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TIM-1 induces T cell activation and inhibits the development of peripheral tolerance

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We have examined the function of TIM-1, encoded by a gene identified as an 'atopy susceptibility gene' (*Havcr2*), and demonstrate here that TIM-1 is a molecule that costimulates T cell activation. TIM-1 was expressed on $CD4^+$ T cells after activation and its expression was sustained preferentially in T helper type 2 (T_H2) but not T_H1 cells. *In vitro* stimulation of $CD4^+$ T cells with a TIM-1-specific monoclonal antibody and T cell receptor ligation enhanced T cell proliferation; in T_H2 cells, such costimulation greatly enhanced synthesis of interleukin 4 but not interferon-γ. *In vivo*, the use of antibody to TIM-1 plus antigen substantially increased production of both interleukin 4 and interferon-γ in unpolarized T cells, prevented the development of respiratory tolerance, and increased pulmonary inflammation. Our studies suggest that immunotherapies that regulate TIM-1 function may downmodulate allergic inflammatory diseases.

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The study of altered phenotypes arising from genetic variation or mutation has provided important insight into the immune system. For example, examination of patients with monogenic diseases such as X-linked agammaglobulinemia or severe combined immunodeficiency has provided fundamental information about the biology of B cells and T cells. Moreover, the study of the specific genes responsible for these diseases, such as those encoding Bruton's tyrosine kinase, responsible for X-linked agammaglobulinemia[1], and interleukin 2 receptor-γ (IL-2Rγ) chain[2], recombination activating genes 1 and 2 (ref. 3), Janus kinase 3 (ref. 4) and IL-7R[5], responsible for severe combined immunodeficiency, has greatly advanced the understanding of disease pathogenesis and the function of the immune system at the molecular level. In a similar way, the study of patients with polygenic disorders such as asthma and allergic disease or autoimmune diseases has improved knowledge of immune responses as well as the function of immunoglobulin E (IgE), mast cells and T helper type 1 (T_H1) and T_H2 lymphocytes in inflammation. Although the identification of specific 'susceptibility genes' for complex traits such as asthma is much more difficult than it is for monogenic traits, the investigation of these susceptibility genes provides essential molecular insight into the regulation of immune responses and inflammation.

Havcr1 (encoding the TIM-1 glycoprotein) was identified as being important in asthma and allergy susceptibility and therefore could provide useful information about the regulation of immune responses[6,7]. The family of genes encoding TIM proteins was positionally cloned using a congenic mouse model of asthma, in which distinct genetic variants encoding TIM-1 were associated with development of T_H2-biased immune responses and the development of allergen-induced airway hyperreactivity (AHR). TIM family members are cell surface glycoproteins with common structural motifs including an IgV domain, a mucin-like domain, a transmembrane helix and a cytoplasmic domain. TIM-1, TIM-2 and TIM-3 contain a predicted intracellular tyrosine phosphorylation motif[6,8]. In humans, *HAVCR1* (encoding the TIM-1 glycoprotein) is an important atopy susceptibility gene; specific alleles are associated with protection against atopy, particularly in people previously exposed to hepatitis A virus (HAV)[7,9], suggesting that TIM-1 controls critical regulatory pathways in the immune system. The relationship between TIM-1, hepatitis A infection and atopy is noteworthy, because TIM-1 functions as the receptor for HAV[10] and because infection with HAV is associated with protection against the development of atopy[11,12]. We hypothesize that HAV interacts differently with specific polymorphic variants of TIM-1 and this interaction alters T cell differentiation, activity or survival in such a way as to protect against the development of atopy. Thus, *Havcr1* as an atopy susceptibility gene integrates environmental and genetic factors crucial for the development of asthma and allergy, but the specific immunological mechanisms by which TIM-1 regulates the immune response are not understood.

The purpose of our studies here was to define the mechanisms by which TIM-1 functions in the immune system, particularly in the context of antigen-specific immune responses. To do this, we generated a TIM-1-specific monoclonal antibody (mAb) that we used in both *in vitro* and *in vivo* experiments. Using this antibody, we

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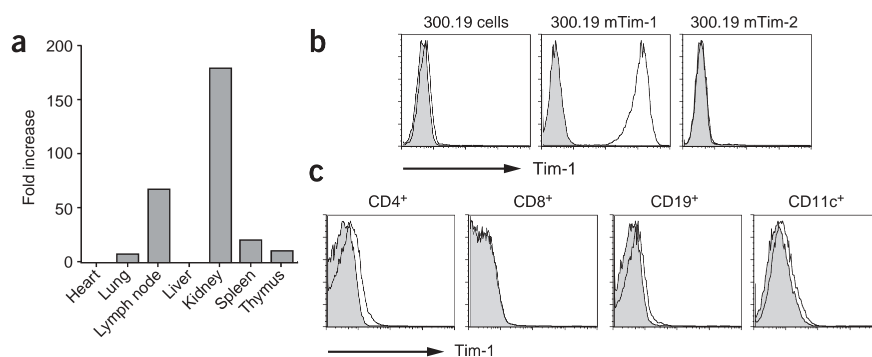

Figure 1 Expression of TIM-1 in mouse lymphoid cells and tissues. (**a**) Quantitative (real-time) PCR analysis of total RNA isolated from various tissues of BALB/c mice. Data are presented as expression of mouse TIM-1 normalized to 18S rRNA and relative to TIM-1 expression in heart tissue. Data are representative of three experiments. (**b**) The 3B3 mAb is specific to TIM-1. Untransfected 300.19 cells (300.19) or 300.19 cells stably transfected with mouse TIM-1 (300.19 mTIM-1) or mouse TIM-2 (300.19 mTIM-2) were stained with 3B3 mAb to TIM-1 (open histograms) or rat IgG isotype control (filled histograms). Data are representative of four experiments. (**c**) TIM-1 expression on $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells and $CD11c^+$ bone marrow–derived DCs. Spleen cells from BALB/c mice were purified with $CD4^+$, $CD19^+$ or $CD8^+$ MACS columns, stained with FITC-conjugated mAbs to cell surface markers (above histograms), costained with phycoerythrin-conjugated 3B3 mAb to mouse TIM-1 or rat IgG2a isotype control (filled histograms) and analyzed by flow cytometry. Data represent expression of TIM-1 on gated populations (open histograms) and are representative of four experiments.

demonstrated that TIM-1 was expressed after activation of naive T cells and on T cells that differentiated in T_H2-polarizing conditions. In addition, we found that engagement of TIM-1 on the surface of $CD4^+$ T cells in combination with T cell receptor (TCR) stimulation provided a potent costimulatory signal that greatly enhanced T cell proliferation and T_H2 cytokine production. Moreover, *in vivo* administration of mAb to TIM-1 greatly increased T cell proliferation and cytokine production and prevented the development of respiratory tolerance, resulting in the development of pulmonary inflammation and AHR in response to antigen challenge. These studies confirm that TIM-1 is essential in regulation of the immune system and suggest that immunotherapies directed at regulating TIM-1 function might be beneficial in controlling allergic inflammatory diseases and asthma.

RESULTS

Expression of mouse TIM-1

We examined expression of TIM-1 in various tissues by quantitative RT-PCR. TIM-1 transcripts were present in high abundance in the lymph node and in low abundance in the spleen, lung and thymus. This expression pattern was consistent with the initial identification of TIM-1 RNA expression by CD4 T cells and concanavalin A–stimulated splenocytes[6]. In addition, our RT-PCR results demonstrated that kidney tissues had high expression of TIM-1 mRNA, perhaps reflecting the expression of TIM-1 in ischemic renal tissue, as has been described in rats[13]. We did not detect TIM-1 in heart or liver tissue (**Fig. 1a**).

To study the cell surface expression pattern and function of mouse TIM-1, we generated mAbs specific for TIM-1 by immunizing a rat with a fusion protein (mouse TIM-1–Ig) containing the immunoglobulin domain of mouse TIM-1. The mAb 3B3 (rat IgG2aK) recognized TIM-1 on 300.19 cells transfected with mouse TIM-1 but did not 'react with' mouse TIM-2 (**Fig. 1b**), mouse TIM-3 or mouse TIM-4 (**Supplementary Fig. 1** online). The mAb 3B3 also blocked interaction between mouse TIM-1–Ig and cells expressing a putative TIM-1 ligand (such as untransfected 300.19 cells; **Supplementary Fig. 2** online), suggesting that 3B3 recognized a critical epitope on TIM-1 required for binding to its ligand.

We identified the lymphoid cells that expressed TIM-1 by staining purified populations of $CD4^+$, $CD8^+$ or $CD19^+$ cells from the spleens of unmanipulated BALB/c mice with the 3B3 mAb to TIM-1. TIM-1 staining was detectable on $CD4^+$ T cells but not on $CD8^+$ T cells.

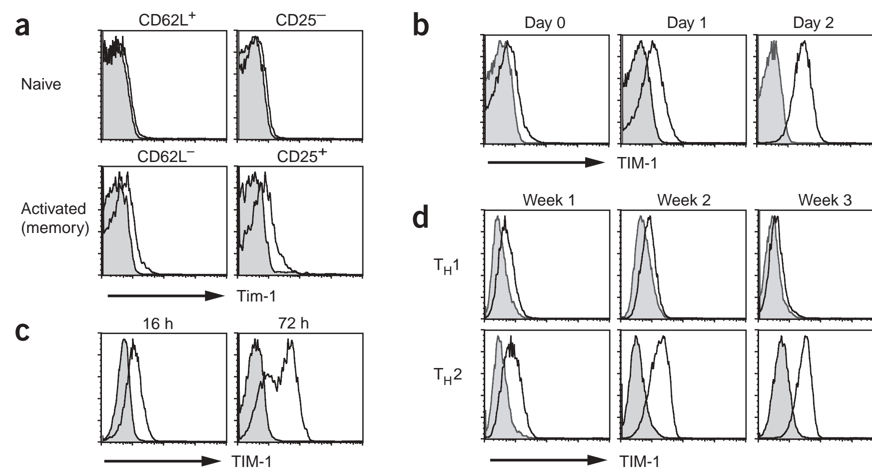

Figure 2 TIM-1 expression on activated T cells. (**a**) $CD4^+$ T cells were purified from spleens of BALB/c mice and were stained with mAb to CD4, CD25 or CD62L and costained for TIM-1 with 3B3 mAb to mouse TIM-1. Cells were analyzed by flow cytometry, gated on $CD4^+$ and surface marker–positive or surface marker–negative populations. (**b**) TIM-1 expression on T cells activated by anti-CD3 and anti-CD28. $CD4^+$ T cells were purified from spleens of BALB/c mice and were incubated with plate-bound anti-CD3 and soluble anti-CD28. Cells were stained at 24 and 48 h for TIM-1 and CD4 and were analyzed by flow cytometry with gating on live $CD4^+$ cells. (**c**) TIM-1 expression after activation with antigen and APCs. Naive DO11.10 $CD4^+$ T cells were incubated with bone marrow–derived DCs and OVA peptide. At 16 and 72 h, cells were stained and analyzed as described in **b**. (**d**) TIM-1 is preferentially expressed on polarized T_H2 cells. Naive DO11.10 cells were stimulated *in vitro* in T_H1- and T_H2-polarizing conditions. After each round of restimulation, T_H1 and T_H2 cells were stained with mAbs to CD4 and TIM-1 as described in **c**. Data represent TIM-1 expression (open histograms) relative to isotype control (filled histograms) and are from one of three similar experiments (**a**,**c**,**d**) or are representative of four experiments (**b**).

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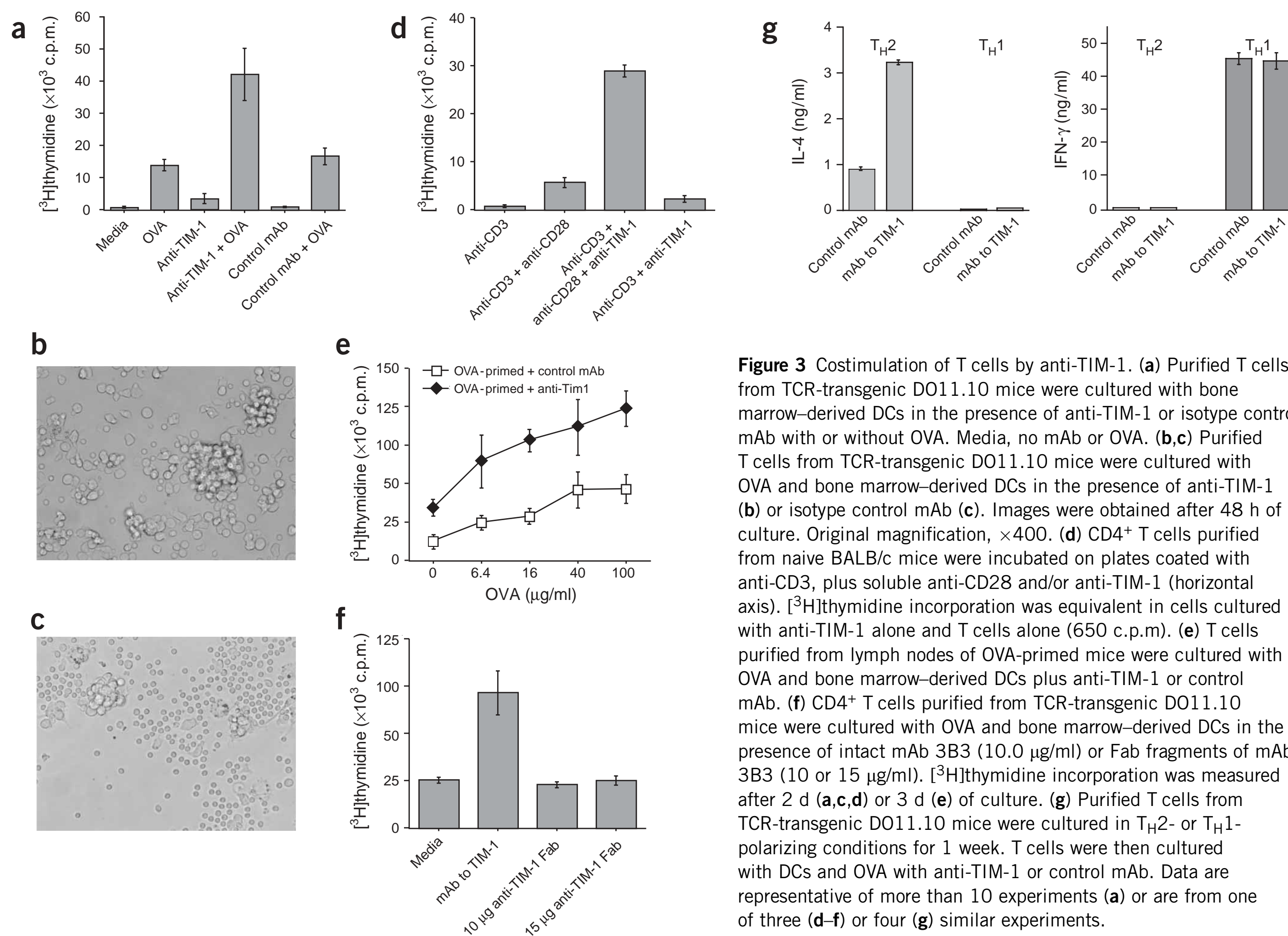

Figure 3 Costimulation of T cells by anti-TIM-1. (**a**) Purified T cells from TCR-transgenic DO11.10 mice were cultured with bone marrow–derived DCs in the presence of anti-TIM-1 or isotype control mAb with or without OVA. Media, no mAb or OVA. (**b**,**c**) Purified T cells from TCR-transgenic DO11.10 mice were cultured with OVA and bone marrow–derived DCs in the presence of anti-TIM-1 (**b**) or isotype control mAb (**c**). Images were obtained after 48 h of culture. Original magnification, ×400. (**d**) $CD4^+$ T cells purified from naive BALB/c mice were incubated on plates coated with anti-CD3, plus soluble anti-CD28 and/or anti-TIM-1 (horizontal axis). [^{3}H]thymidine incorporation was equivalent in cells cultured with anti-TIM-1 alone and T cells alone (650 c.p.m). (**e**) T cells purified from lymph nodes of OVA-primed mice were cultured with OVA and bone marrow–derived DCs plus anti-TIM-1 or control mAb. (**f**) $CD4^+$ T cells purified from TCR-transgenic DO11.10 mice were cultured with OVA and bone marrow–derived DCs in the presence of intact mAb 3B3 (10.0 μg/ml) or Fab fragments of mAb 3B3 (10 or 15 μg/ml). [^{3}H]thymidine incorporation was measured after 2 d (**a**,**c**,**d**) or 3 d (**e**) of culture. (**g**) Purified T cells from TCR-transgenic DO11.10 mice were cultured in T_H2- or T_H1-polarizing conditions for 1 week. T cells were then cultured with DCs and OVA with anti-TIM-1 or control mAb. Data are representative of more than 10 experiments (**a**) or are from one of three (**d**–**f**) or four (**g**) similar experiments.

$CD11c^+$ bone marrow–derived dendritic cells (DCs) and $CD19^+$ B cells had low expression of TIM-1 (**Fig. 1c**).

TIM-1 expression on activated T cells

We next showed that activated but not resting naive $CD4^+$ T cells expressed TIM-1. Splenic activated-memory $CD4^+$ T cells ($CD62L^-$ and $CD25^+$) from unmanipulated mice expressed more TIM-1 than did naive $CD62L^+$ and $CD25^-$ cells (**Fig. 2a**). To study TIM-1 expression on $CD4^+$ T cells in greater detail, we examined the kinetics of TIM-1 expression after activation. Stimulation with antibody to CD3 (anti-CD3) and anti-CD28 increased TIM-1 expression after the first 24 h of culture. At 48 h, TIM-1 expression was increased further (**Fig. 2b**). To examine TIM-1 expression during the course of an antigen-specific response, we cultured T cells purified from TCR-transgenic DO11.10 mice with ovalbumin (OVA) peptide in the presence of bone marrow–derived DCs as antigen-presenting cells (APCs). At 16 h after activation, many of the cells expressed TIM-1, and by 72 h the T cells had high expression of TIM-1 (**Fig. 2c**).

To further understand the kinetics of TIM-1 expression *in vitro*, we analyzed $CD4^+$ T cells cultured in T_H1- or T_H2-polarizing conditions. TIM-1 expression increased in T_H2-polarizing conditions (IL-4 plus mAb to IL-12; **Fig. 2d**). After weekly restimulation of these cells with APCs in the presence of IL-4 and anti-IL-12, T_H2-polarized cells had uniformly high expression of TIM-1. In contrast, TIM-1 expression was low on T cells cultured and restimulated in T_H1-polarizing conditions (IL-12 and mAb to IL-4) over the same time course. This indicates that during differentiation, TIM-1 becomes preferentially expressed on T_H2 cells.

Anti-TIM-1 enhances T cell proliferation

We evaluated the involvement of TIM-1 in T cell stimulation by culturing $CD4^+$ T cells purified from TCR-transgenic DO11.10 mice with OVA and low numbers of bone marrow–derived DCs as APCs. Proliferation of T cells in response to OVA and APCs was greatly increased in the presence of anti-TIM-1 compared with control mAb (**Fig. 3a**). The effect of anti-TIM-1 could be visualized in the cultures, such that after 48 h of culture, the DO11.10 T cells in cultures containing anti-TIM-1 clustered and increased in size (**Fig. 3b**) to a much greater extent than did those of cultures that contained an isotype control mAb (**Fig. 3c**).

The presence of the 3B3 mAb to TIM-1 also greatly enhanced the proliferation of $CD4^+$ T cells purified from unmanipulated BALB/c mice and cultured with mAb to CD3 and mAb to CD28 (**Fig. 3d**). This indicated that the effect of anti-TIM-1 did not depend on any other molecule present on the surface of APCs. To determine if TIM-1 also enhanced responses of primed memory T cells, we purified T cells from lymph nodes of OVA-primed mice and cultured them with OVA and bone marrow–derived DCs. Proliferation was greatly increased in the presence of anti-TIM-1 at all concentrations of OVA used for *in vitro* restimulation (**Fig. 3e**).

Anti-TIM-1 could enhance T cell proliferation by crosslinking TIM-1 and delivering a positive signal or by blocking the interactions

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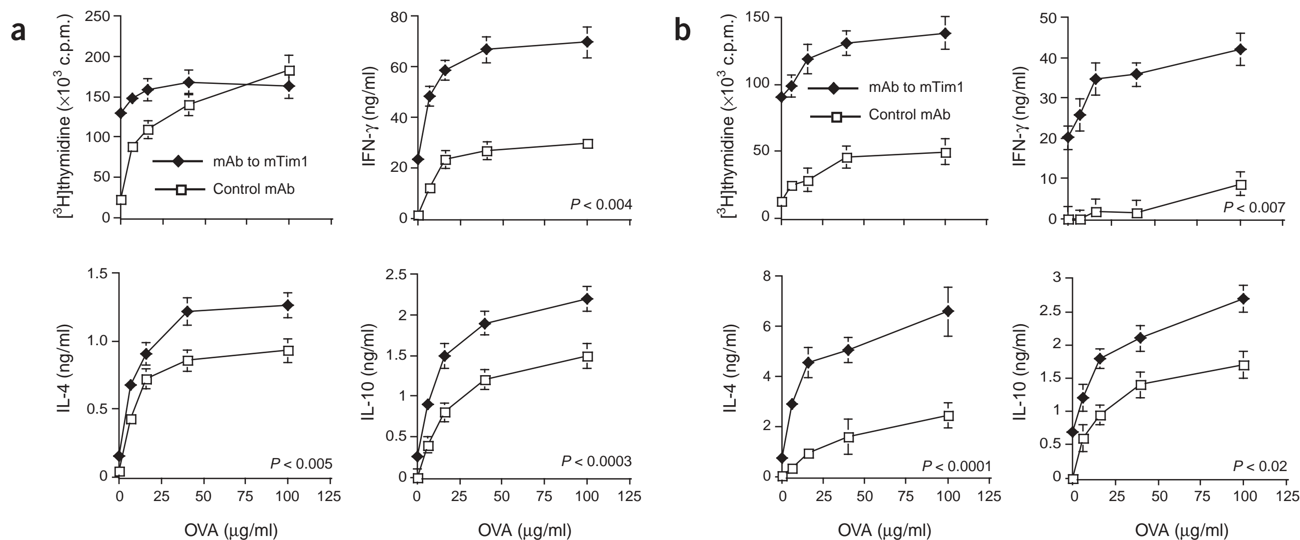

Figure 4 Anti-TIM-1 enhances T cell responses *in vivo*. (**a**) Mice received anti-TIM-1 (500 μg) or isotype control mAb intraperitoneally on day 0 and were immunized subcutaneously with OVA (300 μg) in CFA 8 h later. Lymph nodes were removed after 9 d and cells were cultured with OVA. Proliferation was measured after 48 h by [^{3}H]thymidine incorporation and supernatants were obtained after 4 d for cytokine ELISA. Data are plotted with standard deviation bars and are from one experiment of three. T cells from mice treated with anti-TIM-1 produce significantly more IFN-γ, IL-4 and IL-10 than do those from control mice. (**b**) CD4$^+$ T cells from lymph nodes of OVA-immunized mice that received 3B3 mAb to TIM-1 or isotype control mAb intraperitoneally as described in **a** were purified with MACS columns and were cultured with OVA and bone marrow–derived DCs from naive mice as APCs. Proliferation was measured after 48 h by [^{3}H]thymidine incorporation and supernatants were collected after 4 d for cytokine ELISA. Data are presented with standard deviation bars and are one experiment of three. T cells from mice treated with anti-TIM-1 produce significantly more IFN-γ, IL-4 and IL-10 than do those from control mice. *P*-values were determined as described in Methods.

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with a ligand that delivers a negative signal. To distinguish these possibilities, we treated T cells with monomeric Fab of the mAb to TIM-1. T cell proliferation in response to OVA and DCs was enhanced in the presence of intact 3B3 mAb to TIM-1 but was not enhanced in the presence of monovalent Fab fragments of mAb to TIM-1, strongly suggesting that costimulation of T cells by TIM-1 delivers a positive signal to the T cell, which enhances proliferation (**Fig. 3f**). It is possible that TIM-1 provides a negative signal to T cells, but this is unlikely because monovalent Fab fragments of the mAb to TIM-1 bound to TIM-1 (**Supplementary Fig. 3** online) but did not activate CD4 T cells or replicate the stimulatory effects of the intact mAb.

We next investigated the function of TIM-1 in cytokine production by T cells previously cultured in T_H2 or T_H1 conditions. We cultured DO11.10 T cells for 1 week in T_H2-polarizing conditions, then washed the cells and restimulated them with bone marrow–derived DCs and OVA. In the presence of anti-TIM-1, IL-4 production by these T_H2 cells was greatly enhanced (approximately threefold) compared with that of control mAb (**Fig. 3g**). No interferon-γ (IFN-γ) was detectable in these cultures of the T_H2 cells in the presence or absence of anti-TIM-1. Moreover, anti-TIM-1 had no effect on IFN-γ production in DO11.10 T cells polarized in T_H1 conditions, which have low expression of TIM-1, at any concentration of antigen (**Supplementary Fig. 4** online). These results indicate that stimulation by TIM-1 enhances the proliferation of TIM-1-expressing T cells and, in particular, increases IL-4 but not IFN-γ production in T_H2 cells.

Anti-TIM-1 administration increases T cell responses

Because TIM-1 costimulation enhanced *in vitro* CD4$^+$ T cell responses, we examined the effect of administration of anti-TIM-1 *in vivo*. We gave mice anti-TIM-1 or control mAb (500 μg, intraperitoneally) and immunized them with OVA in complete Freund's adjuvant (CFA) 8 h later. We removed lymph nodes after 9 d and cultured them with OVA. Cells from mice treated with anti-TIM-1 showed vigorous *in vitro* proliferation to OVA as well as higher background proliferation in the absence of OVA *in vitro*, compared with those from mice treated with control mAb (**Fig. 4a**). In addition, T cells from mice treated with anti-TIM-1 showed greatly enhanced production of IFN-γ, IL-4 and IL-10 (**Fig. 4a**). Exposure to antigen at the time of anti-TIM-1 administration was required for the costimulatory effect of anti-TIM-1, because cells from mice treated with anti-TIM-1 in the absence of OVA immunization did not produce cytokines or proliferate *in vitro* in the presence or absence of OVA (data not shown).

It is possible that other cell types present in the lymph nodes of mice treated with anti-TIM-1 could be required for the enhanced prolifera-

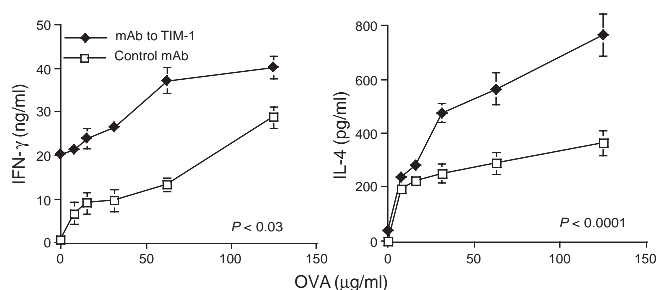

Figure 5 Anti-TIM-1 enhances T cell responses of HBA mice. Mice received anti-TIM-1 (500 μg) or isotype control mAb intraperitoneally on day 0, and 8 h later were immunized subcutaneously with OVA (300 μg) in CFA. Lymph nodes were removed after 9 d and cells were cultured with OVA. Supernatants were obtained after 4 d for cytokine ELISA. Data are representative of three experiments, with standard deviation bars. T cells from mice treated with anti-TIM-1 produce significantly more IFN-γ and IL-4 than do those from control mice. *P*-values were determined as described in Methods.

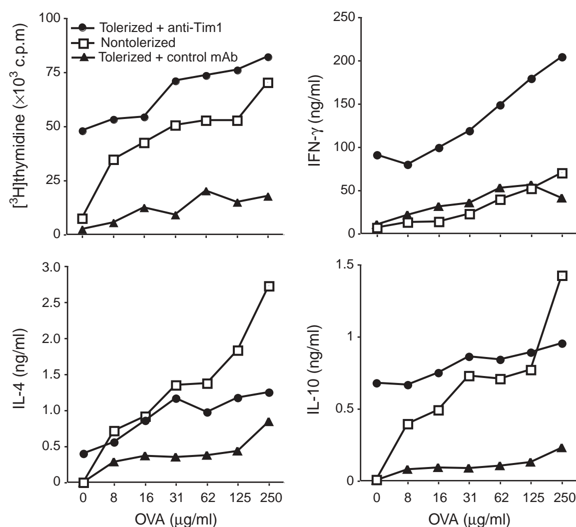

Figure 6 Anti-TIM-1 inhibits tolerance induction *in vivo*. BALB/c mice injected on days 0 and 4 with anti-TIM-1 (100 µg) were exposed on days 0, 1 and 2 to intranasal OVA (Tolerized) or PBS (Nontolerized). All mice were subsequently challenged intraperitoneally with OVA in alum (day 12). Spleen cells were collected after 9 d and were cultured with OVA (concentration, horizontal axes). [^{3}H]thymidine incorporation was measured after 3 d of culture and supernatants were obtained after 4 d for cytokine ELISA. Data are one representative experiment of four.

tion and cytokine production found in these cultures. To address this, we purified CD4$^+$ T cells from lymph nodes of OVA-primed mice treated *in vivo* with anti-TIM-1 or control mAb and cultured the cells with bone marrow–derived DCs and OVA. T cells purified from mice treated with anti-TIM-1 showed significantly enhanced proliferation and production of IFN-γ, IL-4 and IL-10 compared with that of mice treated with control mAb (**Fig. 4b**). These results demonstrate that administration of mAb to TIM-1 *in vivo* at the time of immunization stimulates enhanced responses of CD4$^+$ T cells, characterized by increased proliferation and cytokine production.

The TIM family of genes was initially identified by positional cloning using a congenic mouse model of asthma, in which distinct genetic variants of *Havcr1* were associated with differences in IL-4 and IL-13 production. After immunization with OVA, BALB/c mice develop T_H2-biased immune responses, whereas the congenic C.D2-Es-HBA (HBA) mouse responds to OVA immunization with the production of lower concentrations of IL-4 and IL-13 (ref. 6). Therefore, we determined whether anti-TIM-1 had similar effects on cytokine production in T cells from HBA mice and from BALB/c mice. T cells from HBA mice were similar to those of BALB/c mice in that both had high expression of TIM-1 after activation (**Fig. 2** and **Supplementary Fig. 5** online). In addition, cytokine production in T cells from both BALB/c and HBA mice was enhanced with anti-TIM-1. Thus, treatment of HBA mice with anti-TIM-1 during immunization substantially increased IFN-γ and IL-4 production in lymph node cells restimulated *in vitro* with OVA (**Fig. 5**). These results indicate that costimulation of TIM-1 in CD4 T cells from both BALB/c and HBA mice provides a positive signal that enhances cytokine production.

Anti-TIM-1 inhibits respiratory tolerance induction

Respiratory exposure to allergen induces T cell tolerance and protection against the development of AHR. As TIM-1 costimulation activates T cells *in vitro* and *in vivo* (**Figs. 3–5**), we examined the effects of anti-TIM-1 on the development of tolerance in mice exposed intranasally to OVA. T cells from control mice challenged intraperitoneally with OVA in aluminum potassium sulfate (alum) proliferated vigorously after *in vitro* restimulation with antigen (**Fig. 6**). In contrast, T cells from mice exposed intranasally to OVA and subsequently challenged with OVA in alum proliferated poorly in response to OVA. However, the administration of 100 µg of anti-TIM-1 before and after intranasal exposure to OVA (before challenge with OVA in alum) reversed the induction of tolerance and restored a vigorous response to OVA. Cytokine production was also affected by treatment with anti-TIM-1. Splenocytes from tolerized mice produced lower concentrations of IL-4 and IL-10 than did splenocytes of nontolerized mice. Tolerization did not greatly modify IFN-γ. Administration of

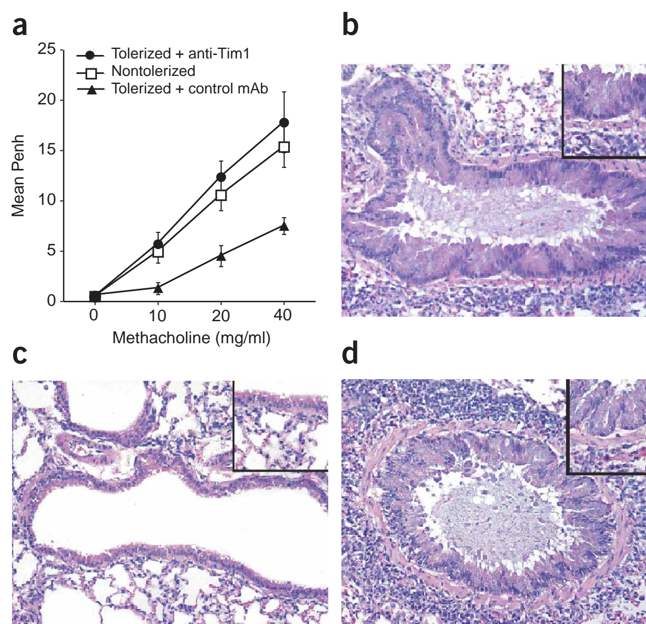

Figure 7 Anti-TIM-1 inhibits tolerance induction in an AHR model. (**a**) BALB/c mice injected on days 0 and day 4 with anti-TIM-1 (100 µg) were exposed on days 0, 1 and 2 to intranasal OVA (Tolerized) or PBS (Nontolerized). All mice were subsequently sensitized intraperitoneally with OVA in alum (day 12), then were challenged intranasally with OVA on days 20, 21 and 22 for induction of AHR, measured after challenge with increasing concentrations of methacholine. Data represent peak Penh values averaged among sensitized mice in each group, from three independent experiments. (**b**–**d**) Lung histopathology of mice treated with mAb to TIM-1. Tissues from mice were inflated and fixed in formalin, sectioned, and stained with hematoxylin and eosin. Insets, high-power magnification of the bronchiolar lining cells. Original magnification, ×200 (main images) and ×600 (insets). (**b**) Tissue from a nontolerized mouse challenged intraperitoneally with OVA in alum shows notable airway inflammation with intraluminal accumulation of mucous and prominent peribronchiolar and perivascular (not shown) inflammatory cell infiltrates composed of eosinophils and lymphocytes. Epithelial cells show abundant intracytoplasmic mucin (inset). (**c**) Tissue from a tolerized mouse sensitized and challenged with OVA shows normal bronchioles without inflammation. Epithelial cells show normal, low columnar and ciliated epithelium. (**d**) Lung of mouse treated with anti-TIM-1 during initial respiratory exposure to OVA (tolerization phase) followed by sensitization and challenge with OVA shows conspicuous peribronchiolar inflammation, consisting of eosinophils and lymphocytes, and expansion of lumens by mucous. The lung epithelial cells contain large amounts of intracytoplasmic mucin.

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anti-TIM-1 abrogated these tolerizing effects of intranasal antigen, such that splenocytes from mice treated with anti-TIM-1 produced IFN-γ, IL-4 and IL-10 at concentrations comparable to or greater than those of nontolerized mice (**Fig. 6**), indicating that treatment with anti-TIM-1 abrogated respiratory tolerance induction.

The development of respiratory tolerance prevents the subsequent development of AHR[14,15]. Because the genes encoding TIM proteins were identified using AHR as a 'screen phenotype', we determined the effect of anti-TIM-1 on T cell tolerance and AHR. Intranasal exposure to OVA on days 0, 1 and 2 induced T cell tolerance that inhibited the development of AHR assessed on day 21 (**Fig. 7a**). Whereas the development of AHR was associated with severe airway inflammation (**Fig. 7b**), the tolerized mice showed greatly reduced airway inflammation (**Fig. 7c**). However, treatment of the mice with anti-TIM-1 (on days 0 and 4) during the initial exposure to OVA prevented tolerance induction, and OVA challenge induced severe AHR that was accompanied by considerable airway inflammation[14] (**Fig. 7d**). Thus, TIM-1 costimulation is important in T cell activation, resulting in the prevention of peripheral T cell tolerance and in the development of AHR and airway inflammation after antigen challenge.

DISCUSSION

In these studies we have examined the immunological effects of TIM-1, which has been identified as being encoded by an important atopy susceptibility gene in both mice and humans[6,7]. We found that recently activated $CD4^+$ T cells had high expression of TIM-1 and that crosslinking of TIM-1 on $CD4^+$ T cells using anti-TIM-1 provided a stimulatory signal that greatly enhanced T cell proliferation as well as production of IL-4 and IFN-γ. TIM-1 expression was maintained on T_H2-polarized cells but not on T_H1-polarized cells, and crosslinking of TIM-1 on T_H2 cells greatly increased T_H2 cytokine production and proliferation. Indeed, *in vivo* administration of anti-TIM-1 provided a potent T cell costimulatory activity, which can be used as an effective adjuvant to enhance T cell immunity. These studies indicate that TIM-1 represents a previously unknown and important costimulatory molecule that considerably affects CD4 T cell function.

The TIM family[6] is thought to represent a previously unidentified family of molecules that regulate population expansion and effector functions of T cells. T cells require at least two signals for optimal activation, the first provided by TCR triggering and the second, by T cell costimulatory molecules that are now categorized into three families with distinct and overlapping functions. These families include the CD28 family of receptors (CD28, CTLA-4, ICOS and PD-1)[16], the CD40L–tumor necrosis factor family (CD40L (CD154), OX40 (CD134) and 4-1BB (CD137))[17] and the CD2 family (CD2, SLAM (CD150) and 2B4 (CD244))[18]. TIM-1 costimulation functions in concert with TCR and CD28 signaling, potently augmenting the signals provided by the TCR and CD28 for T cell proliferation and cytokine production. TIM-3, preferentially expressed on T_H1 cells, is another member of the TIM family that is important in the development of autoimmune disease and tolerance. TIM-3 may provide a negative costimulatory signal to the T cell, as the administration of a blocking antibody to TIM-3 enhances the clinical and pathological severity of experimental autoimmune encephalomyelitis[19] and TIM-3-deficient mice are resistant to induction of high antigen dose tolerance[20]. Therefore, TIM-1 and TIM-3 may together constitute a unique family of costimulatory molecules.

After the TIM family of genes was postitionally cloned, we examined TIM-1 function by generating a mAb specific for TIM-1. The mAb to TIM-1 we used here was indeed specific for TIM-1 and did not cross-react with TIM-2, TIM-3 or TIM-4, and therefore we are confident that the effects demonstrated with this mAb were specific for TIM-1 and were not influenced by the effects of related TIM molecules. Using this mAb in conjuction with T cell receptor ligation, we found that crosslinking of cell surface TIM-1 provided a positive activation signal directly to T cells. Use of mAb to TIM-1 alone in the absence of TCR signaling had no effect, indicating that TIM-1 provides a strong costimulatory signal for T cell activation that augments TCR and CD28 signaling. It is possible that TIM-1 provides a negative signal to T cells that might be blocked by mAb to TIM-1 (which might block the interaction of TIM-1 with its putative ligand). We believe that this is unlikely, as monovalent Fab fragments of the mAb to TIM-1 did not replicate the stimulatory effects of the intact mAb to TIM-1. Evidence suggests that TIM-4 is a ligand of TIM-1 and that TIM-4–Ig costimulates T cell population expansion *in vitro*[21], which also supports this idea.

In T_H2-polarized cells, which preferentially expressed TIM-1, costimulation through TIM-1 greatly enhanced IL-4 but not IFN-γ synthesis, whereas in T_H1 cells, which had only low expression of TIM-1, TIM-1 costimulation did not affect cytokine synthesis. However, because TIM-1 is expressed early after activation in undifferentiated peripheral $CD4^+$ T cells, costimulation by TIM-1 increased production of both IFN-γ and IL-4 in these unpolarized cells. The effect on cytokine production in nonpolarized T cells may be in part due to population expansion of the responding $CD4^+$ T cells, as TIM-1 costimulation greatly increased $[^3H]$thymidine incorporation and seemed to increase T cell viability (data not shown). The induction of CD4 T cell population expansion and the support of both IFN-γ and IL-4 production by TIM-1 costimulation in such cells suggest that agents such as anti-TIM-1 that act on TIM-1 early during immune responses might function as strong adjuvants to boost antigen-specific immune responses and reverse antigen-specific tolerance. The administration of anti-TIM-1 during the induction of respiratory tolerance completely reversed antigen-specific unresponsiveness and was associated with heightened production of IFN-γ, IL-4 and IL-10. $CD4^+$ T cells obtained from mice treated with anti-TIM-1 and cultured *in vitro* produced IL-4 and IFN-γ spontaneously, and this production increased after stimulation with antigen, indicating the high degree of *in vivo* immune stimulation. However, *in vivo* treatment with anti-TIM-1 in the absence of antigen had no effect on T cell cytokine production (data not shown), consistent with the fact that TIM-1 crosslinking in the absence of TCR ligation had very limited effects. The effect of TIM-1 is distinct from that of TIM-3, which may enhance the development of peripheral T cell tolerance[20,22].

TIM-1 and TIM-4 contain mucin domains, which are heavily glycosylated, and both TIM-1 and TIM-4 transfectants show a notable tendency for aggregation and adherence (data not shown), suggesting that homotypic interactions occur. Administration of a TIM-1–Ig fusion protein has been shown to enhance the production of T_H2 cytokines[21]. A possible explanation for this is that the initiation of a TIM-1–Ig–TIM-1–TIM-4 complex could result in activation via TIM-1, preferentially expressed on T_H2 cells.

Because polymorphic variants of TIM-1 are associated with T_H2 responses in mice and in humans[6,7], we examined the effects of anti-TIM-1 on the development of T_H2 responses in BALB/c mice and in congenic HBA mice. In both strains of mice, anti-TIM-1 increased cytokine production (IFN-γ, IL-4 and IL-10), consistent with the ability of the mAb to TIM-1 to enhance immune responses and to abolish respiratory tolerance. The similar effect in both BALB/c and HBA mice might be expected, however, because the TIM-1 IgV domain, which is targeted by the mAb to TIM-1, is identical in

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BALB/c and HBA mice, allowing the mAb to bind equally well to and costimulate T cells from both BALB/c and HBA mice. After immunization with antigen, T cells from BALB/c mice produce more IL-4 and induced more severe airway disease than do those from HBA mice[6], which may be because of the reduced length of the mucin domain of TIM-1 expressed in HBA mice (shorter by 23 amino acids in HBA mice) that may limit the interaction of TIM-1 with it natural ligand. The longer mucin domain of the BALB/c TIM-1 molecule may enhance interactions of TIM-1 with TIM-1 ligand and enhance T_H2 responses, presumably because TIM-1 has higher expression on T_H2 than on T_H1 cells.

The limited T_H2 responses in HBA mice suggest that interruption of the interaction between TIM-1 and its ligand might provide useful anti-inflammatory therapy in limiting T_H2-biased immune responses and allergic asthma. Human infection with HAV, a virus that uses TIM-1 as its cellular receptor, is associated with such an anti-inflammatory effect, as HAV infection is associated with a reduced risk for developing asthma and allergic rhinitis[11,12]. Precisely how HAV disrupts TIM-1 signaling is not yet clear, but HAV could interrupt TIM-1 signaling by directly binding to TIM-1. Alternatively, HAV could preferentially infect and consequently cause the deletion of TIM-1-expressing T_H2 cells. Epidemiological studies have indicated that the effect of HAV on atopic disease is long lasting, presumably beginning in young children, who are known to contract the disease more often than older children. Duplication of the 'anti-allergic' or beneficial effects of HAV infection with a noninfectious agent (such as a blocking antibody) might provide very useful therapy for atopic disease. However, the development of an immunotherapy that mimics the beneficial effects of HAV will require much greater understanding of the costimulatory function of TIM-1 and the effects of HAV infection on T cell biology.

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METHODS

Mice and antigen administration. BALB/c mice were purchased from The Jackson Laboratory. OVA-specific TCR–transgenic DO11.10 breeder mice were used as donors of OVA-specific $CD4^+$ T cells. BALB/c congenic HBA mice (C.D2 Es-HBA) were obtained from M. Potter (National Cancer Institute, Bethesda, Maryland) and were bred in the Stanford animal facility (Stanford University, Stanford, California).

Mice were primed subcutaneously with OVA (ICN Biomedical) in CFA or in complex with alum. For induction of tolerance, mice lightly anesthetized with methoxyflurane received intranasal OVA (100 μg; chromatographically purified; Worthington Biochemical) on 3 consecutive days. Control (nontolerized) mice received intranasal PBS. Mice were challenged 10 d later intraperitoneally with 50 μg of OVA in 2 mg of alum in a volume of 0.5 ml. Mice were killed and spleens were removed 9 d after the intraperitoneal immunization. The Stanford University Committee on Animal Welfare (Administrative Panel of Laboratory Animal Care; Stanford, California) approved all animal protocols used in this study.

Induction of AHR. For measurement of AHR, mice were immunized intraperitoneally with 100 μg OVA in complex with alum on day 0 and were immunized intranasally with 50 μg of OVA in 50 μl of PBS after light anesthesia on days 7, 8 and 9. For induction of tolerance, mice were exposed intranasally on days 0, 1 and 2 to OVA (tolerized) or PBS (nontolerized). All mice were subsequently challenged intraperitoneally with OVA in alum (day 12), then were exposed intranasally to OVA on days 20, 21 and 22 for induction of AHR. AHR in response to inhaled methacholine was measured 24 h after the last intranasal dose of OVA (day 10). AHR responses were assessed by measurement of methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics), as described[6].

Quantitative TaqMan RT-PCR. RNA was purified with Trizol from tissues collected from BALB/c mice. RNA (1 μg) was reverse-transcribed in a volume of 20 μl containing 80 U Superscript II, 250 ng random hexamers, 20 nmol of each dNTP and 20 U RNaseOut (all from Invitrogen). For multiplex amplification, 10 ng of cDNA was used in 25 μl of Universal PCR Master Mix (Applied Biosystems) with 300 nmol of each TIM-1 primer (forward, 5′-TGGTTGCCTTCCGTGTCTCT-3′; reverse, 5′-TCAGCTCGGGAATGCACAA-3′) and a 6-carboxyfluorescein– and N,N,N′,N′-tetramethyl-6-carboxyrhodamine-labeled probe (TaqMan probe, 5′-AGATTGAAGCTTTGCAGAACGCAGCG-3′; 200 nmol; Biosearch Technologies). An 18S rRNA primer-probe was used as an internal reference for normalization of well-to-well variability (Applied Biosystems). Thermal cycling conditions were as follows: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Applied Biosystems).

Standard procedures of analysis were followed to achieve relative expression values. Threshold cycles (C_T) were normalized by subtraction of the average 18S rRNA C_T value from the average TIM-1 C_T value to achieve C_T. Then ΔC_T (C_T tissue − C_T calibrator), which represents the relative n values in various tissues compared with that of heart tissue (calibrator), was calculated. The quantitative data are means of triplicate experiments.

Fusion protein and cell transfections. The TIM-1–Ig fusion protein consists of the signal and IgV domains of BALB/c mouse TIM-1 linked to the hinge-CH2-CH3 domains of mouse IgG2a (with four point mutations blocking Fc receptor and complement binding) in the pEF6 vector[23]. This protein was produced in stably transfected Chinese hamster ovary cells, was purified from conditioned media with protein A–Sepharose and was verified to have the predicted TIM-1 N-terminal sequence of YVEVKGVV. The 300.19 cells were transfected by electroporation with a pEF6 plasmid containing mouse TIM-1 cDNA. Cells were selected with blasticidin, sorted twice by flow cytometry for mouse TIM-1 expression with polyclonal rat anti-TIM-1 serum and subcloned.

Generation of monoclonal antibodies. Female Lewis strain rats (Harlan Sprague-Dawley) were immunized subcutaneously with mouse TIM-1–Ig in CFA and were 'boosted' multiple times with mouse TIM-1–Ig in PBS (100 μg). At 1 d after the last boost, lymph node cells were fused with NS1 myeloma cells and were cloned, and the hybridomas were screened by cell surface staining of mouse TIM-1–transfected 300.19 cells and for lack of reactivity with untransfected cells. Twelve anti-TIM-1-specific hybridomas were made. Hybridoma 3B3 (rat IgG2aK) was chosen for further analysis on the basis of its blocking of the interaction of mouse TIM-1–Ig with TIM-1 ligand and for robust staining. After purification with protein A–Sepharose, 3B3 was coupled to fluorescein isothiocyanate (FITC) using standard protocols or to phycoerythrin by Zymed Laboratories. Fab fragments of 3B3K.2 were prepared by Telos Pharmaceuticals by digestion with papain and purification of the monovalent Fab fragments.

Flow cytometry. Analytical flow cytometry used a FACScan (Becton Dickinson) and data were processed with the FlowJo Software (Tree Star). For characterization of mAbs to TIM-1, TIM-1-transfected 300.19 cells were incubated with unconjugated mAbs and were stained with phycoerythrin-conjugated goat anti-rat immunoglobulin (Southern Biotechnology Associates). For determining if mAbs to TIM-1 blocked binding of mouse TIM-1 fusion protein, untransfected 300.19 cells were stained with mouse TIM-1–Ig that had been preincubated for 20 min on ice with 3B3 (1 μg) or control mAb. Cells were washed and then were stained with phycoerythrin-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates) for detection of binding of the fusion protein. For detection of TIM-1 expression on freshly isolated cells, lymph node or spleen cells were isolated and Fc receptors were blocked with excess anti-Fc (antibody 2.4G2, purified from hybridoma HB197; American Type Culture Collection). T cells were stained with directly conjugated anti-CD4 (FITC or CyChrome), anti-CD8α (FITC), anti-CD62L (FITC) or anti-CD25 (FITC; PharMingen) and B cells were stained with anti-CD19 (FITC). Cells were costained with phycoerythrin-conjugated 3B3 mAb to mouse TIM-1. Directly conjugated isotype control mAbs were purchased from PharMingen. For staining of activated T cells, $CD4^+$ T cells were positively selected from spleen of unmanipulated BALB/c or HBA mice with MACS CD4 microbeads (Miltenyi Biotec) and were incubated in 24-well plates precoated with 5 μg/ml of 145-2C11 mAb to CD3 (BD Biosciences) plus 10 μg/ml of

soluble 37.51 mAb to CD28. In some experiments, $CD4^+$ T cells purified from TCR-transgenic DO11.10 mice were activated with bone marrow–derived DCs and OVA peptide (0.075 μM) before staining.

T cell lines. OVA-specific T_H1 and T_H2 T cell lines were generated by the incubation of $CD4^+$ T cells purified from spleens of DO11.10 mice with 2.5×10^4 bone marrow–derived DCs and OVA (50 μg/ml) in the presence of 10 ng/ml of recombinant IL-4 (Peprotech) and C17.8 mAb to IL-12 (10 μg/ml; T_H2 conditions) or 0.2 ng/ml of recombinant IL-12 and 11B11 mAb to IL-4 (10 μg/ml; T_H1 conditions). Cells were washed and restimulated at weekly intervals.

***In vivo* antibody treatments.** Mice were injected intraperitoneally with 3B3 mAb to TIM-1 or rat IgG2a isotype control mAb 1 d (tolerance induction experiments) or 8 h (other immunization protocols) before the initial exposure to OVA.

Preparation of bone marrow–derived DCs. Bone marrow–derived DCs were generated as described[24,25] with some modifications[26] and were cultured in DMEM (Sigma) supplemented with 10% FCS (Gemini Bioproducts), 2 mM glutamine, 20 μg/ml gentamicin, and 5 μM β-mercaptoethanol. Bone marrow cells (5×10^6) were cultured in Petri dishes (9 cm in diameter; Nunc) in 10 ml culture medium containing 20–25 U/ml of granulocyte-monocyte colony-stimulating factor. Loosely adherent cells were transferred onto a second dish on day 6 of culture. From day 6 to day 9, these transferred cells were used as a source of DCs.

***In vitro* assays.** Lymph node or spleen cells were collected and were passed through a nylon mesh, and then were stimulated in 96-well plates (2×10^5 to 3×10^5 cells/well) with or without OVA in 0.15 ml of complete DMEM. For measurement of cell proliferation, after 72 h, the cultures were pulsed for 16 h with 0.25 μCi [^{3}H]thymidine, and the incorporated radioactivity was measured in a Betaplate scintillation counter (MicroBeta Trilux; Wallac). For measurement of cytokine production, supernatants were collected after 4 d. In some experiments, $CD4^+$ T cells were positively selected from OVA-primed mice or from TCR-transgenic DO11.10 mice with MACS CD4 microbeads (Miltenyi Biotec). T cells were cultured at a density of 1×10^5 cells/well with 5×10^3 bone marrow–derived DCs/well as APCs and 50 μg/ml of OVA. Photomicrographs were taken after 48 h of culture using a Nikon inverted, phase-contrast microscope. In some experiments, T cells (1×10^5) differentiated in T_H1- or T_H2-polarizing conditions were cultured with DCs and OVA in the presence of 3B3 mAb to TIM-1 or isotype control mAb 111/10 (rat IgG2aK; provided by R. Hodes, National Institutes of Health, Bethesda, Maryland). Supernatants were collected 24 h later for analysis of cytokine production. In some experiments, $CD4^+$ T cells (7.5×10^4) positively selected from spleens of naive BALB/c mice were incubated on plates coated with anti-CD3 (2.5 μg/ml) plus soluble anti-CD28 (5 μg/ml) and/or anti-TIM-1 (12.5 μg/ml).

Cytokine enzyme-linked immunosorbent assay (ELISA). ELISAs were done as described[27]. The mAb pairs used were as follows (capture–biotinylated detection): IFN-γ, R4-6A2–XMG1.2; IL-4, 11B11–BVD6-24G2. Antibodies for IL-13 ELISA were purchased from R&D Systems.

Statistical analysis. Each set of curves for cytokine data in **Figures 4** and **5** were compared by nonlinear regression analysis using GraphPad Prism software.

Note: Supplementary information is available on the Nature Immunology website.

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The authors declare that they have no competing financial interests.

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