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The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity

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Tim-3 is a T helper type 1 (T_H1)–specific cell surface molecule that seems to regulate T_H1 responses and the induction of peripheral tolerance. However, the identity of the Tim-3 ligand and the mechanism by which this ligand inhibits the function of effector T_H1 cells remain unknown. Here we show that galectin-9 is the Tim-3 ligand. Galectin-9-induced intracellular calcium flux, aggregation and death of T_H1 cells were Tim-3-dependent *in vitro*, and administration of galectin-9 *in vivo* resulted in selective loss of interferon-γ-producing cells and suppression of T_H1 autoimmunity. These data suggest that the Tim-3–galectin-9 pathway may have evolved to ensure effective termination of effector T_H1 cells.

In adaptive immunity $CD4^+$ T cells differentiate into T helper 1 (T_H1) and T_H2 cells in response to different antigenic stimulation[1]. T_H1 cells produce interleukin 2 (IL-2) and interferon-γ (IFN-γ), elicit delayed-type hypersensitivity responses and induce cell-mediated immunity against intracellular pathogens, whereas T_H2 cells produce IL-4, IL-5, IL-10 and IL-13, mediate immunoglobulin G_1 (IgG_1) and IgE production and eosinophilic inflammation and promote immunity to extracellular parasites[2]. The cytokines produced by T_H1 and T_H2 cells have opposite effects in immune regulation, cross-regulate each other antagonistically and show reciprocal functional inhibition[3]. However, dysregulated T_H1 and T_H2 responses may lead to pathological consequences. Overactivated T_H1 responses cause organ-specific autoimmune diseases, such as type I diabetes and multiple sclerosis[4,5], whereas uncontrolled T_H2 responses have been linked to allergy and atopy[6,7]. Thus, elucidation of the molecular mechanisms of controlling effector T_H1 and T_H2 cells in related immune responses has both biological and clinical importance.

Tim-3, a T_H1-specific type 1 membrane protein, is not expressed on the surfaces of naive T cells but emerges on the cell surface of fully differentiated T_H1 cells[8]. Tim-3 may regulate T cell responses, as shown by the fact that expression of Tim-3 mRNA is correlated with the activation of effector T_H1 cells in the animal model experimental autoimmune encephalomyelitis (EAE)[8] and by the high expression of the proinflammatory cytokines IFN-γ and tumor necrosis factor in patients with multiple sclerosis[9]. Furthermore, polymorphisms in Tim-3 are associated with the development of airway hyper-reactivity in mice[10] and rheumatoid arthritis in humans[11].

Several lines of indirect evidence suggest that Tim-3 expressed on T cells functions as an inhibitory molecule. First, blocking this pathway with a Tim-3–Ig fusion protein during an ongoing immune response results in hyperproliferation of T_H1 cells, with massive spontaneous production of IFN-γ[12]. Second, administration of Tim-3–Ig abrogates the induction of high-dose antigen-specific tolerance as well as transplantation tolerance induced by antibody to CD40 ligand[12,13]. Those data indicate the importance of Tim-3 and its ligand (Tim-3L) in regulating both peripheral tolerance and the expansion of effector T_H1 cell populations; however, the identity of the Tim-3 ligand has remained unknown.

Here we have identified galectin-9, a member of the galectin family (also called S-type lectins) that is expressed on lymphocytes and other cell types[14], as the Tim-3 ligand. Galectins are a family of carbohydrate-binding proteins that have been linked to a crucial function in regulating immune cell homeostasis and inflammation[15], but the ligands for most of the galectins in the immune system have not been identified. Functional studies suggested that galectin-9 induced cell death in T_H1 cells but not in T_H2 cells and that the galectin-9-induced cell death in T_H1 cells was dependent on Tim-3. *In vivo* administration of galectin-9 in immunized mice specifically reduced the numbers of IFN-γ-producing T_H1 cells. The Tim-3–galectin-9 pathway may therefore have evolved to downregulate effector T_H1 responses.

RESULTS

Tim-3 expression on effector-memory $CD4^+$ T cells

Tim-3 is expressed on T_H1 cells after several rounds of *in vitro* polarization[8]. However, the expression of Tim-3 on T cells *in vivo* has not been analyzed in detail. We examined the phenotype of $CD4^+Tim\text{-}3^+$ T cells *in vivo* and found that almost all the $CD4^+Tim\text{-}3^+$ T cells in naive and immunized mice were $CD44^{hi}$ and $CD62L^{lo}$ (**Fig. 1**). This suggested functional involvement of Tim-3 in regulating effector-memory $CD4^+$ cells *in vivo*.

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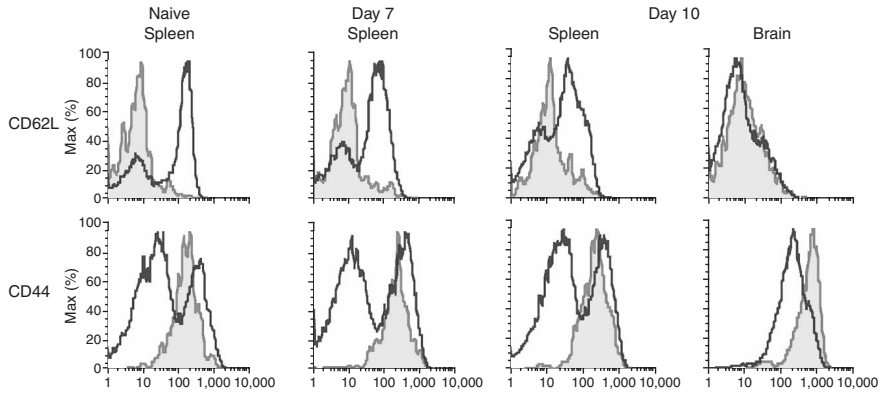

Figure 1 Tim-3 expression on effector-memory T cells *in vivo*. SJL mice were left untreated (Naive) or were immunized for the induction of EAE with a peptide of proteolipid protein (amino acids 139–151) and were killed 7 and 10 d after immunization. Spleens and brains were collected, and single-cell suspensions were prepared by collagenase D digestion and Percoll gradient purification. Cells were stained with antibody to Tim-3 (anti-Tim-3), anti-CD4, anti-CD62L and anti-CD44 and were assessed by flow cytometry for expression of CD62L and CD44 on $CD4^+$ $Tim\text{-}3^+$ cells (filled histograms) relative to total $CD4^+$ cells (open histograms). Data are representative of two independent experiments.

Identification of galectin-9 as the ligand for Tim-3

To identify the ligand for Tim-3, we used two different Tim-3–Ig fusion proteins: a full-length form (flTim-3–Ig), containing both the IgV and mucin domains, and a soluble isoform (sTim-3–Ig), containing the IgV domain but lacking the mucin domain[12]. We used these fusion proteins to screen cell lines for expression of Tim-3L by flow cytometry. Of the cell lines tested, TK-1, a $CD8^+$ mouse lymphoma cell line, had maximum expression of Tim-3L (**Fig. 2a**).

To determine the identity of the specific protein mediating the binding of Tim-3–Ig, we used lysates from surface-biotinylated TK-1 cells in a precipitation assay with Tim-3–Ig fusion proteins, followed by separation by SDS-PAGE. Both Tim-3–Ig fusion proteins precipitated broad bands of over 85 kilodaltons (kDa) and 40–60 kDa (**Fig. 2b**) that were not obtained with the control Tim-2–Ig fusion protein or with human IgG. After treatment of the precipitates with *N*-glycosidase F (PNGase F) to remove *N*-linked oligosaccharides, a 'sharper' band of 35 kDa appeared (**Fig. 2b**), indicating a cell surface membrane protein was specifically recognized by Tim-3. Mass spectrometry analysis of the 35-kDa silver-stained band extracted from the SDS polyacrylamide gel identified the protein as galectin-9.

We isolated two cDNA sequences from TK-1 cells that encoded galectin-9. These corresponded to galectin-9 (Gal-9) and its long isoform (Gal-9L), which has a 31–amino acid insertion in the hinge region of the two carbohydrate-recognition domains (CRDs)[14]. To confirm the interaction between galectin-9 and Tim-3, we subcloned cDNA encoding each galectin-9 isoform into an expression vector that bicistronically expressed enhanced green fluorescent protein (EGFP) along with the protein of interest. Transiently transfected Chinese hamster ovary (CHO) cells expressing either Gal-9 or Gal-9L stained with both flTim-3–Ig and sTim-3–Ig but not with control human IgG (**Fig. 3a**). As an additional control, we stained galectin-9 transfectants with other Tim-Ig fusion proteins (Tim-2–Ig and Tim-4-Ig) and found that only Tim-3–Ig fusion proteins, but not other Tim-Ig fusion proteins, stained galectin-9 transfectants (**Fig. 3b**). To determine whether other members of the galectin family can also interact with Tim-3, we cloned and transiently expressed galectin-4, galectin-3 and galectin-1 in CHO cells. As expected, all cells expressing EGFP galectin-9 stained with flTim-3–Ig and sTim-3–Ig, but only a small percentage of cells with very high expression of galectin-4, galectin-3 and galectin-1 stained with the flTim-3–Ig and sTim-3–Ig fusion proteins (**Fig. 3c**), suggesting low-affinity interactions between Tim-3 and other members of the galectin family.

Galectin-9 binds carbohydrate chains on Tim-3

The interaction of galectins and their receptors is dependent on the CRD and the oligosaccharide chains of the cell surface–associated proteins[16,17]. Therefore, we sought to determine whether the binding of galectin-9 and Tim-3 is based on the same mechanism. Recombinant galectin-9 bound to both full-length and soluble Tim-3–Ig fusion proteins in solution and was inhibited in a dose-dependent way by α-lactose, a competitive substrate that inhibits the interaction between galectins and their receptors (**Fig. 4a**). We obtained similar results when we examined the intracellular staining of galectin-9-transfected CHO cells with sTim-3–Ig in the presence of various concentrations of α-lactose (**Fig. 4b**).

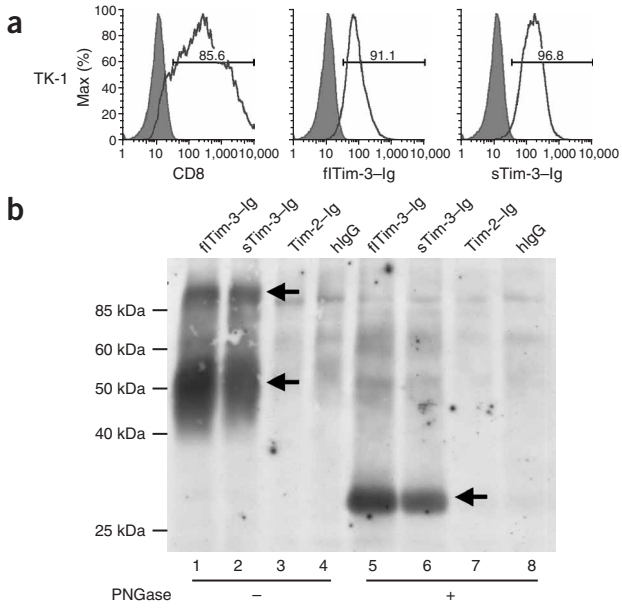

Figure 2 Identification of the Tim-3-binding protein. (**a**) TK-1 cells stained with anti-CD8 and biotinylated flTim-3–Ig, sTim-3–Ig or human IgG (human IgG) control. Numbers above bracketed lines indicate the percentage of cells positive for CD8, flTim-3–Ig or sTim-3–Ig staining. (**b**) Precipitation assays with Tim–Ig fusion proteins or human IgG (hIgG) in lysates of surface-biotinylated TK-1 cells. Bound proteins were separated by SDS-PAGE and detected by immunoblot. A broad band of more than 80 kDa and a band of 40–60 kDa (arrows) are specifically associated with Tim-3–Ig fusion proteins (lanes 1,2) but not with the controls Tim-2–Ig (lane 3) or human IgG (lane 4). After deglycosylation with PNGase F, a 'sharper' band of 35 kDa appears (arrow, lanes 5,6). This band is not present for the controls Tim-2–Ig (lane 7) and human IgG (lane 8). Data are representative of four independent experiments.

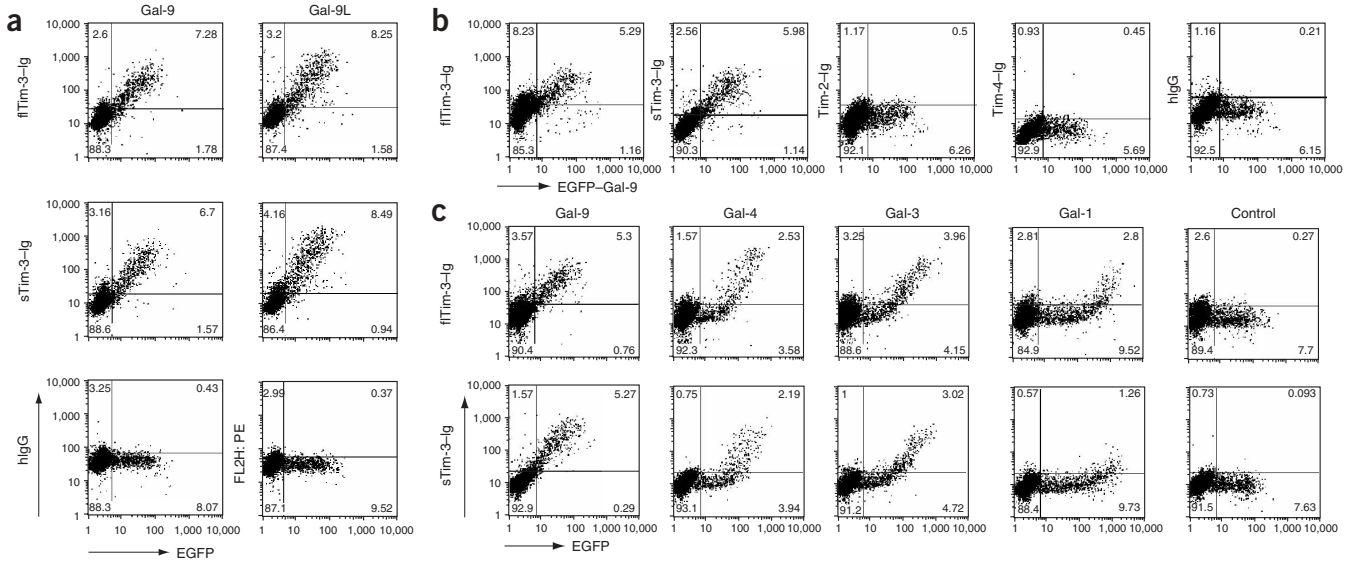

Figure 3 Galectin-9 specifically interacts with Tim-3. (**a**) Flow cytometry with flTim-3–Ig and sTim-3–Ig. Gal-9 and Gal-9L expression plasmids were transiently transfected into CHO cells. As transient transfection does not produce expression of galectins on the cell surface[32], intracellular staining with flTim-3–Ig, sTim-3–Ig and human IgG (nonspecific control) was done 48 h after transfection. (**b**) CHO cells transiently transfected with Gal-9 were stained intracellularly with flTim-3–Ig, sTim-3–Ig, Tim-2–Ig, Tim-4-Ig and human IgG. (**c**) Expression plasmids for mouse Gal-9, Gal-4, Gal-3 and Gal-1 and no plasmid (Control) were transiently transfected into CHO cells, followed by intracellular staining. In all experiments, staining by Tim–Ig fusion proteins is detected by phycoerythrin–anti-human IgG. Numbers in quadrants indicate percent cells in that quadrant. Data are representative of at least three independent experiments.

Galectin-9 has two CRDs in which two conserved amino acids, R64 and R238, are important for carbohydrate recognition[18]. We generated galectin-9 mutants by changing CRD residues R64 and R238 to alanines. Whereas changing a single CRD binding site impaired the capacity of galectin-9 to bind to Tim-3, alteration of both of the CRDs completely abolished the binding of Tim-3–Ig to galectin-9 (**Fig. 4c**). In coprecipitation experiments with untreated or PNGase F–treated (deglycosylated) sTim-3–Ig and galectin-9, only untreated sTim-3–Ig was able to precipitate galectin-9 (**Fig. 4d**), further supporting the idea that it is the carbohydrates present on the sTim-3–Ig that bind to galectin-9.

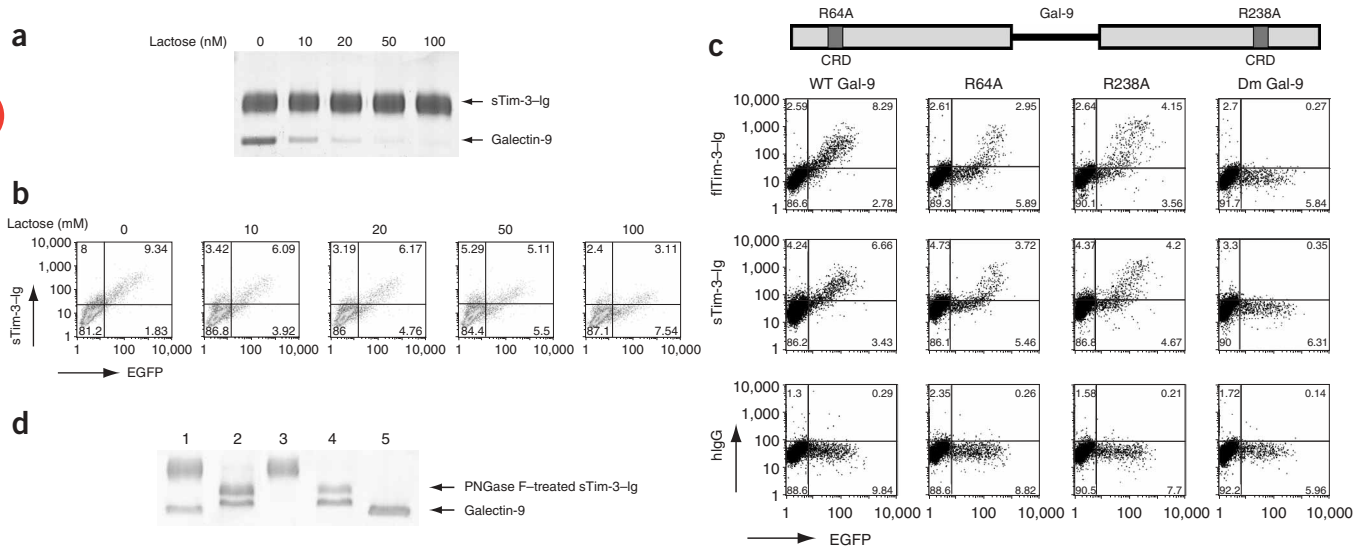

Figure 4 Galectin-9 specifically binds to the carbohydrate chains on Tim-3. (**a**) Precipitation of galectin-9 and Tim-3–Ig fusion proteins. Galectin-9 (2 μg) was mixed with sTim-3–Ig (4 μg) in the presence of increasing concentrations of the competitive substrate α-lactose (above lanes). (**b**) Intracellular staining by sTim-3–Ig of CHO cells transiently transfected with Gal-9, in the presence of increasing concentrations of α-lactose (above plots). (**c**) Flow cytometry of CHO cells expressing wild-type Gal-9 (WT Gal-9) or Gal-9 mutants with alterations in the N-terminal CRD (R64A), C-terminal CRD (R238A) or both CRDs (Dm Gal-9), stained intracellularly with sTim-3–Ig. Numbers in quadrants indicate percent cells in that quadrant. (**d**) Precipitation of Galectin-9 together with untreated sTim-3–Ig or with sTim-3–Ig treated with PNGase F. Lane 1, Galectin-9 plus untreated sTim-3–Ig; lane 2, Galectin-9 plus sTim-3–Ig treated with PNGase F; lane 3, untreated sTim-3–Ig alone; lane 4, sTim-3–Ig treated with PNGase F alone; lane 5, Galectin-9 alone. Data are representative of three independent experiments.

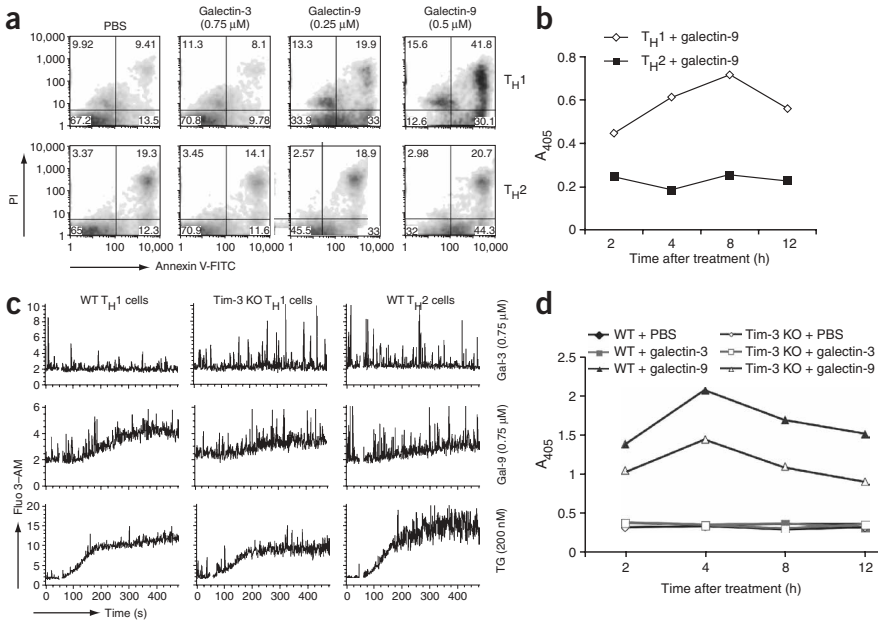

Figure 5 Galectin-9-induced cell death in activated mouse T_H1 cells is mediated by interaction with Tim-3. (**a**) Propidium iodide staining for cell viability. DO11.10 T_H1 and T_H2 cells polarized *in vitro* were purified with Ficoll-Paque and then treated with PBS, galectin-3 or galectin-9. After 8 h, cells were stained with propidium iodide (PI) and annexin V–fluorescein isothiocyanate (Annexin V–FITC). Numbers in quadrants indicate percent cells in each. Propidium iodide–positive populations positive or negative for annexin V are dead or late-stage apoptotic cells; propidium iodide–negative annexin V–positive populations represent early-stage apoptotic cells; double-negative populations are live cells. (**b**) Nucleosome enrichment assay of cell death of wild-type (WT) DO11.10 T_H1 cells and T_H2 cells treated with 0.5 μM galectin-9. A_{405}, absorbance at 405 nm. (**c**) Calcium flux in wild-type and Tim-3-deficient (Tim-3 KO) DO11.10 T_H1 and wild-type DO11.10 T_H2 cells labeled with Fluo-3–AM and treated with galectin-9, galectin-3 or thapsigargin (TG). (**d**) Nucleosome enrichment assay of cell death of wild-type and Tim-3-deficient DO11.10 T_H1 cells treated with 0.5 μM galectin-9, galectin-3 or PBS. Data are representative of three independent experiments.

Galectin-9 induces T_H1 cell death through Tim-3

Blocking Tim-3L *in vivo* by the administration of Tim-3–Ig induces hyperproliferation of effector T_H1 cells[12]; however, the molecular mechanism by which the interaction of Tim-3 with its ligand regulates T_H1 cell population expansion is not known. Galectin-9 can induce cell death of thymocytes and peripheral $CD4^+$ and $CD8^+$ T cells[19,20], and the calcium–calpain–caspase-1 pathway has been linked galectin-9-induced cell death[20]. However, the receptor engaged by galectin-9 on the surface of T cells is not known. To analyze the effect of galectin-9 on T cells, we first determined the effect of galectin-9 on terminally differentiated T_H1 and T_H2 cells derived from naive DO11.10 T cell receptor (TCR)–transgenic T cell precursors. Incubation of T_H1 cells with galectin-9 induced rapid cell death that peaked within 4 to 8 h, with little or no cell death in T_H2 cells (**Fig. 5a,b** and **Supplementary Table 1** online). Thus, galectin-9-induced cell death correlated with Tim-3 expression on T_H1 cells. To test whether the effects of galectin-9 on T_H1 cells were dependent on Tim-3, we used Tim-3-deficient DO11.10 TCR-transgenic mice. The addition of galectin-9 generated a distinct calcium flux in wild-type T_H1 cells. In contrast, Tim-3-deficient T_H1 cells showed substantially less galectin-9-mediated calcium flux (**Fig. 5c**). However, thapsigargin, an inhibitor of endoplasmic reticulum calcium-ATPase[21], triggered immediate calcium efflux from the endoplasmic reticulum lumen in both wild-type and Tim-3-deficient T_H1 cells (**Fig. 5c**), suggesting that there was no intrinsic defect in intracellular calcium mobilization in Tim-3-deficient T_H1 cells and that the galectin-9-induced intracellular calcium flux was dependent on its interaction with Tim-3 on T_H1 cells. Independent analysis of cell death by nucleosome enrichment assay further demonstrated that wild-type T_H1 cells were susceptible to cell death by galectin-9, which was partially abrogated in the Tim-3-deficient T_H1 cells (**Fig. 5d**). As an additional specificity control, we treated cells with galectin-3, which also has a CRD and showed low-affinity binding to Tim-3–Ig (**Fig. 3c**). Galectin-3 was not able to induce either calcium flux (**Fig. 5c**) or cell death (**Fig. 5a,d** and **Supplementary Table 1** online) in T_H1 cells, further supporting the idea of a functional interaction between galectin-9 and Tim-3 in the regulation of T_H1 responses. In the same experiment, T_H2 cells generated from the same precursor did not flux calcium when stimulated with galectin-9. Thus, the cell death assays suggested that a portion of the galectin-9-mediated cell death in T_H1 cells was dependent on the Tim-3–galectin-9 interaction. As galectin-9-mediated cell death of T_H1 cells was not completely abolished in Tim-3-deficient cells, this raises the possibility that galectin-9 may use an additional receptor to induce cell death.

To determine the mechanism by which T_H1 cells undergo cell death, we obtained galectin-9-treated T_H1 and T_H2 cells from DO11.10 TCR-transgenic mice and did live-imaging confocal microscopy of cells labeled with carboxyfluoroscein succinimidyl ester (CFSE). Treatment with galectin-9 induced DO11.10 T_H1 cell aggregation beginning at 15 min after exposure and induced cell death 4 h thereafter. DO11.10 T_H2 cells did not demonstrate this galectin-9-induced aggregation and cell death (**Fig. 6a,b**). Cell death was preceded by and was associated with tight physical engagement between T_H1 cells, leading to the formation of large clusters (**Supplementary Videos 1** and **2** online). In addition, galectin-9-induced cell death in T_H1 cells seemed to involve both apoptotic and necrotic mechanisms, because typical apoptotic bodies as well as necrotic cells were present in the galectin-9-treated cultures (**Fig. 6b**). Cell death was accompanied by a massive increase in cell size, rapid disruption of the cell membrane and loss of cytoplasmic contents, as shown by the loss of CFSE-labeled proteins (**Supplementary Video 3** online). Galectin-9 induced cell aggregation in both wild-type and Tim-3-deficient T_H1 cells, but Tim-3-deficient-T_H1 cells were less vulnerable to galectin-9-triggered cell death than wild-type T_H1 cells, as demonstrated by the reduction in the number of $CFSE^+$ T cells (**Fig. 6c**).

Galectin-9 eliminates IFN-γ-producing T_H1 cells

To determine the *in vivo* effects of galectin-9 on the development of T_H1 responses, we immunized C57BL/6J mice (a T_H1-prone mouse strain) to induce EAE using a myelin oligodendrocyte glycoprotein (MOG) peptide of amino acids 35–55 (MOG(35–55)) in complete Freund's adjuvant (CFA) and treated the mice intraperitoneally with recombinant galectin-9. We then collected splenocytes and analyzed cell proliferation and cytokine production. Although galectin-9 administration *in vivo* did not substantially affect overall T cell

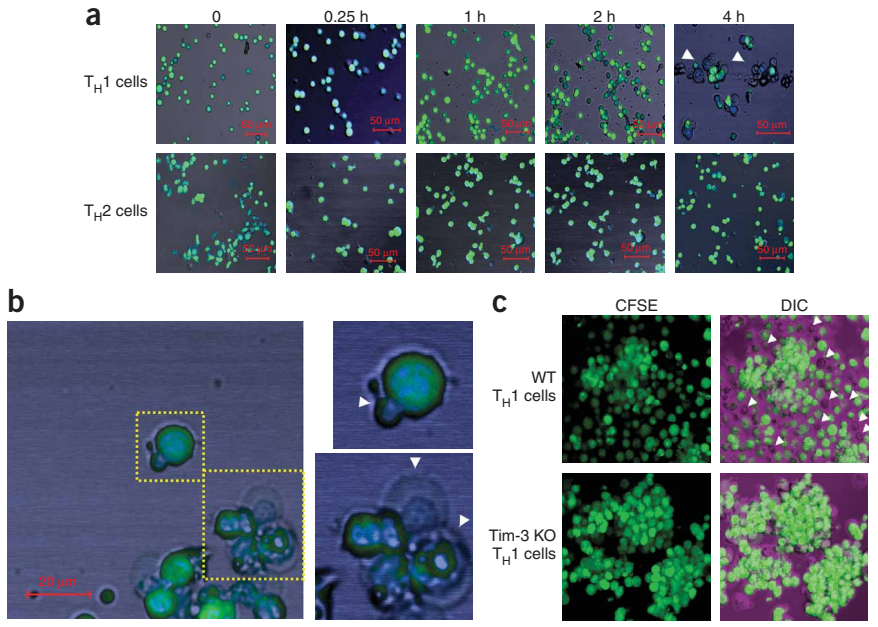

Figure 6 Confocal microscopy of galectin-9-induced cell death in T_H1 cells. Polarized wild-type DO11.10 T_H1, T_H2 and Tim-3-deficient T_H1 cells were purified with Ficoll-Paque and then labeled with CFSE. Live images were recorded from 'time zero' (0) to 4 h after the addition of 0.5 μM galectin-9. (**a**) Galectin-9 specifically induced aggregation and tight contact in wild-type T_H1 cells but not in T_H2 cells. By 4 h, most aggregated cells in galectin-9-treated cultures were dead, with loss of CFSE staining and cell membranes clumped together (arrowheads). Scale bars, 50 μM. (**b**) Tight contact between T_H1 cells triggers cell death, but, unlike pure apoptotic or necrotic cell death, galectin-9-induced cell death produced a mixed morphology of both apoptosis and necrosis (arrowheads). Scale bar, 20 μM. (**c**) Three-dimensional digital reconstructions of confocal images obtained at 0.3-μm or 0.5-μm intervals in the *z*-axes of wild-type and Tim-3-deficient T_H1 cells 3 h after the addition of galectin-9. There was reduction in CFSE fluorescence and cell membranes of dead cells for wild-type T_H1 cells (arrowheads) by differential interface contrast (DIC). Data are representative of two independent experiments.

proliferation, there was a decrease in IFN-γ production of more than 50% in these cultures, as determined by cytokine enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISPOT) assay of cells stimulated *ex vivo* (**Fig. 7a–c**). This demonstrated that the reduction in IFN-γ production was indeed due to a reduced number of IFN-γ-producing cells. However, there was no significant effect on cells producing IL-2 ($P = 0.45$), IL-5 ($P = 1$) or IL-4 ($P = 0.08$) in the mice treated with galectin-9. To determine whether administration of galectin-9 specifically affected the MOG(35–55)-specific IFN-γ-producing T_H1 cells, we immunized C57BL/6J mice with MOG(35–55) in CFA and treated them with galectin-9. We collected and restimulated splenocytes and assessed the frequency of IFN-γ-producing $CD4^+$ T cells by intracellular cytokine staining. The mice treated with galectin-9 lost antigen-specific (MOG(35–55)-specific) IFN-γ-producing $CD4^+$ T cells almost completely, suggesting that galectin-9 targeted antigen-specific T_H1 cells *in vivo* (**Fig. 7d**). These data show that galectin-9 does not delete all peripheral T cells but selectively deletes antigen-specific T_H1 cells and spares T_H2 cells, which is consistent with the selective expression of Tim-3 on terminally differentiated T_H1 but not T_H2 cells.

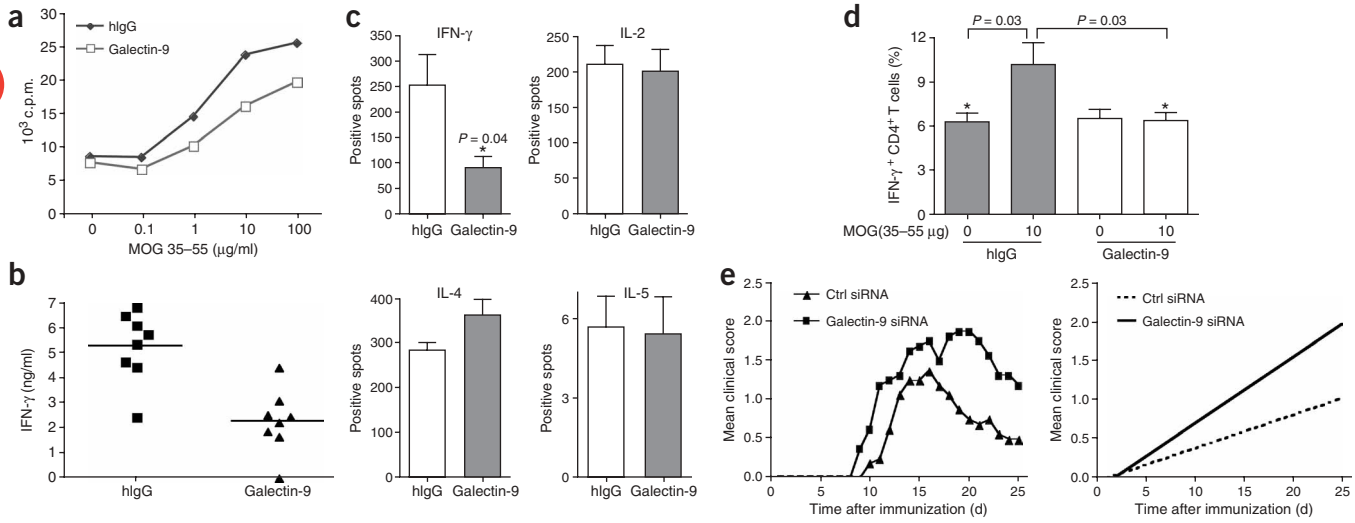

Figure 7 Effect of the modulation of galectin-9 on T_H1 cells *in vivo*. (**a–d**) C57BL/6J mice immunized with MOG(35–55) were treated with 100 μg galectin-9 or human IgG control intraperitoneally everyday from day 3 to day 9 after immunization. Mice were killed and spleen cells were collected for analysis. (**a**) Cell proliferation assay. Data are representative of two independent experiments. (**b**) ELISA for total IFN-γ. Data represent a total of eight mice in each group from two independent experiments. (**c**) ELISPOT for IFN-γ, IL-2, IL-4 and IL-5. Galectin-9, $n = 7$; human IgG, $n = 8$. (**d**) Intracellular IFN-γ staining in $CD4^+$ T cells. Galectin-9, $n = 10$; human IgG, $n = 10$. Data are from three independent experiments. (**e**) SJL mice were injected with control siRNA (Ctrl siRNA; $n = 8$) or galectin-9 siRNA (Gal-9 siRNA; $n = 8$) and then were immunized with a peptide from myelin proteolipid protein (amino acids 139–151) and were monitored for the development of EAE. Left, clinical scores of each group; right, linear regression analysis. $P = 0.00036$, slope of Gal-9 siRNA versus control siRNA regression curve. Data are from two independent experiments.

Table 1 Reduced disease severity and mortality in mice with EAE treated with galectin-9

Treatment	Incidence	Mortality	Onset	Maximal score
PBS	12 of 12	7 of 12 (58%)	13.8 ± 0.8	4.5 ± 0.2
Galectin-9	11 of 11	1 of 11 (9%)*	13.9 ± 1.2	3.3 ± 0.2**

'Onset' indicates the day of disease onset (mean ± s.e.m.); 'Maximal score' is the maximum disease score achieved (mean ± s.e.m.).
*, $P = 0.03$, and **, $P = 0.0005$, versus PBS.

Next we assessed the effect of galectin-9 treatment on the development of disease. We immunized mice to induce EAE and administered recombinant galectin-9 from day 3 to day 9. Treatment with galectin-9 reduced disease severity and mortality (**Table 1**). Because recombinant galectin-9 is inherently unstable and prolonged use *in vivo* induces anaphylactic-like reactions, we also analyzed the effect of loss of galectin-9 on the development of EAE by designing small interfering RNA (siRNA) to suppress galectin-9 expression *in vivo*. We injected galectin-9 siRNA or control siRNA into SJL mice intravenously on days 0 and 7 during disease induction. We deliberately induced disease with a suboptimal amount of encephalitogenic peptide so that the disease in the mice treated with control siRNA would be minimal and the effects of loss of galectin-9 on the development of disease could be more easily discerned. Indeed, EAE was exacerbated in the mice treated with galectin-9 siRNA versus mice treated with control siRNA, suggesting that 'knocking-down' galectin-9 expression during disease induction affected the progression of EAE (**Fig. 7e**). Combined with our results showing that galectin-9 induced cell death in activated T_H1 cells, our data suggest that galectin-9 *in vivo* is able to control proinflammatory effector T_H1 cells.

DISCUSSION

Here we have shown that galectin-9 is a ligand for the T_H1-specific cell surface molecule Tim-3 and that the interaction of these two molecules downregulates T_H1 immunity. Galectin-9 is ubiquitously expressed in a variety of tissues, including lymph nodes and spleen, in both humans and mice[14,22]. Galectin-9 had high expression in the naive immune system both in the spleen and the lymph node (unpublished observations). That raises the issues of how immune responses are started and when galectin-9 is expressed in lymphoid versus target tissues during an autoimmune immune response. Expression of galectin-9 mRNA is downregulated in the lymph node and spleen after activation, thereby providing a 'window' for the generation of T_H1 effector cells. That explains why exogenous administration of galectin-9 resulted in the deletion of IFN-γ-producing cells. In contrast, there was no galectin-9 expression in the central nervous system (CNS) of naive mice, but it was upregulated to peak expression by day 10 after immunization to induce EAE (unpublished observations). This coincided with the peak in T cell infiltration and Tim-3 expression in the CNS of mice with EAE[8]. We propose that the kinetics of galectin-9 expression in peripheral lymphoid tissue versus the CNS after immunization reflects its function, such that expression of galectin-9 in the naive state serves to inhibit the generation of T_H1 responses, the loss of galectin-9 after activation in the lymphoid tissue allows the generation of T_H1 cells and the increase in galectin-9 expression in the CNS deletes pathogenic T_H1 cells and controls inflammation.

According to our confocal image data, there were apoptotic bodies and necrotic-like cells present during galectin-9-induced cell death, suggesting the possible involvement of apoptotic and necrotic cell death. Similarly, galectin-2 can induce both necrosis and apoptosis[23]. In addition to galectins, the Fas–Fas ligand pathway can also induce both apoptotic and necrotic cell death[24], suggesting that necrotic-like cell death is a part of programmed cell death and is actually a very common mechanism for controlling effector T cell homeostasis. Even though galectin-9 induced necrotic-like cell death *in vitro*, we do not believe that treatment with galectin-9 *in vivo* induces more inflammation; instead, the Tim-3–galectin-9 pathway seems to negatively regulate effector T_H1 cells and resolve inflammation.

If the Tim-3–galectin-9 interaction inhibits autopathogenic effector T_H1 cells, it would be expected that Tim-3-deficient mice would develop very severe and protracted inflammation during EAE. At present, Tim-3-deficient mice are available only on the BALB/c background, which is mostly resistant to EAE induction. These mice cannot be crossed onto the EAE-susceptible C57BL/6J background because of polymorphisms in the closely linked genes encoding Tim-1 and Tim-3 in the BALB/c and C57BL/6J strains. Future studies should target the Tim-3 locus directly in C57BL/6J mice. Nevertheless, when we treated MOG(35–55)-immunized C57BL/6J mice with galectin-9, we found that limited exogenous administration of recombinant galectin-9 during EAE reduced mortality and disease severity. Furthermore, a decrease in the expression of galectin-9 by siRNA *in vivo* resulted in exacerbation of EAE, supporting the idea that galectin-9 is involved in controlling the encephalitogenic T cell response *in vivo*. These data support the idea of involvement of the Tim-3–galectin-9 pathway in inhibiting autopathogenic T cell responses *in vivo*.

In contrast to the galectin-9 expressed mainly in the immune system, the galectin-9 long isoform is expressed mainly in the intestine[19]. Both isoforms bind to Tim-3 equally well and induce cell death in T_H1 cells. Therefore, it is possible that whereas the short form of galectin-9 may function in regulating T_H1 responses in the immune compartment, the long isoform of galectin-9 may regulate T_H1 responses in the intestinal mucosa and potentially other mucosal tissue sites. Expression of galectin-9 in the intestinal mucosa and gut-associated lymphoid tissue may be responsible for the inhibition of T_H1 responses and the predominant expression of T_H2 and T_H3 responses in the gut-associated lymphoid tissue[25].

Galectin-9 expression can be induced by IFN-γ or IL-1β in various tissues such as endothelial cells, fibroblasts and astrocytes[26–28]. This fact introduces an interesting paradigm whereby the IFN-γ that induces tissue inflammation also induces an inhibitory ligand (galectin-9) in the target tissue, which deletes T_H1 cells and thereby prevents protracted inflammation in target organs. The finding that galectin-9 was upregulated in the CNS on day 10 after immunization to induce EAE, at a time when T cell infiltration and Tim-3 expression was at its peak, not only supports this model but also is consistent with the observation that the encephalitogenic T cells that induce EAE, once activated in the brain, produce IFN-γ and undergo rapid cell death. This may also mark the beginning of the reduction in inflammation and remission from autoimmune disease[29]. In addition to its proinflammatory effect, IFN-γ also functions in this feedback inhibition and termination of inflammation, as IFN-γ upregulates galectin-9, which in turn terminates T_H1-mediated inflammation. Loss of IFN-γ would disrupt this regulatory loop and could be the underlying mechanism that may explain why IFN-γ-deficient mice develop profound EAE[30]. In conclusion, our data have demonstrated that the interaction between Tim-3 and galectin-9 serves as a mechanism to dampen T_H1 immunity by selective deletion of Tim-3$^+$ T_H1 cells. The Tim-3–galectin-9 pathway may have evolved to control the population expansion and tolerance of T_H1 cells in the immune compartment and to prevent prolonged inflammation in target tissues.

METHODS

Precipitation assays and mass spectrometry analyses. Extracellular membrane–associated proteins on live TK-1 cells were labeled with biotin (EZ-Link Sulfo-NHS-LC-Biotin; Pierce). Whole-cell lysates were prepared and were incubated at 4 °C with 5 μg human IgG, Tim-2–Ig, flTim-3–Ig or sTim-3–Ig in the presence of protein G–agarose beads (Roche). Beads were washed and boiled with 1× SDS-PAGE loading buffer. Supernatants were collected by centrifugation, and half of each was digested with PNGase F (New England Biolabs). Samples were separated by SDS-PAGE and the Tim-3–Ig fusion protein–specific binding proteins were detected by immunoblot. For mass spectrometry, TK-1 cells were subjected to precipitation assays with Tim-3–Ig fusion proteins. Protein samples were separated by SDS-PAGE and were detected by modified silver staining. The 35-kDa band was cut out for matrix-assisted laser desorption–ionization technique-time of flight and tandem mass spectrometry analyses.

Intracellular staining. Mouse cDNA encoding Gal-9 or Gal-9L from TK-1 cells was subcloned into the bicistronic expression vector pIRES2-EGFP (Invitrogen). CHO cells were transfected and then were analyzed for galectin-9 expression by detection of the EGFP signal. For intracellular staining with sTim-3–Ig, Tim-2–Ig, Tim-4-Ig and human IgG, transfected CHO cells were fixed with 2% paraformaldehyde and then were made permeable with buffer containing 0.1% saponin. Phycoerythrin-conjugated anti-human IgG was used as the secondary detection reagent in flow cytometry.

T_H1-T_H2 polarization. $CD4^+CD62L^{hi}$ T cells from wild-type or Tim-3-deficient DO11.10 TCR-transgenic mice were polarized into T_H1 and T_H2 cells as described[8]. Cells were considered fully polarized into T_H1 or T_H2 populations after three rounds of polarization.

Purification of recombinant mouse galectin-9. Expression plasmids of Gal-9 were generated in vector pTrcHis2A (Invitrogen) and were transformed into BL21 *Escherichia coli* (Invitrogen). Protein induction and purification were done as described[19] with some modifications. PBS containing 80 mM α-lactose and 0.5 mM dithiothreitol was used as the elution buffer as well as the control reagent for *in vitro* and *in vivo* analyses (referred to as 'PBS' here). Purified galectin-9 was applied to Detoxi-Gel (Pierce) for removal of endotoxin contamination. Typical protein yield was between 1 and 1.5 mg per liter bacterial culture.

Cell death analyses. Tim-3-deficient DO11.10 T_H1 cells and wild-type DO11.10 T_H1 and T_H2 cells were stimulated with irradiated splenocytes from BALB/c mice and 10 μg/ml of ovalbumin peptide (amino acids 323–339). After 60–72 h, cells were purified by Ficoll-Paque (Pharmacia) and were plated for 2, 4, 8 and 12 h at a density of 2×10^5 cells/well in the presence of recombinant galectin-9 (0.25 μM, 0.5 μM or 0.75 μM), recombinant galectin-3 (0.75 μM) or PBS. Cell death was analyzed by staining with annexin V–fluorescein isothiocyanate and propidium iodide (Annexin V-FITC Apoptosis Detection Kit I; BD Biosciences) or by nucleosome enrichment assay (Cell Death Detection ELISA^{PLUS} kit; Roche).

Confocal microscopy and live imaging. Polarized wild-type T_H1, T_H2 and Tim-3-deficient T_H1 cells from DO11.10 TCR-transgenic mice were labeled with 1 μM CFSE according to the protocol from the manufacturer (Molecular Probes) and then cultured in MatTek culture dishes. Cells were placed in a Zeiss Live Imaging Microincubator at 37 °C and 5% CO_2 for monitoring of the response of CFSE-labeled cells to 0.5 μM galectin-9. Data were recorded over 4 h with a Zeiss laser-scanning confocal microscope and results were analyzed with LSM 510 software (Carl Zeiss). Sixty images at 20-second intervals were recorded at each time point.

Calcium mobilization. Live cells were labeled with 10 μM Fluo-3–acetoxymethylester (Fluo-3–AM; Molecular Probe) as described with some modifications[20]. For determination of baseline, calcium signals were recorded for 45 s by flow cytometry without any stimuli. After 45 s, stimuli were added and calcium fluxes were measured for a total of 8 min. Calcium signals were recorded on a FACSCalibur.

Proliferation assay, ELISA, ELISPOT assay and IFN-γ intracellular staining. C57BL/6J mice (Jackson Laboratory) were immunized with 100 μg MOG(35–55) (MEVGWYRSPFSRVVHLYRNGK) in CFA (Difco). Mice were injected intraperitoneally every day from day 3 to day 9 with 100 μg recombinant galectin-9 or with human IgG as a control (Sigma Aldrich). Proliferation assays were done as described[12]. Detection of IFN-γ by ELISA and of IFN-γ, IL-2, IL-4 and IL-5 by ELISPOT assay was done as described[12,31].

For IFN-γ intracellular staining, splenocytes were plated at a density of 5×10^5 cells/well in round-bottomed 96-well plates with or without 10 μg/ml of MOG(35–55). On day 7, cells were stimulated for 4 h with 5 ng/ml of phorbol 12-myristate 13-acetate and 500 ng/ml of ionomycin in the presence of GolgiStop (BD Bioscience). Intracellular staining for IFN-γ in $CD4^+$ T cells was assessed by flow cytometry. All animal experiments were in compliance with the approval of the Harvard Medical Area Standing Committee on Animals (protocol 696).

Induction of EAE. For treatment with recombinant galectin-9 *in vivo*, female C57BL/6J (Jackson Laboratory) mice were immunized with 100 μg MOG(35–55) in CFA supplemented with 400 μg *Mycobacterium tuberculosis* (Difco). Mice received 100 ng pertussis toxin (List Biological Laboratories) intravenously on days 0 and 2. Recombinant galectin-9 (100 μg) or PBS was injected intraperitoneally every day from day 3 to day 9.

For Gal-9 siRNA knockdown, female SJL/J mice (Jackson Laboratories) were immunized with a suboptimal dose (60 μg) of a peptide consisting of amino acids 139–151 of myelin proteolipid protein (HSLGKWLGHPDKF) in CFA supplemented with 400 μg *M. tuberculosis*. Mice were injected intravenously with 100 ng pertussis toxin on day 0 and were injected intravenously on days 0 and 7 with 50 μg control siRNA or Gal-9 siRNA (Dharmacon). The sequence of each siRNA was 5′-GGCCAGAGCUUCUCG GUGU-3′ (mouse Gal-9) and 5′-UAGCGACUAAACACAUCAA-3′ (control). Disease progression was monitored and assigned scores as follows: limp tail, 1; uneven gait and hindlimb weakness, 2; total hindlimb paralysis, 3; weak forelimb and hindlimb paralysis; forelimb and hindlimb paralysis, 4; and moribund, 5.

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

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Immunology* website for details).

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