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Competing interests statement

The authors declare competing financial interests: details accompany the paper on *Nature's* website (http://www.nature.com).

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Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease

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Activation of naive CD4$^+$ T-helper cells results in the development of at least two distinct effector populations, Th1 and Th2 cells[1–3]. Th1 cells produce cytokines (interferon (IFN)-γ, interleukin (IL)-2, tumour-necrosis factor (TNF)-α and lymphotoxin) that are commonly associated with cell-mediated immune responses against intracellular pathogens, delayed-type hypersensitivity reactions[4], and induction of organ-specific autoimmune diseases[5]. Th2 cells produce cytokines (IL-4, IL-10 and IL-13) that are crucial for control of extracellular helminthic infections and promote atopic and allergic diseases[4]. Although much is known about the functions of these two subsets of T-helper cells, there are few known surface molecules that distinguish between them[6]. We report here the identification and characterization of a transmembrane protein, Tim-3, which contains an immunoglobulin and a mucin-like domain and is expressed on differentiated Th1 cells. *In vivo* administration of antibody to Tim-3 enhances the clinical and pathological severity of experimental autoimmune encephalomyelitis (EAE), a Th1-dependent autoimmune disease, and increases the number and activation level of macrophages. Tim-3 may have an important role in the induction of autoimmune diseases by regulating macrophage activation and/or function.

In addition to their distinct roles in disease, Th1 and Th2 cells cross-regulate each other's expansion and functions. Thus, preferential induction of Th2 cells inhibits autoimmune diseases[7,8], and

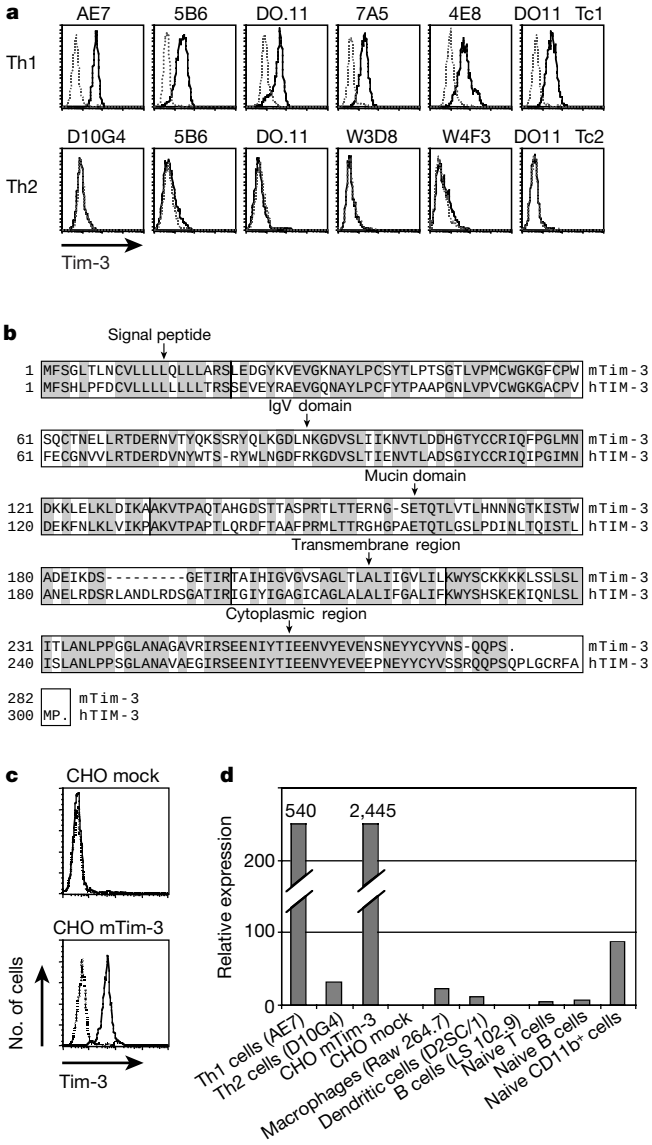

Figure 1 Cloning of a Th1-specific cell surface protein, Tim-3. **a**, Th1, Th2, Tc1 and Tc2 cells were stained with monoclonal antibody to Tim-3 (solid line) or rat IgG isotype control (dotted line). **b**, Deduced amino-acid sequence of murine and human Tim-3. Shading indicates regions of homology. IgV, variable region of immunoglobulin. **c**, CHO cells transfected using either Tim-3 cDNA (CHO mTim-3) or vector alone (CHO mock). Stable puromycin-resistant cells were stained with monoclonal antibody to Tim-3 (solid line) or rat IgG isotype control (dotted line). **d**, Total RNA from various cell lines and cells purified from SJL mice was isolated and transcribed to cDNA by reverse transcription, and cDNA was used for Taqman PCR. The figure shows expression of Tim-3 RNA relative to control GAPDH expression.

predominant induction of Th1 cells can regulate induction of asthma, atopy and allergies[9,10]. Several groups have reported the association of chemokine and co-stimulatory receptors with Th1 (refs 11–14) and Th2 (refs 12, 13, 15–18) cells; however, the nature of the differences in expression of most of these molecules is quantitative.

To identify new Th1-specific cell surface proteins, we immunized Lewis and Lou/M rats with Th1 T-cell clones and lines, including the established Th1-specific clone AE7 and *in vitro* differentiated Th1 cell lines derived from 5B6 (ref. 19) and DO11.10 T-cell receptor (TCR) transgenic mice. A panel of approximately 20,000 monoclonal antibodies was generated and screened on Th1 and Th2 cells. Two of the monoclonal antibodies (8B.2C12 and 25F.1D6) that selectively stained Th1 cells were further characterized. These

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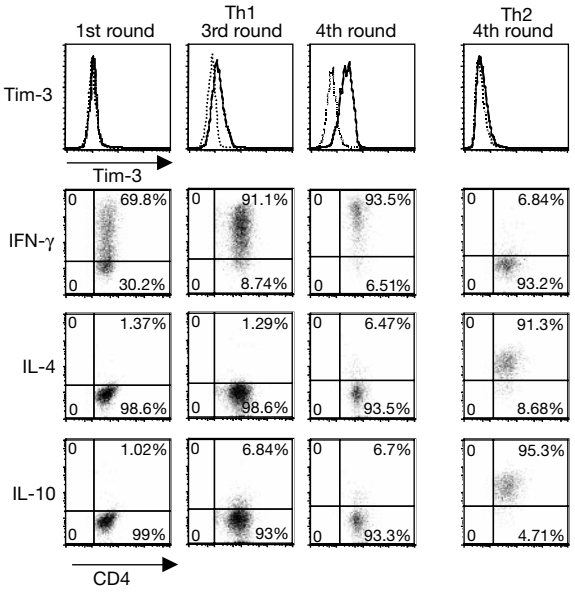

Figure 2 Tim-3 is detected on the surface of Th1 DO11.10 T cells after the third round of stimulation. Naive DO11.10 T cells were stimulated *in vitro* under Th1- and Th2-polarizing conditions. After each round of restimulation, Th1 and Th2 cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin for the induction of cytokines and then stained with monoclonal antibodies to Tim-3 and CD4, and cytokine expression was detected by intracellular staining. Histograms represent Tim-3 expression on $CD4^+$ cells (dotted line, isotype control; solid line, specific staining). Dot plots (logarithmic scale) represent cytokine expression in $CD4^+$ cells.

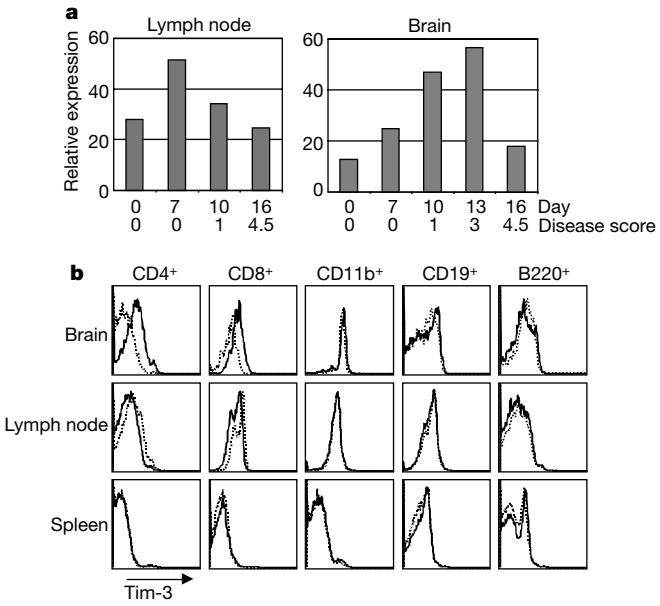

Figure 3 Tim-3 is expressed on $CD4^+$ and $CD8^+$ T cells during the course of EAE. SJL mice were immunized with PLP 139–151 peptide for the induction of EAE. Mice were killed at various time points after immunization, and spleen, brain and lymph nodes were removed and tested for the expression of Tim-3. **a**, Total RNA was isolated and transcribed to cDNA by reverse transcription and cDNA was used for Taqman PCR. The figure shows expression of Tim-3 relative to control GAPDH. **b**, On day 10, cells from brain, spleen and lymph nodes were stained with monoclonal antibodies to Tim-3, CD4, CD8, CD11b, CD19 and B220. Histograms represent Tim-3 expression on different cell populations (dotted line, isotype control; solid line, specific staining).

monoclonal antibodies recognize a cell surface protein present on established $CD4^+$ Th1 cells and $CD8^+$ Tc1 (cytotoxic) cells, but absent on $CD4^+$ Th2 cells and $CD8^+$ Tc2 cells (Fig. 1a).

By gene-expression cloning[20], we identified a complementary DNA (Tim-3; T-cell immunoglobulin- and mucin-domain-containing molecule) encoding a type I membrane protein of 281 amino acids whose extracellular domain consists of an immunoglobulin variable-region-like domain followed by a mucin-like domain consisting of 31% serine and threonine residues. The cytoplasmic region contains six tyrosines, one of which is part of a tyrosine phosphorylation motif (RSEENIY). The extracellular domain contains four sites for *N*-linked and five sites for *O*-linked glycosylation. The human homologue of Tim-3 was identified through genomic database searches and polymerase chain reaction using reverse transcribed RNA (RT-PCR). It has a 63% amino-acid identity to murine Tim-3 overall, with 77% identity in the cytoplasmic domain, including conservation of the tyrosine phosphorylation motif (Fig. 1b). Database searches show that Tim-3 is related to kidney injury molecule-1 (Kim-1; also known as HAVcr-1), the receptor for human hepatitis A virus, and Tim-2; all of which have a similar immunoglobulin and mucin structure. All three family members (Kim-1, Tim-2 and Tim-3) are located on human chromosome 5q33.2 and mouse chromosome 11. A Tim-3 cDNA expression construct was used to stably transfect Chinese hamster ovary (CHO) cells. Flow cytometry and immunoprecipitation analysis of the stable transfectants confirmed that the cloned protein is Tim-3, and that it is expressed at the cell surface (Fig. 1c and data not shown).

Expression of this gene in various cell lines (Th1, Th2, dendritic, macrophage and B cells) and SJL T-, B- and $CD11b^+$ cells was determined by quantitative RT-PCR. Tim-3 transcripts were present at the highest level only in Th1 cells, although *in vivo*-derived $CD11b^+$ cells also showed low level of expression (Fig. 1d). Flow cytometric analysis using anti-Tim-3 antibodies confirmed that Tim-3 is not expressed on naive T cells, B cells, macrophages or dendritic cells (data not shown). These data suggest that the molecule recognized by these antibodies is expressed mainly on differentiated Th1 or Tc1 cells and not on other haematopoietic cell types, at least at the protein level. To examine the kinetics of Tim-3 protein expression during T-cell differentiation, naive DO11.10

Table 1 EAE in mice treated with anti-Tim-3 or control antibody

	Clinical EAE				Histological EAE			
Treatment	Incidence	Mortality	Day of onset	Mean maximal score	Incidence	Meningeal foci	Parenchymal foci	Total foci
Anti-Tim-3	28/29 (97%)	12/29* (41%)	11.5 ± 0.4†	3.6 ± 0.3‡	14/16 (88%)	53.2 ± 11.7	64.6 ± 14.9	117.8 ± 26.1
PBS	18/18 (100%)	2/18 (11%)	11.8 ± 0.6	2.7 ± 0.3	11/11 (100%)	33.5 ± 7.5	25.5 ± 9.4	59.1 ± 16.2
rIgG	23/26 (88%)	2/26 (8%)	15.4 ± 1.7	2.3 ± 0.3	13/15 (87%)	32.3 ± 8.3	38.5 ± 9.9	66.9 ± 17.8

*$P < 0.01$ when compared with the group treated with rat IgG (rIgG), and $P < 0.05$ when compared with the group treated with PBS.
†$P < 0.05$ when compared with the group treated with rIgG.
‡$P < 0.01$ when compared with the group treated with rIgG, and $P < 0.05$ when compared with the group treated with PBS.

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TCR transgenic T cells were activated *in vitro* under Th1- or Th2-polarizing conditions. Tim-3 is not expressed on Th2 cells but is detectable on Th1 cells after the third round of *in vitro* polarization (Fig. 2). Tim-3 is therefore expressed at a late stage of T-cell differentiation, suggesting that Tim-3 may not contribute to T-cell differentiation as such, but may have a function in the transport and/or effector functions of Th1 cells.

We next examined the expression of Tim-3 during the development of experimental autoimmune encephalomyelitis (EAE), a Th1-mediated autoimmune disease of the central nervous system (CNS). We immunized EAE-susceptible SJL mice with the encephalitogenic proteolipid protein (PLP) 139–151 peptide. Using quantitative RT-PCR, we detected that in the lymph node, Tim-3 messenger RNA was upregulated after immunization with expression peaking at 7 days after immunization, just before onset of EAE. Expression in the lymph nodes was then downregulated as the disease progressed. In the brain, expression of Tim-3 mRNA steadily increased and peaked at the onset of the disease (around day 10–13). Tim-3 mRNA expression was then downregulated to near basal levels at the maximal disease score (Fig. 3a). This expression pattern was confirmed at the protein level by flow cytometric analysis. Whereas Tim-3 is expressed on very few $CD4^+$ T cells (less than 2%) in the periphery after immunization, Tim-3 is specifically expressed on most of the $CD4^+$ and $CD8^+$ T cells present in the CNS at the onset of clinical signs of disease (Fig. 3b). The number of Tim-3^+ T cells decreased in the CNS as the disease progressed. These data suggest that Tim-3 is expressed *in vivo* on T cells and may have an important function in the initiation of EAE.

To study the function of Tim-3 *in vivo*, we tested the effect of anti-Tim-3 antibody administration on the development of EAE. SJL mice immunized with PLP 139–151 peptide were administered anti-Tim-3 antibody and monitored for the development of EAE. Mice treated with anti-Tim-3 developed a hyperacute and atypical EAE, with increased weight loss, malaise, ataxia and paralysis of the forelimbs, but without hindlimb paralysis and while sometimes retaining tail tone. Although disease onset was normal, progression was accelerated and resulted in significantly more severe clinical disease and increased mortality compared with the control group treated with control rat immunoglobulin-γ (IgG) (Table 1). Histologically, mice treated with anti-Tim-3 generally showed typical findings of acute EAE, but with increased numbers of inflammatory foci both in the meninges and parenchyma compared with the control group treated with rat IgG (Table 1). Animals treated with anti-Tim-3 that were killed immediately after the onset of clinical signs had a preponderance of neutrophils and mononuclear cells in the CNS inflammatory infiltrates with focal perivascular fibrin deposition, indicating vascular damage and a hyperacute inflammatory response (Fig. 4a, b). Animals that were killed at day 30 showed more extensive demyelinating lesions than animals treated with rat IgG (Fig. 4c–f). The demyelinating lesions in mice treated with anti-Tim-3 were filled with activated macrophages with detectable myelin fragments (Fig. 4d, inset). Activated macrophages are the cells primarily responsible for demyelination in EAE[21]; furthermore, depletion of activated macrophages leads to an inhibition of EAE[22]. Thus, it is possible that activated macrophages induced by anti-Tim-3 antibody treatment are responsible for the hyperacute disease phenotype and enhanced inflammation and demyelination.

To understand the function of Tim-3 *in vivo* and its observed role in EAE, we examined spleen and lymph node cells from SJL mice that were immunized with PLP 139–151 peptide and treated with anti-Tim-3 antibody or rat IgG control antibody. Whereas control mice treated with rat IgG showed a low basal (background) proliferative response (1,000-5,000 c.p.m.) and a dose-dependent increase in proliferation with the addition of specific antigen, the spleen cells from mice treated with anti-Tim-3 had 6–10 times the basal response in the absence of antigen, although the response to specific antigen was very similar (Fig. 5a). Flow cytometric analysis of spleens from mice treated with anti-Tim-3 revealed a 2–3-fold increase in $CD11b^+$ cell population (data not shown).

To confirm whether $CD11b^+$ cells in mice treated with anti-Tim-3 were proliferating—and thereby contributing to the basal proliferative responses—5-bromodeoxyuridine (BrdU) was administered in the drinking water of immunized SJL mice treated with anti-Tim-3 or rat IgG control antibody. Flow cytometric analysis of the splenocytes from mice treated with anti-Tim-3 compared with rat IgG revealed an increase in the number of both BrdU positive and BrdU negative cells only in the $CD11b^+$ population (Fig. 5b). Two-thirds of these $CD11b^+$ cells incorporated BrdU and expressed higher levels of major histocompatibility complex (MHC) class II (Fig. 5b and data not shown), suggesting that these activated $CD11b^+$ cells may have a principal role in the high basal response of mice treated with anti-Tim-3.

To determine the role of T cells and non-T cells, especially the $CD11b^+$ cells, in the basal proliferative response, we purified T and non-T cells from spleen cells of mice treated with anti-Tim-3 or rat IgG2a. Although there was only a modest basal proliferative response from purified T cells, B cells or $CD11b^+$ monocytes alone from mice treated with anti-Tim-3, the co-culturing of both B cells and $CD11b^+$ cells with the anti-Tim-3-treated T cells recapitulated the high basal response (Fig. 5c). In comparison, the addition of B cells, monocytes or both B cells and monocytes to T cells from mice treated with rat IgG2a produced only an additive effect (Fig. 5c). When criss-cross proliferation experiments were

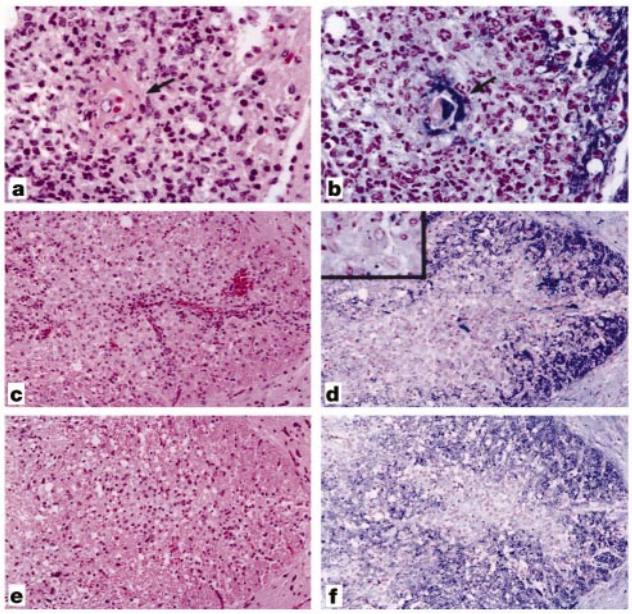

Figure 4 Treatment with anti-Tim-3 antibody enhances EAE. **a**, **b**, Inflammatory/demyelinating lesions in the spinal cord of a mouse treated with anti-Tim-3 on day 12 after immunization, at the peak of clinical disease. The infiltrate consists of a mixture of neutrophils and mononuclear cells. Perivascular fibrin deposition (arrow) indicates vasculitis and vascular injury. A portion of an intact peripheral nerve root (dark blue staining in **b**) is on the right side, whereas most of the CNS myelin in the field is lost. Magnification ×411. **c**, **d**, Extensive demyelination associated with a perivascular mononuclear cell infiltrate in the posterior columns of the spinal cord of a mouse treated with anti-Tim-3 killed on day 30 after transfer. Inset, sheets of macrophages with phagocytosed myelin fragments (blue dots). Magnification ×137; inset, ×411. **e**, **f**, A similar infiltrate in the posterior columns of the spinal cord of a mouse treated with rat IgG killed on day 30 after immunization shows fewer macrophages and larger areas of intact (blue staining) myelin. Magnification ×137. **a**, **c**, **e**, Staining with haematoxylin and eosin; **b**, **d**, **f**, Klüver–Barrera staining for myelin.

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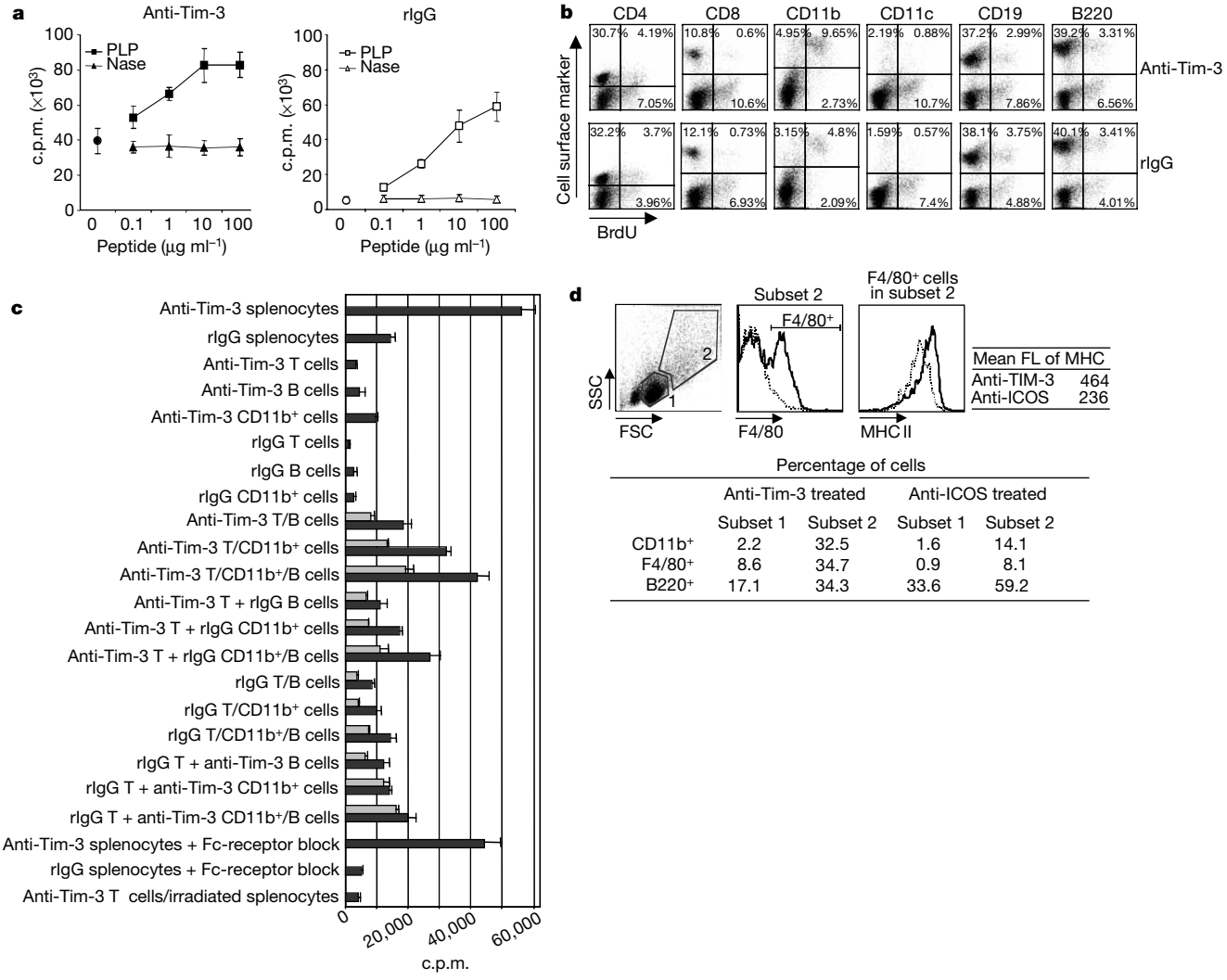
conducted, the addition of T cells treated with anti-Tim-3 to rat IgG2a-treated B cells plus CD11b$^+$ cells increased the basal proliferative response; however, maximal basal proliferation required the presence of both anti-Tim-3-treated T cells and non-T cells. This basal proliferative response cannot be lowered by incubating the splenocytes with Fc-receptor (crystallizable fragment receptor, FcR)-blocking antibodies, suggesting that FcR-mediated crosslinking can be excluded as the mechanism for increasing basal response. The synergistic effect of the interaction between T cells and non-T cells is confirmed further, as addition of irradiated non-T cells to anti-Tim-3-treated T cells does not reconstitute the same high basal response. To determine whether a cognate interaction was required between T cells and non-T cells for the increase in the basal responses, we separated T cells and non-T cells with a permeable membrane, which inhibits cell contact, and measured proliferative responses. As shown in Fig. 5c, separation of anti-Tim-3-treated T cells from anti-Tim-3-treated non-T cells resulted in a marked decrease in the proliferative responses, indicating that a cognate interaction between T and non-T cells is necessary.

To address directly whether T cells expressing Tim-3 regulate the expansion and activation of macrophages and other non-T cells, we adoptively transferred Tim-3$^+$, Th1 5B6 transgenic T cells (with specificity for PLP 139-151 peptide) to naive SJL recipients, which were then immunized with PLP 139-151 peptide and treated with anti-Tim-3 or anti-ICOS antibody (as a T-cell antibody binding control). Flow cytometric analysis of spleen cells three days after transfer showed a marked increase in the number of activated CD11b$^+$/ F4/80$^+$ macrophages in mice treated with anti-Tim-3,



Percentage of cells

	Anti-Tim-3 treated		Anti-ICOS treated	
	Subset 1	Subset 2	Subset 1	Subset 2
CD11b$^+$	2.2	32.5	1.6	14.1
F4/80$^+$	8.6	34.7	0.9	8.1
B220$^+$	17.1	34.3	33.6	59.2

Figure 5 Treatment with anti-Tim-3 antibody induces macrophage activation. **a**, SJL mice immunized with PLP 139–151 peptide and given anti-Tim-3 or rat IgG (rIgG). Mice were killed on day 10, and spleens were removed. The cells were stimulated *in vitro* with increasing concentrations of PLP 139–151 peptide or neuraminidase 101–120 (Nase) peptide as control antigen. Proliferation was measured after 48 h by ^{3}[H]-thymidine incorporation. **b**, SJL mice were given BrdU in their drinking water (0.8 mg ml^{-1}) after immunization with PLP 139–151 peptide and anti-Tim-3 or rat IgG antibody treatment. Mice were killed on day 10 and splenocytes were stained with monoclonal anitbodies to CD4, CD8, CD11b, CD11c, CD19, B220 and BrdU. Dot plots (logarithmic scale) represent BrdU incorporation in the different cell populations. **c**, SJL mice immunized with PLP 139–151 peptide and given anti-Tim-3 or rat IgG2a treatment. Mice were killed on day 10. T, B and CD11b$^+$ cells were purified from the spleen. Either whole splenocytes or T cells (10^5) were incubated in the presence or absence of 2×10^5 B cells, 2×10^5 CD11b$^+$ cells or irradiated splenocytes, and the proliferative response (black bars) was measured (^{3}[H]-thymidine incorporation in triplicate wells). In a separate set of wells (grey bars), the purified T cells and non-T cells were separated by a permeable 0.2-μm Anapore membrane to eliminate cognate interaction. **d**, SJL mice were injected with 5×10^6 Tim-3-expressing, Th1 5B6 transgenic T cells specific for the PLP 139–151 peptide, then immunized with PLP 139–151 peptide. The recipients were also injected with anti-Tim-3 or anti-ICOS (as control) antibody on days 0 and 2 (subset 1 and 2, respectively). Spleen cells were removed on day 3 and the cells were stained with monoclonal antibodies to CD11b, F4/80, B220 and MHC class II. FSC, forward scatter; SSC, side scatter; FL, fluorescence.

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but not in mice treated with anti-ICOS. There was a 2–3-fold increase in the number of $CD11b^+/F4/80^+$ cells present in subset 2 (Fig. 5d). These $F4/80^+$ macrophages also expressed higher levels of MHC class II, indicating that they are activated to a greater extent.

Taken together, these data indicate that a cognate interaction between non-T cells and Tim-3-expressing Th1 cells is affected by anti-Tim-3 treatment, resulting in the expansion and activation of $CD11b^+/F4/80^+$ macrophages. Several possible mechanisms may explain our finding: (1) anti-Tim-3 may crosslink Tim-3 protein on the surface of differentiated Th1 cells *in vivo* and amplify the production of pro-inflammatory cytokines (IFN-γ and TNF), which in turn may induce activation of macrophages; (2) anti-Tim-3 antibody could enhance migration of differentiated Th1 cells into the brain where these cells may increase the cellular influx of macrophages from the circulation; (3) anti-Tim-3 could block a cognate interaction of Tim-3 with its potential inhibitory ligand on macrophages, thus leading to enhanced macrophage activation in the presence of pro-inflammatory cytokines produced by Th1 cells.

Furthermore, as Th1 and Th2 cells cross-regulate each other's functions, expression of Tim-3 on Th1 cells and subsequent transport of Tim-3-bearing Th1 cells to tissue sites might also have a role in the regulation of asthma and atopy. Recent studies have shown that Hba mice (BALB/c congenic mice carrying an interval on chromosome 11 from DBA/2) that are resistant to development of airway hyperreactivity show a strong linkage to the Tim-3/Kim locus and express polymorphic *Tim-3* and *Tim-1* genes[23]. In summary, we have described a molecule that is selectively expressed on the surface of Th1 cells and have shown that engagement of this molecule during T-cell activation results in macrophage activation and increased severity of an autoimmune disease. These data, together with the results in asthma-resistant mice, suggest that the *Tim* gene family may have an important role in the regulation of autoimmunity and allergies. □

Methods

Generation of Th1-specific monoclonal antibodies

Lewis and Lou/M female rats (Harlan Sprague–Dawley) were immunized three times by a combination of subcutaneous injection with either $1–5\times10^7$ Th1-polarized T-cell clones and/or lines. The rats were boosted and four days later spleen cells were fused with myeloma cells (American Type Culture Collection number CRL8006) using polyethylene glycol 1450 and selection in HAT medium[24]. The supernatants from the fusion plate wells were screened by flow cytometry on Th1 and Th2 cells. All hybridoma wells that gave a positive shift on Th1, but not Th2, T-cell clones were expanded and subcloned twice by limiting dilution.

Cloning procedure

A eukaryotic expression library was constructed using mRNA from the AE7 Th1 clone and the pAXF vector. We carried out library screening by expression cloning according to the method developed in ref. 20. Immunoselected individual plasmids were transfected into COS cells followed by indirect immunofluorescence staining with anti-Tim-3 antibody (8B.2C12). Positive clones were sequenced.

In vitro T-cell differentiation

CD62L high, CD44 low sorted naive DO11.10 T cells were stimulated *in vitro* with irradiated BALB/cJ spleen cells and OVA 323–339 peptide (10 μg ml^{-1}, Quality Controlled Biochemicals) for 7 days in the presence of mIL-12 (5 ng ml^{-1}, Pharmingen) and anti-mIL-4 (10 μg ml^{-1}) for Th1 differentiation and mIL-4 (10 ng ml^{-1}, R&D Systems) and anti-mIL-12 (10 μg ml^{-1}, Pharmingen) for Th2 differentiation. The cultures were supplemented with recombinant murine IL-2 (10 ng ml^{-1}) on days 2 and 4. All cells were collected on day 10 and used for further analysis.

Induction of EAE

Female SJL mice (4–8 weeks old) (Jackson Laboratory) were injected subcutaneously in each flank with 25–75 μg PLP 139–151 peptide (HSLGKWLGHPDKF) (Quality Controlled Biochemicals) in complete Freund's adjuvant (Difco) supplemented with 400 μg *Mycobacterium tuberculosis* (Difco). Each mouse was also injected intravenously with 100 ng pertussis toxin (List Biological Laboratories) in 0.1 ml PBS. Mice were injected intraperitoneally every other day beginning on day 0 and continuing for 10–16 days with either 100 μg anti-Tim-3 (endotoxin activity of 0.2–0.8 EU (endotoxin units) mg^{-1}) or 100 μg control rat IgG or PBS. Mice were examined daily for signs of EAE, which were graded as follows: flaccid tail, 1; uneven gait and impaired righting reflex, 2; total hindlimb paralysis, 3; fore- and hindlimb paralysis, 4; and moribund, 5. At the peak of the disease or at the end of the experiment, brains and spinal cords were removed and fixed in 10% formalin and examined histopathologically for inflammation and demyelination.

Proliferation assays

Female SJL mice (4–8 weeks old) were injected subcutaneously in each flank with 50 μg PLP 139–151 peptide emulsified in complete Freund's adjuvant. Mice were injected intraperitoneally every other day with either 100 μg anti-Tim-3 or 100 μg control rat IgG or PBS. Mice were killed on day 10, and spleens and lymph nodes were removed. Cells were plated at 5×10^5 cells per well in round-bottomed 96-well plates with PLP 139–151 peptide or neuraminidase 101–120 peptide (EALVRQGLAKVAYVYKPNNT) (Nase; Quality Controlled Biochemicals) added at 0–100 μg ml^{-1} for 48 h, and plates were pulsed with 1 μCi ^{3}[H]-thymidine per well for 12 h. We measured the incorporated radiolabelled thymidine using a Beta Plate scintillation counter (Wallac).

Quantitative Taqman RT-PCR

RNA was reverse-transcribed to cDNA. We performed real-time quantitative RT-PCR using the Taqman system (Applied Biosystems). The expression levels of Tim-3 and internal reference GAPDH were measured by multiplex PCR using probes labelled with 6-carboxyfluorescein (FAM) or VIC (Applied Biosystems), respectively. The primers and probes were designed in the 3′ untranslated region of the 2.8-kilobase transcript using Primer Express v1.0 software (Applied Biosystems). The Tim-3 sequences are as follows: FAM probe, 5′-ACAGCTGCCTGCCCAGTGCCC-3′; forward primer, 5′-GCCGGTGGACCTCAGTTTC-3′; reverse primer, 5′-TGGGAGCCAGCACAGATCA-3′. We purchased the GAPDH primer/probe set from Applied Biosystems. The simultaneous measurement of Tim-3–FAM and GAPDH–VIC permitted normalization of the amount of cDNA added per sample. We performed PCRs using the Taqman Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System. A comparative threshold cycle (C_T) was used to determine gene expression relative to the no-tissue control (calibrator). Hence, steady-state mRNA levels were expressed as an *n*-fold difference relative to the calibrator. For each sample, the Tim-3 C_T value was normalized using the formula $\Delta C = C_{TTim\text{-}3} - C_{TGAPDH}$. To determine relative expression levels, the following formula was used: $\Delta\Delta_{C_T} = \Delta_{CT(1)sample} - \Delta_{CT(1)calibrator}$, and the value used to plot relative Tim-3 expression was calculated using the expression $2^{-\Delta\Delta C_T}$.

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Competing interests statement

The authors declare that they have no competing financial interests.

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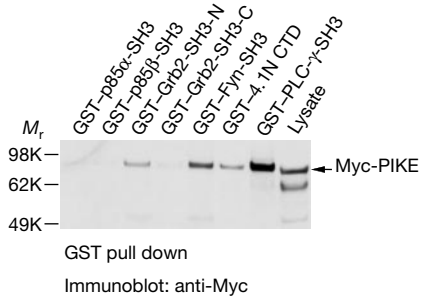

Figure 1 Binding of Myc-PIKE to different GST–SH3 domain fusion proteins. GST–SH3 fusion proteins were coupled to glutathione Sepharose beads and incubated with HEK 293 cell lysates transfected with Myc-PIKE. The beads were washed extensively and bound proteins eluted by boiling in Laemmli sample buffer. The samples were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) on a 4–12% gel, and visualized by immunoblotting with anti-Myc antibody. Similar levels of various GST fusion proteins were used (see Supplementary Information Fig. 1). The relative molecular mass (M_r) is indicated along the left. CTD, C-terminal domain.

Phospholipase Cγ1 is a physiological guanine nucleotide exchange factor for the nuclear GTPase PIKE

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Phospholipase Cγ1 (PLC-γ1) hydrolyses phosphatidylinositol-4,5-bisphosphate to the second messengers inositol-1,4,5-trisphosphate and diacylglycerol. PLC-γ1 also has mitogenic activity upon growth-factor-dependent tyrosine phophorylation[1,2]; however, this activity is not dependent on the phospholipase activity of PLC-γ1, but requires an SH3 domain[3,4]. Here, we demonstrate that PLC-γ1 acts as a guanine nucleotide exchange factor (GEF) for PIKE (phosphatidylinositol-3-OH kinase (PI(3)K) enhancer). PIKE is a nuclear GTPase that activates nuclear PI(3)K activity, and mediates the physiological activation by nerve growth factor (NGF) of nuclear PI(3)K activity[5]. This enzymatic activity accounts for the mitogenic properties of PLC-γ1.

PIKE possesses proline-rich domains, which typically bind to SH3 domains of other proteins[6–8]. To identify PIKE-interacting proteins, we conducted pull-down experiments exploring potential interactions between PIKE and SH3 domains of proteins such as the p85 subunit of PI(3)K, Grb2, Fyn and PLC-γ (Fig. 1; see also Supplementary Information Fig. 1). The most robust interaction occurs with the SH3 domain of PLC-γ, although substantial binding is also evident for Fyn and the amino-terminal SH3 domain of Grb2; however, we observed no binding with p85α or -β. The carboxy-terminal domain of protein 4.1N, which binds PIKE through a non-SH3 domain[5], also binds PIKE in these experiments.

To test whether these different SH3 domains have any effect on activation of the GTPase PIKE, we monitored the influence of their glutathione *S*-transferase (GST)–SH3 fusion proteins on the binding by PIKE of [^{35}S]GTP-γS and [^{3}H]GDP (Fig. 2a, b). PLC-γ-SH3 (residues 790–850) markedly augments the binding by PIKE of [^{35}S]GTPγ-S and accelerates the dissociation of [^{3}H]GDP. By contrast, Fyn and Grb are inactive. A mutation at position 842 of proline to leucine in PLC-γ-SH3, which abolishes association with its target protein[9], also diminishes its interaction with PIKE (Supplementary Information Fig. 2a). Consequently, GST–PLC-γ1-SH3 (P842L) fails to stimulate binding by PIKE of [^{35}S]GTPγ-S or the dissociation of [^{3}H]GDP (Fig. 2a, b). Selectivity for the SH3 domain of PLC-γ is evident from the enhancement of [^{3}H]GDP dissociation from PIKE elicited by GST fusion proteins containing only the SH3

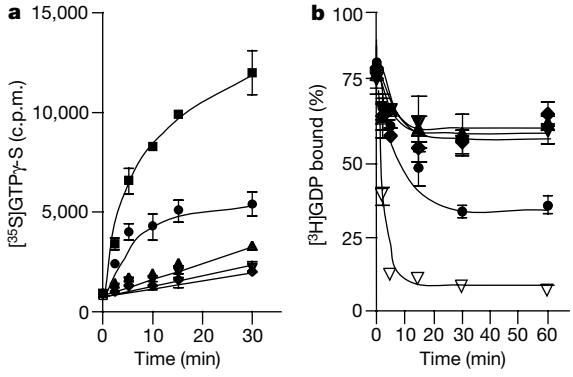

Figure 2 The SH3 domain of PLC-γ functions as a nucleotide exchange factor for PIKE. **a**, GST–PLC-γ-SH3 stimulates GTP binding to PIKE. PIKE (50 nM) was incubated with 50 nM GST–PLC-γ-SH3 (squares), GST–PLC-γ-SH3 (P842L) (circles), GST–Fyn-SH3 (diamonds), GST–Grb2-SH3-N (triangles) protein or control buffer (inverted triangles) for 30 min at 37 °C. [^{35}S]GTPγ-S (2 μCi ml^{-1}) (3 μM) was added to start the assay. At the indicated time points, [^{35}S]GTPγ-S bound to PIKE was measured by a filter-binding assay. c.p.m., Counts per minute. **b**, Effects of purified GST–PLC-γ-SH3 fusion protein on the kinetics of GDP dissociation from PIKE. His-PIKE (50 nM) was preloaded with 0.5 μM [^{3}H]GDP for 30 min at 30 °C. Purified GST–PLC-γ-SH3 (open, inverted triangles), GST–PLC-γ-SH3 (P842L) (filled circles), GST–Fyn-SH3 (filled diamonds), GST–Grb2-SH3-N (filled triangles) proteins (50 nM each) or control buffer (filled, inverted triangles) were added together with 0.5 mM unlabelled GTP at the start of the assay. At the time intervals indicated, the PIKE-bound radioactivity was measured by a filter-binding assay. Stimulation of GDP dissociation is expressed as the percentage of [^{3}H]GDP bound to PIKE before the addition of unlabelled GTP (see Supplementary Information). Standard error is indicated (n = 3).

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