, at the Stanford Transgenic Research Facility, Stanford University (Stanford, CA). After backcrossing to BALB/c for 10 generations, we found that both female and male Tg mice developed normally and had normal numbers of CD4+ or CD8+ T cells (CD3+) and B cells (CD19+) in spleen and lymph nodes, as well as normal ratios of CD4+, CD8+, and double-positive T cells in thymus compared with wild-type (WT) BALB/c mice (Supplemental Fig. 2A). We saw no difference in the number of splenic DCs (CD11c+) or macrophages (CD11b+) in Tg mice compared with WT mice (data not shown). Peritoneal macrophages (PMs) from both WT and TIM-4 Tg mice expressed high levels of TIM-4 (Supplemental Fig. 2B). TIM-4 was expressed at higher levels on both CD107− and CD107+ subsets of CD11c+ DCs in the TIM-4 Tg compared with WT (Supplemental Fig. 2C), but was not detectable on plasmacytoid DCs (data not shown). TIM-4 was expressed at moderate levels on CD19+ B cells from TIM-4 Tg mice, which is expected due to the MHC class II Eα promoter used in the transgene construct. In contrast, TIM-4 was not detected on splenic CD19+ B cells from WT mice (Supplemental Fig. 2B), or on resting or activated T cells from Tg or WT mice (Supplemental Fig. 2B and data not shown).

In vitro assays

Lymph node or spleen cells were collected and restimulated with OVA, as described (6). In some experiments, CD4+ T cells were purified by positive selection using MACS CD4 microbeads (Miltenyi Biotech), and were depleted of remaining APCs by adherence on 100-nm tissue culture dishes for 2 h at 37°C. As indicated, T cell responses were measured by culturing 5 × 10^6 CD4+ T cells with 5 μg/ml anti-CD28 on a 96-well plate previously coated with 1–3 μg/ml anti-CD3. MLRs were performed by stimulating 5 × 10^6 CD4+ T cells with irradiated (2000 rads) splenocytes from C57BL/6 mice, and proliferation was measured on day 2. B cells used as APCs were prepared by positive selection from spleen of (TIM-4 Tg × MD4) or (WT × MD4) mice using CD19 MACS microbeads or goat anti-mouse Ig-coated plates, followed by depletion of adherent cells. Cells were treated with 50 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, washed three times, and used as APCs. B cells from HEL Tg mice were cultured with a HEL-OVA conjugate, prepared as described (18). Cytokine and anti-OVA (ELISAs) were performed, as described (20, 21).

Adoptive transfer of DO11.10 Rag−/− T cells

DO11.10 Tg Rag−/− T cells were positively selected from the spleens of DO11.10 Tg mice by labeling with CD4+ magnetic beads (Miltenyi Biotech) and sorting on an Automacs. Each recipient received 10^6 DO11.10 Rag−/− T cells by i.v. injection. One day later, mice were immunized with OVA/aluminum potassium sulfate (alum) i.p. For analysis of regulatory T (Treg) cells, cells were stained with CD25 FITC, KJ1-26 allophycocyanin, and CD44 allophycocyanin-A750. Cells were then fixed and analyzed for Foxp3 expression using the biosciences Foxp3 PE intracellular staining kit (eBioscience, San Diego, CA). Alternatively, for analysis of T cell activation and number, cells were stained with CD44 FITC, KJ1-26 PE, CD45 PE-Texas Red, CD62L allophycocyanin, CD4 A700, and TCRβ allophycocyanin-A750. In some experiments, T cells were labeled with CFSE (Molecular Probes, Eugene, OR), as described (22), prior to transfer. Analysis of FACS data was performed using FlowJo software (Tree Star, Ashland, OR).

Time course of T cell expansion in vitro

Protocol was based on experiments by Hawkins et al. (23) Briefly, a naive T cell population was isolated from DO11.10 Rag−/− spleens using CD4+ MACS beads. T cells were labeled with 2 μM CFSE for 3 min, and the reaction was quenched with 40% FBS/PBS. A total of 5 × 10^6 T cells were incubated with 5 μg/ml plate-bound anti-CD3, 2 ng/ml IL-2, and 10^3 3T3-Vector or 3T3–TIM-4 cells. Cells were then harvested every 12 h and assayed for T cell expansion in triplicate wells. The 3T3 cells, which are adherent, remain bound to the wells under these conditions. The few 3T3 cells that detach with the T cells are larger than the T cells and off the scale of the forward light scatter plot with the gain used and are not counted. To obtain cell counts, BD Calibrate beads were added to culture wells prior to harvest. Cell counts were then determined by multiplying the number of beads per well times the ratio of cellular events to bead events, performing the calculation P0=exp((k1 × T)+k2 × exp((-(k3 × T)+k4 × T))). Time courses were performed using GraphPad Prism Software. P2 represents a population of cells that does not divide and die at an exponential (exp) rate with a constant k2. P1 represents a starting population of cells that divides with an exponential growth coefficient k1. T is time in hours. This equation was fit to the 3T3-Vector curve to give values for P1 and P2 of 8.456 and 41.544 respectively. Using these values for P1 and P2, the equation was then fit to the 3T3–TIM-4 curve.

Preparation of apoptotic cells

Preparation of apoptotic cells and assays for engulfment of apoptotic cells were performed essentially as described (12, 24). Thymocytes isolated from 4- to 5-wk-old WT BALB/c mice were incubated with 10 μM dexamethasone (Sigma-Aldrich) in RPMI 1640 without FCS for 3 h. Cells were washed and apoptosis was confirmed by annexin V-FITC and propidium iodide staining (BD Pharmingen, San Diego, CA). The U937 human monocytic cell line was incubated with 100 μM etoposide (Sigma-Aldrich) in RPMI 1640 without FCS for 5 h and washed with PBS.

Assays for engulfment of apoptotic cells

For measurement of phagocytosis by TIM-transfected 3T3 cells, 2.5 × 10^4 3T3 cells were cultured in complete DMEM (10% FBS, 1% glutamine) in 24-well plates for 24 h. The 3T3 cells were then washed and preincubated with mouse (m) TIM-4 mAb or control mAb for 15 min. Apoptotic thymocytes labeled with pHrodo (1 μM; Invitrogen, Carlsbad, CA) or Cell-Tracker Green (CMFDA; 4 μM) according to the manufacturer’s instructions were added to the 3T3 cells in the ratio of 20:1. After 90 min of incubation at 37°C, adherent 3T3 cells were washed twice with PBS and 0.1 mM EDTA and were detached from plates by PBS containing 2 mM EDTA. Cells were again washed and resuspended in PBS, including 2%
FBS, for FACS analysis. For annexin V blocking, thymocytes were re-suspended in annexin V binding buffer (BD Pharmingen) and incubated with 0.1 mg/ml annexin V for 1 h at room temperature. Thymocytes were then added to the 3T3 cells at a ratio of 5:1 for 30 min. Resident peritoneal macrophages (10⁵) were cultured in 24-well plates or on 25-mm glass coverslips (Warner Instruments, Hamden, CT) in 6-well plates for 2 h at 37°C, and washed with PBS. mTIM-4 mAb or isotype control mAb was added to the culture for 10 min prior to addition of CMFDA (Molecular Probes, Invitrogen)-labeled apoptotic U937 cells for 30 min or apoptotic thymocytes for 15 min at 37°C. Cells were then washed with PBS. TIM-4–3T3 cells and PMNs were gated by forward and side scatter.

Apopotic thymocytes in vivo and spleen immunohistochemistry

CellTracker (CMFDA)-labeled, apoptotic thymocytes (20 × 10⁶) were injected i.v. into WT BALB/c mice that were treated with 400 μg anti-TIM-4 (2H12) or isotype control i.p. 4 h before the transfer of apoptotic cells. After 30 or 60 min of incubation, mice were sacrificed, and spleens were collected, immediately placed into OCT, and snap frozen in liquid nitrogen. Frozen sections of 4 or 6 μm were cut by Leica CM3050 Cryostat and treated with anti-CD169 (AbD Serotec, Oxford, U.K.) or with anti–TIM-4 (2H12) and anti-rat Ig-Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA). Samples were analyzed on a Zeiss Axiophot microscope with a Spot camera (Diagnostic Instruments, Sterling Heights, MI). ImageJ 1.38× (National Institutes of Health, http://rsb.info.nih.gov/ij) analysis program was used to count and quantify the amount of apoptotic cells in the spleen sections.

Confocal microscopy

Confocal images were acquired using a TE-2000 inverted microscope (Nikon Eclipse, Tokyo, Japan) with attached Orca AG camera (Hamamatsu Photonics, Hamatsu, Japan). Trajectories were done by Slidebook software.

Induction and measurement of AHR. Mice were sensitized with 50 μg OVA adsorbed in 2 mg alum on days 0 and 14 i.p. Mice were then exposed to 50 μg OVA or saline intranasally on days 14, 25, 26, and 27. On day 28, airway resistance was directly measured with invasive plethysmography (BUXCO Research Systems, Wilmington, NC) in which anesthetized and tracheostomized mice were mechanically ventilated using a modified version of a described method (25, 26).

Influenza infection

Mice were infected with 1.2 × 10⁴ PFU of influenza strain Mem71 (H3N1) in atlantic fluid (27, 28). At the indicated times, lungs were collagenase digested and stained with CD45, Thy1.2, CD8 mAbs, and H-2Kb tetramer for Influenza nucleoprotein (NP) 147–155 (TYQRTRALV) obtained from the National Institutes of Health Tetramer facility. Isotype or anti–TIM-4 mAb 2H12 (500 μg) was administered i.p. on days 0, 5, and 10.

Results

Blockade of TIM-4 results in increased numbers of Ag-specific T cells following the peak of the immune response

We and others have shown based on binding and crystallographic studies that both human and mouse TIM-4 are receptors for PtdSer, and that TIM-4–expressing cells bound and/or engulfed apoptotic cells expressing PtdSer (12, 13, 29). To investigate the role of TIM-4 in immune responses in vivo, we generated mAbs against TIM-4 in vitro with OVA. Splenocytes from anti–TIM-4–treated mice showed greater proliferation to OVA as well as greatly enhanced production of IL-4 and IFN-γ compared with control mAb (Fig. 2A). These findings are consistent with a recent report that showed enhanced T cell responses following immunization of TIM-4−/− mice (16). To track OVA-specific T cells, we transferred DO11.10 T cells (5 × 10⁶/animal) and found that 7 d after immunization the number of OVA-specific T cells was increased by treatment with anti–TIM-4 mAb compared with control mAb-treated mice, as assayed by staining with the DO11.10 anti-TCR clonotypic Ab KJ1-26. By day 9, anti–TIM-4–
treated mice had significantly higher numbers of DO11.10 T cells per spleen than control mAb-treated mice ($p < 0.01$) (Fig. 2B). Expression of activation markers CD44 and CD62L was assayed at day 5, the peak of the DO11.10 T cell response, and on day 9, when higher numbers of DO11.10 T cells were observed in anti–TIM-4–treated mice (Fig. 2C). The effector/memory phenotype (expression of CD44 and CD62L) of these cells was not different in the isotype and anti–TIM-4–treated groups; DO11.10 T cells were CD44 high at both time points, and the percentage of DO11.10 T cells that expressed low levels of CD62L was also equivalent between the two groups (Fig. 2D).

We also showed that addition of anti–TIM-4 treatment at the peak of the immune response, rather than at the initiation of the response, still led to significantly higher numbers of DO11.10 T cells per spleen on day 9 in anti–TIM-4–treated mice compared with that in control-treated mice ($p < 0.05$) (Fig. 2E). This suggested that TIM-4 functioned at least in part during the contraction phase of the immune response.

To confirm the effect of TIM-4 mAb treatment on the number of Ag-specific cells during an immune response, we infected mice with $1.2 \times 10^5$ PFU of influenza A strain Mem71 (H3N1) intranasally. The number of FLU NP147–155 tetramer-specific CD8+ positive cells in the lung peaked at day 10 in both anti–TIM-4 and isotype-treated mice, but peak levels were significantly increased ($p < 0.05$) in anti–TIM-4 mAb-treated mice. On day 14, the NP-specific tetramer+ population contracted, but anti–TIM-4 mAb treatment limited the reduction in NP-specific cells compared with mice treated with isotype control (Fig. 2F). Representative FACS plots gated on CD45+TCR$\beta^+$ cells of one mouse from each group are shown (Fig. 2G). Mock-infected control mice...
had undetectable levels of NP147–155-specific CD8⁺ cells in the lung. Together, these findings suggest that TIM-4 may have an important role in regulating the magnitude of the immune response.

**Blocking TIM-4 in vivo inhibits clearance of apoptotic cells**

We determined whether treatment with anti–TIM-4 mAb reduced the clearance of apoptotic cells in vivo. Following i.v. transfer of CMFDA-labeled apoptotic thymocytes, apoptotic cells accumulated in the splenic marginal zone, peaking at 20 min, after which the apoptotic cells were cleared. Apoptotic cells colocalized in the marginal zone with cells expressing TIM-4 and with cells expressing CD169 (Fig. 3A), a marker for marginal metallophilic macrophages located in the splenic marginal zone, which have been shown to play a critical role in clearance of apoptotic cells (30). There was a dramatic delay in apoptotic cell clearance in mice treated with TIM-4 mAb, and injected apoptotic cells accumulated in marginal zone areas of the spleen (Fig. 3B). At 60 min after transfer, greater than twice the number of apoptotic cell corpses remained in the marginal zone of TIM-4–treated than in control Ab-treated mice (p < 0.0001) (Fig. 3C).

TIM-4 blockade prevented the uptake of apoptotic cells by resident PMΦs in vitro. PMΦs (CellTracker orange labeled, orange) avidly phagocyted apoptotic U937 cells (CMFDA labeled, green), as assessed by confocal microscopy (Fig. 3D, 3E). TIM-4 mAb 21H12 reduced phagocytosis of apoptotic cells by PMΦs by 88%, whereas mAb QT3.14 moderately reduced phagocytosis (by ~70%), as assessed by flow cytometry (Fig. 3F). TIM-1 mAb 3B3 had no effect on phagocytosis by PMΦs, which do not express TIM-1. These findings suggest an important role for TIM-4 in clearance of apoptotic cells in vivo, particularly in the region of splenic marginal zone macrophages, which have been shown to be critical in induction of peripheral tolerance and prevention of autoimmunity (30).

**Activated T cells are engulfed by TIM-4–expressing phagocytic cells**

We hypothesized that TIM-4–expressing phagocytic cells engulf activated, apoptotic T cells during the course of an immune response, and thereby regulate the number of Ag-specific T cells that persist after Ag activation. To model the in vivo function of TIM-4, we used a modification of an in vitro system described by Hawkins et al. (23), in which the number of anti-CD3–activated T cells could be accurately followed in cultures containing TIM-4–expressing phagocytic cells (TIM-4–transfected 3T3 cells). After a slight decrease in T cell numbers during the first 36 h of culture (Fig. 4A), the number of T cells in cultures containing control 3T3 cells increased logarithmically from 48 to 90 h of culture, whereas the number of T cells in cultures containing TIM-4–transfected 3T3 cells did not increase significantly. At 90 h, the number of T cells in cultures with 3T3–TIM-4 cells was only 60% of that in the 3T3-vector control cultures (Fig. 4A). The reduced T cell number in the cultures with TIM-4–transfected 3T3 cells was not due to reduced T cell proliferation, because CFSE plots showed that the number of T cell divisions was equivalent to that in control cultures at every time point (Fig. 4B and data not shown). Furthermore, the cultures containing TIM-4–transfected 3T3 cells had far fewer apoptotic T cells (Fig. 4C), providing further evidence that the 3T3–TIM-4–expressing cells engulfed and cleared PtdSer-expressing cells.

Apoptotic T cells were generated by activating CD4⁺ T cells with anti-CD3 and anti-CD28 mAb for 9 d. These apoptotic T cells were avidly engulfed by TIM-4–3T3, but not control 3T3 cells. This phagocytosis was blocked with TIM-4 mAb 21H12, but not control mAb (Fig. 4D), demonstrating that T cells expressing PtdSer are engulfed, and this process is mediated by TIM-4. Together, these experiments provide strong evidence that TIM-4–expressing cells are able to reduce the number of activated...
FIGURE 4. TIM-4 mediates phagocytosis of apoptotic and activated T cells. A–C, A total of 5 × 10⁴ T cells was cultured with 5 μg/ml plate-bound anti-CD3, 2 ng/ml IL-2, and 10⁴ 3T3-Vector or 3T3-TIM-4 cells and analyzed for T cell expansion. A, T cell counts with SD are shown. B, CFSE plots at 90 h show equivalent number of T cell divisions, as analyzed by the proliferation function in FlowJo. C, Forward scatter-side scatter plots show gates for BD Calibrite beads, apoptotic cells, and live cells. D, Purified CD4⁺ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 9 d and then labeled with CMFDA. Untransfected or mTIM-4–transfected 3T3 cells were pretreated with TIM-4 mAb or control mAb, incubated with labeled apoptotic CD4⁺ T cells, and analyzed by FACS. Data are presented as percentage of engulfment, indicating the percentage of transfected cells that have phagocytosed apoptotic cells. One experiment representative of three is shown.

T cells, and support the idea that TIM-4–expressing phagocytic cells can regulate the number of Ag-specific T cells by removing PtdSer-expressing apoptotic and activated T cells.

TIM-4 Tg mice demonstrate reduced secondary responses

To further examine the role of TIM-4 in immune responses, we generated TIM-4 Tg mice using a TIM-4 cDNA construct under control of the MHC class II I-Eα promoter. TIM-4 Tg mice developed normally and had similar lymphocyte populations to WT in spleen, lymph node, and thymus (Supplemental Fig. 2A). TIM-4 Tg mice expressed TIM-4 at higher levels on I-Ad⁺ populations in spleen, lymph node, and thymus (Supplemental Fig. 2A). TIM-4 Tg mice developed normally and had similar lymphocyte populations to WT when immunized with OVA and thymus (Supplemental Fig. 2A). TIM-4 Tg mice immunized with OVA at higher levels on I-Aβ⁺ populations including CD8α⁻ and CD8α⁺ subsets of CD11c⁺ DCs, and CD19⁺ B cells (Supplemental Fig. 2B, 2C). Nine days after immunization of the TIM-4 Tg mice with OVA in IFA, in vitro proliferation of lymph node cells to OVA was significantly reduced compared with that of WT control mice (Fig. 5A). In addition, lymph node cells from the OVA-immunized TIM-4 Tg mice produced less IL-4 and IFN-γ compared with that of WT mice (Fig. 5A). Similar results were obtained using an additional TIM-4 Tg founder line. TIM-4 Tg mice also demonstrated significantly reduced responses compared with WT when immunized with OVA i.p. in a different adjuvant, alum (Supplemental Fig. 3A), indicating that TIM-4 Tg mice have decreased secondary T cell responses to Ag regardless of adjuvant or route of immunization. In addition, when lymph node cells were assayed 4 wk following immunization of mice with OVA in alum, T cells from TIM-4 Tg mice had reduced proliferative responses compared with WT (Supplemental Fig. 3B), suggesting that T cells from TIM-4 Tg mice have reduced memory responses.

To determine whether multiple boosts with Ag would increase or overcome the low responses observed in TIM-4 Tg mice, we immunized WT and TIM-4 Tg mice with OVA (10 μg) in alum i.p. on day 0 and boosted on days 14 and 21. The low OVA response in TIM-4 Tg mice was not overcome with multiple immunizations and remained low (Fig. 5B). Moreover, when the TIM-4 Tg mice were immunized multiple times, production of IL-4 and IFN-γ was nearly abolished compared with WT controls (Fig. 5B). TIM-4 overexpression primarily affected T cells and not B cells, as no significant difference was detected in serum Ab titers of OVA-specific IgG1, IgG2a, and IgE Abs from the TIM-4 Tg mice versus WT mice (Supplemental Fig. 3C).

TIM-4 Tg mice exhibit reduced AHR in a murine model of asthma

CD4 Th cells have been shown to be a critical effector cell in several murine models of asthma (31). TIM-4 Tg mice develop reduced AHR when sensitized and challenged intranasally with the model protein Ag OVA. Indeed, invasively measuring lung resistance to increasing doses of methacholine showed that OVA-sensitized and challenged TIM-4 Tg mice (●) exhibited significantly reduced AHR compared with sensitized and challenged WT mice (▲, p < 0.01, two-way ANOVA, Fig. 5C). These results show that reduced CD4 T cell responses in the TIM-4 Tg lead to a concomitant reduction in AHR in a murine model of asthma.

To demonstrate that the reduced T cell response in the TIM-4 Tg mice was due to decreased T cell activity and not reduced APC activity, we showed that CD4⁺ T cells purified from TIM-4 Tg mice immunized once with OVA in alum or OVA in CFA i.p. proliferated poorly in vitro in response to OVA and irradiated spleen cells from an unimmunized WT mouse compared with T cells from WT mice. In addition, CD4⁺ T cells purified from TIM-4 Tg mice showed greatly reduced cytokine production compared with T cells from WT mice (Fig. 5D, 5E). These results confirm that the reduced Ag-specific in vitro recall response to OVA in TIM-4 Tg mice was due to reduced Ag-specific T cell function rather than reduced APC function.

The reduced secondary T cell response to OVA observed in the TIM-4 Tg mice was also not due to intrinsic defects in the T cells, as purified CD4⁺ T cells from TIM-4 Tg mice proliferated normally on in vitro stimulation with anti-CD3 and anti-CD28, or when stimulated with irradiated splenocytes from C57BL/6 mice as alloantigen (Supplemental Fig. 4A). In addition, DCs from TIM-4 Tg mice were able to stimulate primary responses of naïve T cells equally as well as DCs from WT mice. Thus, DCs from Tg or WT mice pulsed with OVA induced similar levels of IL-2 or IFN-
γ cytokine production by CD4+ DO11.10 T cells in vitro (Supplemental Fig. 4B). Furthermore, B cells from TIM-4 Tg mice did not inhibit T cell activation, as B cells from TIM-4 Tg mice crossed to Ig receptor Tg mice specific for hen egg lysozyme (HEL) (17, 18) were able to activate DO11.10 T cells and induce similar levels of proliferation and IL-2 production as HEL-specific B cells from control mice, when HEL-OVA was used as Ag (Supplemental Fig. 4C).

**Generation of Treg cells is not enhanced in TIM-4 Tg mice**

The development of Treg cells is a potential mechanism that limits T cell responses. The reduced secondary T cell responses to OVA observed in the TIM-4 Tg mice were not due to development of OVA-specific (adaptive) Treg cells, because OVA-specific Treg cells (KJ1-26, Foxp3+ cells) were not increased in popliteal lymph nodes 9 d after immunization of recipient TIM-4 Tg mice compared with WT mice (0.62 and 0.51%, respectively) (Fig. 6A) following transfer of T cells from DO11.10 Rag−/− mice. The number of natural CD25+ Treg cells (CD4+, Foxp3+) was also similar in both the TIM-4 Tg and WT recipient mice (13.3 and 11.1%, respectively), suggesting that neither adaptive nor natural Treg cells were responsible for the reduced secondary immune responses of TIM-4 Tg mice (Fig. 6A).

Whereas Treg percentages were comparable, we noted that at 9 d after transfer, the total number of OVA-specific DO11.10 cells present in TIM-4 Tg mice was reduced compared with that in WT recipients, 2.1×10⁴ (0.30%) in TIM-4 Tg versus 3.2×10⁴ (0.53%) in WT (Fig. 6A). To examine whether the initial in vivo expansion of T cells in TIM-4 Tg mice in response to Ag was normal, we labeled OVA-specific CD4+ T cells from WT DO11.10 mice with CFSE, adoptively transferred the labeled T cells into TIM-4 Tg and WT mice, and immunized the recipients with OVA. This allowed us to visualize the early response of T cells in these mice, by monitoring cell division in the TIM-4 Tg versus WT following immunization. We found that cell cycle progression in the responding OVA-specific T cells was similar in WT and Tg recipients on days 3 and 5 after immunization (Fig. 6B and data not shown), indicating that the initial division of Ag-specific T cells occurs normally in TIM-4 Tg mice. These results are similar to the in vitro findings from Fig. 4A and 4B, and indicate that TIM-4 may affect cell numbers without affecting progression through cell division.

**FIGURE 5.** TIM-4 Tg mice immunized with OVA demonstrate greatly reduced secondary T cell responses. A, TIM-4 Tg or WT BALB/c mice were immunized with OVA (300 μg) in IFA s.c. Lymph nodes were removed after 9 d and cells were cultured with OVA, as indicated. B, WT or TIM-4 Tg mice were immunized three times with 10 μg OVA in alum i.p. on days 0, 14, and 21. On day 26, mice were sacrificed and splenocytes were restimulated with OVA in vitro. C, WT and TIM-4 Tg mice were immunized on days 0 and 14 with saline or 50 μg OVA in alum i.p. On days 14 and 24–27, mice received 50 μg OVA or saline intranasally. Airway resistance was measured using invasive BUXCO. One representative experiment of three is shown. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test to compare WT and Tg OVA groups, n = 5. D and E, WT and TIM-4 Tg mice were immunized with 50 μg OVA in alum i.p. (D) or in CFA i.p. (E). Spleens were removed after 9 d, and CD4+ T cells were purified and cultured with OVA and irradiated splenocytes from a WT mouse. Results are shown as the mean ± SD. **p < 0.01.
The ability of the remaining CD4$^+$ T cells to proliferate.

T cells that persist during and following the contraction phase of an immune response are known as memory T cells. The contraction phase is a process where the CD4$^+$ T cells are activated, proliferate, and then undergo apoptosis to reduce the number of activated cells. However, a subset of these cells persists and can be activated again by antigen-specific stimulation.

**FIGURE 6.** Following secondary immunization, TIM-4 Tg mice have reduced numbers of Ag-specific T cells without alteration in function or generation of Foxp3$^+$ Treg. A, CD4$^+$ T cells purified from TCR Tg DO11.10 Rag$^{-/-}$ mice were adoptively transferred (10$^5$ cells/mouse) to WT or TIM-4 Tg mice. Mice were immunized with OVA (50 μg) in alum, and 9 d later the percentage of CD4$^+$Foxp3$^+$ Treg cells in the popliteal lymph node was determined by flow cytometry by first gating on TCR$^+$ cell population. Two representative mice of four are shown. B, DO11.10 T cells were CFSE labeled and adoptively transferred (3 × 10$^5$ cells/mouse) to WT or TIM-4 Tg mice. Recipients were immunized after 15 h with OVA (50 μg) in alum i.p. Spleens were removed after 5 d, and proliferation of OVA-specific T cells was detected as dilution of CFSE, by gating on CD4$^+$ KJ1-26$^+$ population. One experiment of two is shown. C, WT and TIM-4 Tg mice received 10$^5$ DO11.10 Rag$^{-/-}$ T cells i.v., and were immunized i.p. with OVA in alum on days 1 and 8. Spleens were harvested on day 15, and the number of DO11.10-specific cells was quantified. Pooled data of two experiments are shown. D, Cells from C were stained with the TCR clonotype-specific Ab KJ1-26, CD4, TCR$^+$, CD44, and CD62L. CD4 and KJ1-26 plots (top panels) show cells gated on CD4$^+$ TCR$^+$ cells. E, KJ1-26$^+$CD4$^+$ cells from D were sub gated and plotted for CD44 and CD62L. Mean fluorescent intensity of these markers reveals no significant differences between WT and TIM-4 Tg recipient mice (p > 0.05, Student t test, mean + SEM shown). D011.10 T cells were not detected in control WT mice that did not receive DO11.10 T cells. F, WT and TIM-4 Tg mice received DO11.10 cells i.v. and were immunized with OVA in alum. Seven days later, DO11.10 T cells were sorted from pooled spleens of recipient mice, and 5 × 10$^5$ DO11.10 T cells were cultured with 5 × 10$^6$ irradiated WT splenocytes pulsed with OVA 323–339 peptide. One experiment of two is shown. *p < 0.05, Student t test, mean + SEM shown.

**Discussion**

In these studies, we demonstrated that TIM-4, a cell surface PtdSer receptor expressed on APC and macrophage populations, but not T cells, regulates T cell responses through, to our knowledge, a novel mechanism involving the clearance of PtdSer-expressing, apoptotic-predisposed T cells. Using both protein Ag and infection models, we found that treatment with a TIM-4 mAb that blocked TIM-4 binding to PtdSer greatly increased secondary T cell responses as measured by proliferation and cytokine production, as well as the number of Ag-specific CD4 and CD8 T cells. Conversely, overexpression of TIM-4 on APCs in a Tg mouse model resulted in decreased secondary T cell responses and decreased Ag-specific T cell numbers. We also showed that TIM-4–expressing phagocytic cells rapidly engulfed apoptotic T cells, a process that could be specifically blocked by mAb against TIM-4. These results strongly suggest that TIM-4 regulates immunity through a pathway in which TIM-4–expressing APCs control the clearance of Ag-specific T cells, thereby regulating the size of the Ag-specific memory T cell population.

Engulfment of apoptotic cells has been studied previously in the context of self-tolerance, in which the prevention of autoimmunity requires engulfment of autoantigen-expressing somatic cells by DCs, which then induce T cell tolerance to self Ags (32, 33). Removal of cells undergoing programmed cell death has also been...
studied in the context of normal physiological processes in which dying cells are silently removed without inflammation. In contrast, little is known of the molecular mechanisms by which apoptotic T cells are cleared during an Ag-specific immune response (34, 35). It is well known that after immunization, Ag-reactive T cells expand exponentially, but that >90% of the T cells die by apoptosis during the contraction phase, mediated by expression of Bim and Fas (36–38), leaving a population of memory T cells, generally ∼10% of the peak number, but still >100-fold above the initial frequency (39–41). The number of remaining Ag-reactive memory T cells depends on the extent of the expansion and contraction phases of the immune response, and determines the rapidity and strength of subsequent memory responses. We suggest that TIM-4 and PtdSer control immune responses by regulating the clearance of Ag-specific T cells during the late expansion and early contraction phases of immune responses.

During the course of the immune response, expression of PtdSer by T cells occurs at two stages, as follows: shortly after cell activation (42, 43), when it is exposed and reinternalized, and during apoptosis at levels that remain high as a consequence of signaling through receptors of apoptosis, such as FAS and TRAIL, or due to insufficient survival signals via common γ-chain cytokine receptors. The expression of PtdSer on the external surface of the plasma membrane is a key signal for recognition of cells by phagocytes, and T cells expressing PtdSer over a threshold level are rapidly removed by phagocytic cells (3). T cells expressing levels of PtdSer below this threshold would normally continue to survive and progress into the memory T cell pool, but in TIM-4 Tg mice, even T cells expressing low levels of PtdSer are likely to be removed by phagocytes overexpressing TIM-4. We believe that TIM-4–mediated engulfment of PtdSer-expressing cells is an important mechanism that controls the size of the Ag-specific memory T cell pool, by regulating the fraction of Ag-specific cells that are selected for removal.

The marginal zone of the spleen is a major site of clearance of PtdSer-expressing cells (30), in which TIM–4–expressing CD169+ metallophilic macrophages reside (14, 44). These macrophages play a critical role in regulating immune responses because the failure of these macrophages to remove apoptotic cells impairs tolerance (30, 33). In our studies, we demonstrated that transferred apoptotic cells localized to the splenic marginal zone, which also stained with TIM-4 and CD169 mAbs, and treatment with anti–TIM-4 mAb in vivo delayed clearance of apoptotic cells in this site. In contrast, a recent study found that in vitro uptake of apoptotic cells by isolated splenic macrophages or DCs was not affected by TIM-4 deficiency, although engulfment of apoptotic cells by isolated peritoneal cells from TIM–4–deficient mice was greatly reduced compared with WT (16). However, in these studies, isolated splenic cells are not likely to replicate the complex events that occur in the tissue microenvironment of the splenic marginal zone, where a specific population of TIM–4+ marginal metallophilic macrophages engulfs apoptotic cells.

The restricted expression of TIM-4 may explain the role of TIM-4 on regulating the number of Ag-specific T cells. TIM–4 is expressed by CD8+ and CD8+ DCs, as well as by macrophages (12) in the marginal zone of the spleen (14, 44), and in lymph nodes (data not shown), but not in resting or differentiated T cells (14) (Supplemental Fig. 2). In contrast, other PtdSer receptors, such as MFG-E8 and GAS6, are widely expressed by somatic cells (32). This suggests that TIM-4 differs from MFG-E8 and GAS6 by specifically regulating immune function by targeting T cells passing through the lymphoid tissues, where TIM-4 is preferentially expressed.

We showed that secondary T cell responses in TIM-4 Tg mice were diminished compared with that in WT mice and remained low following multiple Ag boosts. Moreover, we demonstrated that the reduced secondary T cell responses in TIM–4 Tg mice were not due to the development of increased numbers of either Ag-specific adaptive or natural Treg cells. Because secondary responses were decreased in TIM–4 Tg mice, but enhanced with a blocking TIM-4 mAb (Fig. 2), we suggest that TIM–4–expressing macrophages inhibit immune responses by removing Ag-specific apoptotic T cells during the contraction phase of the immune response.

We and others demonstrated that both human and mouse TIM-4 bind PtdSer (12, 13), a finding confirmed by the cocrystallization of TIM–4 with PtdSer (29). TIM–4 was previously reported to be a ligand for TIM–1 (11). Both TIM-1 and TIM–4 may have several different ligands depending on conditions (45), and the physiological ligands and interactions of TIM–1 and of TIM–4 under different conditions are still being determined. Miyaniishi et al. (13) have shown that TIM-1 and TIM–4 can bind to separate sites on the surface of an exosome, and this bridge can give the appearance of an interaction. Exosomes are nanovesicles secreted by many cell types and expose PtdSer on their outer leaflet and are derived from intracellular structures of the endosomal pathway called multivesicular bodies (46). Thus, TIM-4 binds PtdSer, but also may bind to TIM–1, although the TIM–1–TIM–4 binding may be of lower affinity (47) and/or mediated by bridging PtdSer on exosomes or apoptotic bodies (13).

Because both TIM–1 and TIM–4 bind PtdSer (12), the possibility exists that TIM–1 might also be involved in engulfment of apoptotic cells in vivo. Only APCs, such as peritoneal macrophages and DCs, express high levels of TIM–4, whereas activated T cells and differentiated Th2 cells express TIM–1 and do not express TIM–4 (6). Whereas expression of TIM–1 by kidney epithelial cells facilitates phagocytosis (12, 48), we found that expression of TIM–1 by T cells allows T cells to bind apoptotic cells, but does not enable T cells to engulf apoptotic cells (24), suggesting that the engulfment machinery that is required for phagocytosis is not present in T cells. The tyrosine-signaling motifs present in the cytoplasmic domain of TIM–1 might allow the T cell to respond to the health of the APCs or surrounding tissue by signaling in response to the level of PtdSer on interacting cells. These observations suggest that TIM–4, but not TIM–1, mediates the clearance of apoptotic T cells and regulates the size of the T cell memory population.

We and others recently showed that TIM–3 is also a receptor for PtdSer (24, 49), which binds PtdSer with a lower affinity than TIM–1 and TIM–4 (24), and that TIM–3–expressing cells could bind and/or engulf apoptotic cells (24, 49). TIM–3, which also binds galectin-9 (50), is expressed on differentiated Th1 cells (7), and engagement of TIM–3 by galectin-9 has been shown to lead to Th1 cell death and decreased IFN–γ production (50). TIM–3 is also expressed by mast cells (51) and by subpopulations of DCs and macrophages (52). TIM–3–expressing DCs were shown to phagocytose apoptotic cells and cross-present apoptotic cell–associated Ag to CD8+ T cells (49). Outcome of phagocytosis mediated by TIM–3– or TIM–4–expressing APCs may differ, as TIM–3 has a tyrosine kinase-signaling motif in the cytoplasmic domain, but TIM–4 does not (5). Further studies are needed to elucidate the role of TIM–3–mediated recognition of apoptotic cells in regulation of immune responses.

TIM–4 lacks the intracellular tyrosine phosphorylation motifs present in mouse TIM–1, TIM–2, and TIM–3 that have been shown to participate in T cell signaling (53–55). The role of TIM–4, which regulates the extent of secondary T cell responses, is quite distinct from that of TIM–2 and TIM–3, which provide down-modulatory
signals that directly attenuate Th2- and Th1-type responses, respectively (7, 53), and is distinct from that of TIM-1, which costimulates and enhances T cell proliferation and cytokine production. Nevertheless, each of the TIM family members regulates some aspect of T cell survival, suggesting that the TIM gene family evolved to regulate immune responses by determining the fate of T cells.

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Disclosures

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References


