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## Differential ICAM-1 Isoform Expression Regulates the Development and Progression of Experimental Autoimmune Encephalomyelitis

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### Abstract

Intercellular adhesion molecule-1 (ICAM-1) functions in leukocyte trafficking, activation, and the formation of the immunological synapse. ICAM-1 is a member of the immunoglobulin superfamily of adhesion proteins, which share a similar structure of repeating Ig-like domains. Many genes in this family, including ICAM-1, show alternative splicing leading to the production of different protein isoforms, although little functional information is available regarding the expression patterns, ligand interactions, and functions of these isoforms, especially those arising from the ICAM-1 gene. In this study, we show using different lines of mutant mice (*Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>*) that alterations in the expression of the alternatively spliced ICAM-1 isoforms can significantly influence the disease course during the development of EAE. *Icam1<sup>tm1Jcgr</sup>* mutant mice, unlike *Icam1<sup>tm1Bay</sup>* mutants, do not express isoforms containing the Mac-1 binding domain and had significantly attenuated of EAE. In contrast, *Icam1<sup>tm1Bay</sup>* mice developed severe EAE in both active and adoptive transfer models compared to both *Icam1<sup>tm1Jcgr</sup>* and wild type mice. We also observed that T cells from *Icam1<sup>tm1Bay</sup>* mice displayed increased proliferation kinetics and produced higher levels of IFN- $\gamma$  compared to *Icam1<sup>tm1Jcgr</sup>* and wild type mice. Thus, our investigations show that the alternatively spliced ICAM-1 isoforms are functional, and play key roles during the progression of CNS inflammation and demyelination in EAE. Furthermore, our findings suggest that these isoforms may also play key roles in controlling the development of inflammatory diseases such as multiple sclerosis, possibly through differential engagement with ICAM-1 ligands such as Mac-1.

### Keywords

ICAM-1; adhesion molecules; MS/EAE; alternative splicing

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## 1. Introduction

Intercellular adhesion molecule-1 (ICAM-1, CD54)<sup>4</sup> is a type 1 membrane-bound glycoprotein that plays a critical role in leukocyte emigration, lymphocyte activation, and other immune and inflammatory responses (Harlan et al., 1992; Lebedeva et al., 2005; Springer, 1994). ICAM-1 is expressed on a wide variety of cell types, including most leukocyte subsets, endothelial cells, and in the central nervous system, on microglia, astrocytes, and oligodendrocytes (Bo et al., 1996; Lee and Benveniste, 1999; McMurray, 1996; Roebuck and Finnegan, 1999). Initially described as a monomeric protein, ICAM-1 is composed of repeating immunoglobulin-like (Ig) domains and is expressed as a homodimer in a “bent” conformation (Chen et al., 2007; Kirchhausen et al., 1993; Miller et al., 1995). This adhesion molecule can bind to many different ligands, including the  $\beta_2$  integrins LFA-1, Mac-1, and p150/95, as well as fibrinogen, and hyaluronan (Diamond et al., 1991; Languino et al., 1995; McCourt et al., 1994; Springer, 1994). ICAM-1, like other members of the immunoglobulin superfamily of adhesion molecules such as VCAM-1, PECAM-1, and MAdCAM-1, has also been shown to undergo alternative splicing and there are at least seven ICAM-1 isoforms that have now been described (Giorelli et al., 2002; King et al., 1995; Leung et al., 1997; Ochietti et al., 2002; Osborn et al., 1992; Robledo et al., 2003; van Den Engel et al., 2000; Wakatsuki et al., 1995; Werner et al., 2001; Yan et al., 1995). However, these alternatively spliced isoforms have been poorly characterized and little information exists regarding their expression patterns, ligand binding, and functions during inflammatory responses.

Several different lines of gene-targeted mutant mice have been generated that are amenable for functional analyses of these alternatively spliced ICAM-1 isoforms (Dunne et al., 2003; Sligh et al., 1993; Xu et al., 1994). The first two ICAM-1 mutants were developed in the 1990s and used replacement constructs designed to insert the neomycin selection gene into exon 4 (*Icam1<sup>tm1Jcgr</sup>*), which encodes for IgG domain 3, or exon 5 (*Icam1<sup>tm1Bay</sup>*), which encodes for IgG domain 4 of this adhesion molecule (Sligh et al., 1993; Xu et al., 1994). Although these mutations were originally thought to result in complete loss of ICAM-1 (null), it was subsequently discovered that these mice expressed different subsets of the alternatively spliced isoforms normally found in murine cells, but not the full-length ICAM-1 molecule (King et al., 1995; van Den Engel et al., 2000). ICAM-1 knockout mice completely deficient or null for all isoform expression (*Icam1<sup>tm1Alb</sup>*) were subsequently generated by deleting the entire coding sequence (Dunne et al., 2003). Previously, we analyzed these ICAM-1 null mutant mice in the MS model, experimental autoimmune encephalomyelitis (EAE), and observed a markedly attenuated disease phenotype (Bullard et al., 2007). These results significantly differed from earlier EAE studies involving *Icam1<sup>tm1Bay</sup>* mice, in which an exacerbated form of the disease was observed compared to wild type mice (Samoilova et al., 1998), even though both studies used similar induction methods and both mutations were bred onto the same inbred strain background.

To further investigate the roles of alternatively spliced ICAM-1 isoforms in regulating CNS inflammation and demyelination, we analyzed and compared the EAE phenotypes in MOG-induced EAE using *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* mice in both active and adoptive transfer EAE. We found that *Icam1<sup>tm1Bay</sup>* mutant T cells can induce severe EAE following adoptive transfer and that encephalitogenic T cells from these mice promote CNS inflammation and demyelination even in the absence of ICAM-1 expression on other cell types. In contrast, *Icam1<sup>tm1Jcgr</sup>* mice developed both active and adoptive transfer EAE with a phenotype intermediate to that of *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Alb</sup>*. These findings suggest that the alternatively spliced ICAM-1 isoforms are functional, and that the full-length molecule is not necessary to promote leukocyte infiltration and activation during the development of EAE. Our studies further indicate that expression of individual ICAM-1 isoforms or specific

combinations of these molecules can significantly regulate immune and inflammatory responses in the CNS and possibly other tissues.

## 2. Materials and Methods

### 2.1. Mice

*Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* C57BL/6J (N10) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and have been previously described (Sligh et al., 1993; Xu et al., 1994). Both mutant lines of mice express at least three of the ICAM-1 isoforms found in wild type mice, and two are common to each mutant strain (Fig. 1). These two mutant lines differ in at least one isoform, although both have the potential to express ICAM-1 isoforms that lack domains 3 and 4 (Figure 1B and C). ICAM-1 null mutant mice (*Icam1<sup>tm1Alb</sup>*) were backcrossed at least 8 generations onto C57BL/6 (Dunne et al., 2003). Only mice homozygous for the particular ICAM-1 mutation were used for these studies, and inbred C57BL/6 mice were used as controls for all experiments. All studies were performed with approval from the UAB IACUC.

### 2.2. Induction of active and adoptive transfer EAE

For active EAE, control, *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* mice were immunized with MOG peptide<sub>35-55</sub> as previously described (Bullard et al., 2007). MOG peptide was synthesized by standard 9-fluorenyl-methoxycarbonyl chemistry and was >95% pure as determined by reversed phase-HPLC (Biosynthesis, Lewisville, TX). Onset and progression of EAE clinical signs was monitored daily (30 days) using a standard clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund; 6, dead. Only mice with a score of at least 2 (flaccid tail) observed for 2 or more consecutive days were judged to have onset of EAE. A cumulative disease index (CDI) was calculated from the sum of the daily clinical scores observed between day 7 and day 30. For transferred EAE, spleens of control, *Icam1<sup>tm1Jcgr</sup>*, or *Icam1<sup>tm1Bay</sup>* donors were removed two to three weeks following induction of active EAE, and prepared as previously described (Szalai et al., 2002). Adoptive transfer EAE was induced by injecting  $\sim 5 \times 10^6$  purified T cells into wild type or mutant recipient mice as described in the Results section. Reciprocal transfers were performed by injecting wild-type encephalitogenic T cells into *Icam1<sup>tm1Jcgr</sup>*, *Icam1<sup>tm1Bay</sup>*, or wild type mice. Mice were scored daily for 18 days using the identical system as described above.

### 2.3. Histopathology

Mice with actively induced EAE were sacrificed at 30 days p.i. by CO<sub>2</sub> inhalation, and spinal columns were removed, fixed in 10% buffered-formalin and paraffin embedded. Sections (5 $\mu$ m thick) from the cervical, thoracic and lumbar spinal cord were cut and either stained with hematoxylin and eosin for overall lesion evaluation and characterization of inflammatory responses or with Luxol fast blue for evaluation of demyelination. The extent of inflammation and demyelination was scored based on lesion size (0-4) and lesions were evaluated for lymphocyte accumulation, neutrophil infiltration, demyelination, axonal degeneration and gliosis (0-4). Tissues were evaluated without identification as to experimental group. Severity scores were calculated as the mean over all segments of the products of the intensity scores multiplied by the extent scores for each lesion characteristic (inflammation, axonal degeneration, gliosis, and demyelination). The means of the individual lesion characteristic severity scores were summed to give the overall severity score.

## 2.4. T cell proliferation and cytokine and chemokine production

Antigen-specific T cell proliferation assays were performed as previously described (Szalai et al., 2002). Single cell suspensions from spleens obtained 14 days after EAE induction were cultured in triplicate in 96-well plates at  $5 \times 10^5$  cells/well with increasing concentrations of MOG<sub>35-55</sub> peptide. After 48 h, cultures were pulsed with <sup>3</sup>H-thymidine for 18 h and incorporation of thymidine was measured. The *in vitro* cytokine assays were performed essentially as described for the proliferation assay. Duplicate cultures were either left untreated or stimulated with MOG peptide alone (5 μg/ml). Culture supernatants were collected at 48 hrs for use in cytokine ELISAs. ELISA kits for murine cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-10, IL-12, IL-17 and TGF-β) were purchased from R&D Systems (Minneapolis, MN). Each assay was performed according to the manufacturer's instructions. Cytokine production by cultures of wild type, *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* cells, is reported as the percentage of wild type cytokine production from four mice per group.

## 2.5. T regulatory cell isolation and suppression assay

CD4<sup>+</sup>CD25<sup>+</sup> cells were isolated or depleted from spleens and lymph nodes of wild type, *Icam1<sup>tm1alb</sup>*, *Icam1<sup>tm1Jcgr</sup>*, and *Icam1<sup>tm1Bay</sup>* mice using the Miltenyi regulatory T cell isolation kit, as per the manufacturer's instructions. Isolated CD4<sup>+</sup>CD25<sup>+</sup> populations contained greater than 95% Foxp3 expressing cells. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells ( $5 \times 10^4$ ) from control mice were stimulated in round bottom 96-well plates using anti-CD3 and CD28 antibodies along with CD4<sup>+</sup>CD25<sup>+</sup> Tregs from wild type or different ICAM-1 mutants ( $1 \times 10^5$ ) as previously described (Pandiyani et al., 2007). Cells were pulsed after 48 hr with <sup>3</sup>H-thymidine (1 μCi) for 18 hrs, harvested and thymidine incorporation determined.

## 2.6. Statistics

Statistical significance between control and *Icam1<sup>tm1Jcgr</sup>* or *Icam1<sup>tm1Bay</sup>* mice for active and transferred EAE experiments was calculated using the Wilcoxon signed rank test, for proliferation assays the student's t-test was used. Results of evaluations for inflammation and demyelination were analyzed using analysis of variance for main effects and Tukey's test for pair-wise mean comparisons.

## 3. Results

### 3.1. Active EAE induction is attenuated disease in *Icam1<sup>tm1Jcgr</sup>* mice

We first analyzed and compared the EAE phenotypes in mice homozygous for the *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* mutations in order to determine whether there were differences in the initiation and progression of disease. We found that the overall course of active EAE in *Icam1<sup>tm1Jcgr</sup>* mice was significantly attenuated compared to wild type mice (Fig. 2A and Table 1; cumulative disease index or CDI of 31 vs. 58,  $p=0.0004$ , Wilcoxon signed rank test). There was no significant difference in the onset and initial course of EAE between genotypes; however, *Icam1<sup>tm1Jcgr</sup>* mice showed a lack of progression to severe disease. In addition, disease-related mortality was also reduced in *Icam1<sup>tm1Jcgr</sup>* mice compared to wild type mice (16% versus 30%, Table 1). As reported previously (Samoilova et al., 1998), we observed that *Icam1<sup>tm1Bay</sup>* mice developed EAE with similar onset and severity as that of wild type mice during at early timepoints post-immunization (Fig. 2B, Table 1), and showed an overall increase in the CDI severity scores during the chronic phases ( $p=0.0008$ , Wilcoxon signed rank test). Concomitant with the severe disease, we observed a significant increase in mortality in *Icam1<sup>tm1Bay</sup>* mice compared to wild type controls (44% versus 20%, Table 1), nearly twice that previously reported (Samoilova et al., 1998).

Histological evaluation of spinal cord sections taken at 30 days post-immunization (active EAE) revealed an overall increase in the severity of lesions in *Icam1<sup>tm1Bay</sup>* mutants compared to that of *Icam1<sup>tm1Jcgr</sup>*. H&E (Fig. 3A) and LFBPAS (Luxol fast blue + periodic acid-Schiff) (Fig. 3B) stained sections from *Icam1<sup>tm1Bay</sup>* mice showed a pattern of demyelinating leukomyelitis, most severely affecting the outer ventrolateral white matter tracts. The inflammatory cell response was mixed, and included neutrophils, macrophages, and lesser numbers of lymphocytes, as well as perivascular cuffing, primarily by lymphocytes. Within affected areas, scattered myelin sheaths were dilated and apparently empty (“moth-eaten” appearance), and a few of these contained swollen homogeneous eosinophilic (degenerating) axons. Similar manifestations were present in *Icam1<sup>tm1Jcgr</sup>* mice, to a lesser degree for demyelination.

### 3.2. Adoptive transfer EAE studies

To determine if the observed phenotypes in the mutants were due to ICAM-1 isoform expression on T cells or other cell types we performed transferred EAE. Surprisingly, we found that antigen-specific *Icam1<sup>tm1Bay</sup>* T cells induced severe EAE in naïve wild type recipients (Fig. 4A, Table 2). The CDI was significantly elevated in *Icam1<sup>tm1Bay</sup>* to wild type transfers compared to wild type-to-wild type transfers (49.3 vs. 35.9,  $p=0.0002$ , Wilcoxon ranked sign test), however there were no other significant differences in the disease parameters analyzed. These data suggest that the combination of ICAM-1 isoforms expressed by *Icam1<sup>tm1Bay</sup>* T cells can promote severe demyelinating disease and that these cells are highly pathogenic. To further examine the potential of these cells to promote severe disease, we transferred the *Icam1<sup>tm1Bay</sup>* T cells into EAE-resistant *Icam1<sup>tm1Alb</sup>* mice that lack expression of all isoforms on APCs, endothelial cells, and other cell types (Dunne et al., 2003). We observed that *Icam1<sup>tm1Bay</sup>* T cells were capable of inducing CNS inflammation and demyelination in *Icam1<sup>tm1Alb</sup>* mice following transfer, with no significant differences in the CDI, disease onset or the acute phase course of disease compared to wild type-to-wild type transfers (Fig. 4B, Table 2). This induction is remarkable however, since transfer of wild type MOG-specific T cells into *Icam1<sup>tm1Alb</sup>* mice fails to produce disease, as we have previously shown (27). Next we performed the reciprocal transfer in which wild type MOG-specific T cells were transferred into naïve *Icam1<sup>tm1Bay</sup>* mice to investigate the roles of these isoforms on other ICAM-1 expressing cell types. We observed disease comparable to control wild type-to-wild type transfers run in parallel with no significant difference in disease onset or incidence (Fig. 4C, Table 2). These findings significantly contrast with our previous observations in which encephalitogenic wild type T cells were not capable of inducing disease in *Icam1<sup>tm1Alb</sup>* mice (Bullard et al., 2007).

Unlike the transferred EAE findings made in the *Icam1<sup>tm1Bay</sup>* mice, we found that transfer of MOG-specific T cells from wild type mice to *Icam1<sup>tm1Jcgr</sup>* mice resulted in significantly attenuated EAE (Fig 5A, Table 2,  $p=0.012$ , Wilcoxon ranked sign test). Differences in the disease course between the two groups of mice were observed only in the chronic phase of disease, similar to the course of active EAE in *Icam1<sup>tm1Jcgr</sup>* mice. Transfer of MOG-specific *Icam1<sup>tm1Jcgr</sup>* T cells to naïve wild type mice also induced attenuated EAE, but the difference was not statistically significant ( $p=0.094$ , Wilcoxon signed rank test; Fig. 5B, Table 2). These data indicate that the combination of ICAM-1 isoforms expressed by *Icam1<sup>tm1Jcgr</sup>* T cells, although capable of promoting disease development, do so with a phenotype intermediate to that of *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Alb</sup>* mice (Bullard et al., 2007).

### 3.3. *Icam1<sup>tm1Bay</sup>* T cells have increased proliferative capacity and an altered IFN- $\gamma$ production

We next performed T cell *in vitro* proliferation assays to test whether the EAE phenotypes observed in the *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Jcgr</sup>* mice were associated with alterations in the

proliferative capacity of these cells. T cells from *Icam1<sup>tm1Bay</sup>* mice showed significantly enhanced proliferation capacity following MOG stimulation when compared to wild type T cells ( $p=0.04$ , paired t-test). Conversely, T cells from *Icam1<sup>tm1Jcgr</sup>* mice showed similar proliferation kinetics as those derived from wild type mice with EAE ( $p=0.28$ , ns; Fig. 6). To determine if there were major shifts in the production of several key T cell cytokines between *Icam1<sup>tm1Bay</sup>*, *Icam1<sup>tm1Jcgr</sup>*, and wild type T cells, we examined for expression differences in splenic-derived T cells from all three genotypes. Surprisingly, the only significant change observed was in the production of IFN- $\gamma$  between *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Jcgr</sup>* mice relative to wild type controls (Fig. 7). IFN- $\gamma$  production by *Icam1<sup>tm1Bay</sup>* mice was elevated 50% over that of wild type mice, but reduced (<50% of wild type mice) in *Icam1<sup>tm1Jcgr</sup>* mice. *Icam1<sup>tm1Bay</sup>* mice also had modest elevations of TNF- $\alpha$  and TGF- $\beta$  compared to wild type mice, however the remaining cytokines we examined were either reduced (IL-2, IL-4, IL-10, IL-17) or similar to that of wild type mice (IL-12). These data suggest that differences in IFN- $\gamma$  production may play a dominant role in regulating disease severity in these ICAM-1 mutants, but we cannot rule out the role of other cytokines based on these studies.

### 3.4. Analysis of T regulatory cell function

Regulatory T cells (Tregs) have been shown to play an important role in modulating the severity of EAE (Anderton and Liblau, 2008). The expression of the unique subsets of ICAM-1 isoforms in *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Jcgr</sup>* could potentially alter the suppressive functions Tregs and contribute to the unique disease phenotypes seen in these mice. To assess this possibility, we performed *in vitro* Treg assays using Tregs isolated from different ICAM-1 mutant mice. As expected, wild type Tregs readily suppressed effector T cell proliferation in response to antigen-independent stimulation using anti-CD3 and anti-CD28 antibodies. We also found that *Icam1<sup>tm1Alb</sup>* and *Icam1<sup>tm1Jcgr</sup>* Tregs were able to suppress wild type effector T cells with comparable efficiency to wild type Tregs (Fig. 8A). In contrast, *Icam1<sup>tm1Bay</sup>* CD4<sup>+</sup>CD25<sup>+</sup> cells suppress wild type effector T cell proliferation with significantly greater efficiency than wild type Tregs (Fig. 8A) indicating that the exacerbated disease seen in the *Icam1<sup>tm1Bay</sup>* mice is not due to a deficiency in regulatory T cell function. To determine if alterations in ICAM-1 isoform expression on responder T cells (CD4<sup>+</sup>CD25<sup>-</sup>) influence the ability of these cells to undergo Treg-mediated suppression, we stimulated *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* effector T cells, *in vitro*, in the presence of wild type Tregs and analyzed proliferation levels. Wild type Tregs were capable of suppressing both *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* effector T cells (Fig. 8B). These studies collectively, at least at the *in vitro* level, suggest that ICAM-1 isoforms expressed on Tregs or responder cells is not critical for suppressive activity of these cells.

## 4. Discussion

Multiple forms of ICAM-1 exist in mammals due to alternative RNA splicing, and current evidence suggests that these ICAM-1 variants can differ in their expression levels, ligand binding, and possibly in their functions during CNS inflammatory processes (Giorelli et al., 2002; King et al., 1995; Ochietti et al., 2002; Robledo et al., 2003; van Den Engel et al., 2000; Werner et al., 2001). Our analyses of different ICAM-1 mutant mice in the EAE model suggests that specific ICAM-1 isoforms expressed on T cells and other cell types, despite low levels of expression, can significantly contribute to disease development independent of the expression of the full-length molecule. Alternatively spliced isoforms are not unique to the ICAM-1 gene, and have been described for many different genes in the immune system, including other adhesion molecules, CTLA-4, and CD45 (Lynch, 2004; Tchilian and Beverley, 2006). Furthermore, studies to date suggest that many of these genes are functionally active, and may in some cases promote the development of EAE, MS, and

other inflammatory diseases (Dawes et al., 2006; Tchilian and Beverley, 2006; Ueda et al., 2003).

Our findings in the *Icam1<sup>tm1Bay</sup>* mice suggest that specific ICAM-1 isoforms expressed on T cells and other cell types can significantly contribute to disease development independent of the expression of the full-length molecule. In active EAE experiments, our results mimicked those of Samoilova et al (Samoilova et al., 1998), and transfer of wild type encephalitogenic T cells into *Icam1<sup>tm1Bay</sup>* recipients induced a similar disease course to that wild type control transfers. Unexpectedly, severe disease was also induced when *Icam1<sup>tm1Bay</sup>* encephalitogenic T cells were transferred into wild type or *Icam1<sup>tm1Alb</sup>* recipients. These results indicate that specific alternatively spliced ICAM-1 isoforms on T cells alone, in the absence of ICAM-1 expression on other cell types, can readily drive disease development. In contrast to these findings we observed dramatically different EAE phenotypes in *Icam1<sup>tm1Jcgr</sup>* mice. Active EAE in *Icam1<sup>tm1Jcgr</sup>* mice was markedly attenuated compared to wild type mice, although disease severity in this ICAM-1 mutant mouse was increased compared to *Icam1<sup>tm1Alb</sup>* mice, which we previously showed develop only mild or no disease (Bullard et al., 2007). Transferred EAE experiments involving these mutant mice showed phenotypes similar to that observed in active EAE (Fig. 5).

There are many possible mechanisms that could contribute to the phenotypic differences observed in between ICAM-1 mutant and wild type mice. Our current and previous findings suggest an important role for ICAM-1 on T cells and, thus differential isoform expression may alter the proliferative capacity of these cells during the course of EAE (Bullard et al., 2007). Indeed, proliferation of T cells from *Icam1<sup>tm1Bay</sup>* mice was modestly elevated on restimulation with MOG peptide, which may at least partially explain increased disease severity. In contrast, T cells from *Icam1<sup>tm1Jcgr</sup>* mutants proliferated comparably to wild type mice, while our previous studies of ICAM-1 null mice in identical assays showed that complete loss of ICAM-1 isoforms significantly attenuates MOG-induced restimulation (Bullard et al., 2007). This suggests that isoforms expressed by *Icam1<sup>tm1Jcgr</sup>* mutants represent a minimal complement of ICAM-1 molecules required for T cell proliferation.

We also examined for differences in T cell effector functions between *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* mice in terms of T cell-produced cytokines and regulatory T cell function. Neither group of mice produced a cytokine profile consistent with any T helper cell subset that matched their respective disease phenotypes. Although the proinflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$ , IL-12 and IL-17 in *Icam1<sup>tm1Jcgr</sup>* mice with EAE were reduced relative to those of control mice, the levels of anti-inflammatory cytokines including IL-4 and IL-10 were also reduced, while TGF- $\beta$  levels were essentially identical to that seen in *Icam1<sup>tm1Bay</sup>* mice. In contrast, IFN- $\gamma$  was highly elevated during EAE in *Icam1<sup>tm1Bay</sup>* mice compared to wild type mice, while in *Icam1<sup>tm1Jcgr</sup>* mice, levels of this cytokine were markedly reduced. In the absence of a cytokine profile that provides a clear mechanistic explanation for the phenotypic differences between the ICAM-1 mutant mice, we investigated whether Treg numbers or suppressive functions were different. Similar to *Icam1<sup>tm1Alb</sup>* mice, both mutant strains had normal numbers of Tregs (data not shown) and were fully capable of suppressing T cell activation (Figure 8). These data indicate that ICAM-1 plays little to no role in Treg function, although we cannot rule out that the expression of other combinations of ICAM-1 isoforms on Tregs may be important. It is interesting to note that LFA-1, a receptor that frequently pairs with ICAM-1 in mediating many leukocyte functions, is critical for Treg function but does so independent of ICAM-1 expression on the responder T cells (Wohler et al., 2009).

Different ICAM-1 isoform expression may modulate the course of not only EAE but other biological process through alterations in ligand binding or intracellular signaling on

endothelial cells, APCs or T cells. Previous studies have shown that LFA-1 binds to Ig-like domain 1, while Mac-1 interacts with Ig-like domain 3 (Casasnovas et al., 1998; Diamond et al., 1991). Interestingly, all isoforms identified to date retain domain 1, while only a few contain the domain 3 (King et al., 1995); (Giorelli et al., 2002; Ochietti et al., 2002; Robledo et al., 2003; van Den Engel et al., 2000; Werner et al., 2001) (Wakatsuki et al., 1995). *Icam1<sup>tm1Jcgr</sup>* mice express the three smallest ICAM-1 isoforms (Fig. 1) and do not have any known binding sites for Mac-1, and this may account for the dramatic phenotypic differences observed (Diamond et al., 1991). It is interesting to note that several ICAM-1 isoforms expressed in *Icam1<sup>tm1Bay</sup>* mice retain the Mac-1 binding domain (Fig. 1). This raises the possibility that Mac-1/ICAM-1 interactions may serve as an activation rheostat on certain cell types and alter intracellular signaling in T cells and/or APCs. Nevertheless, it is not yet clear how this particular set of ICAM-1 isoforms expressed in the *Icam1<sup>tm1Bay</sup>* mice mediates severe demyelinating disease. Interpretation of these studies as well as others is complicated by our poor understanding of ICAM-1 isoform biology. For example, it is not known in any mammalian species whether all ICAM-1 isoforms are similarly expressed on different cell types under both homeostatic and inflammatory conditions, or if specific isoforms have distinct functions. Further studies of alternatively spliced ICAM-1 isoforms with respect to their expression patterns, ligand interactions and signaling capabilities are necessary to address these many questions.

Adhesion molecules have long been therapeutic targets in animal models of demyelinating disease (Engelhardt and Briskin, 2005; Miller et al., 2003; von Andrian and Engelhardt, 2003). Our results argue that an ICAM-1-mediated therapeutic approach warrants significant but cautious investigation. Exacerbated disease in *Icam1<sup>tm1Bay</sup>* mice suggests that enhanced disease could be a possible therapeutic side effect in MS patients treated with anti-ICAM-1 antibodies. Despite this possibility, anti-ICAM-1 therapy may have advantages over other adhesion molecule immunotherapeutics. For example, since ICAM-1 is not a ligand for  $\alpha 4$ -integrins, the serious clinical side effects associated with Tysabri, the anti- $\alpha 4$ -integrin antibody may be less of a concern (Engelhardt and Briskin, 2005; Miller et al., 2003; von Andrian and Engelhardt, 2003). Furthermore, unlike LFA-1, ICAM-1 does not appear to be critical for Treg function (Wohler et al., 2009), therefore anti-ICAM-1 antibody therapy may be less immunosuppressive in modulating demyelinating disease.

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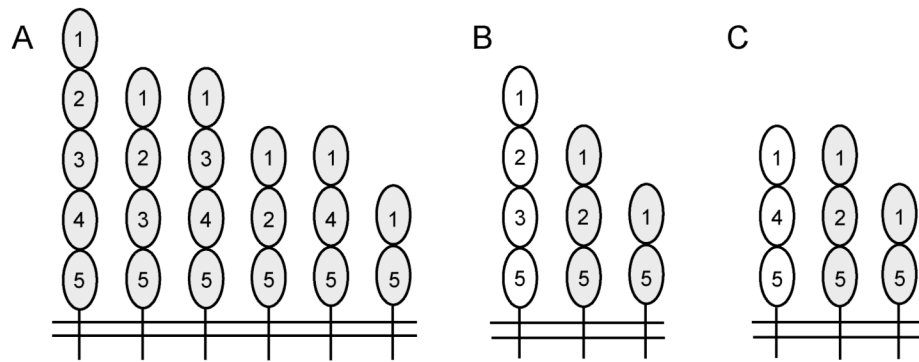
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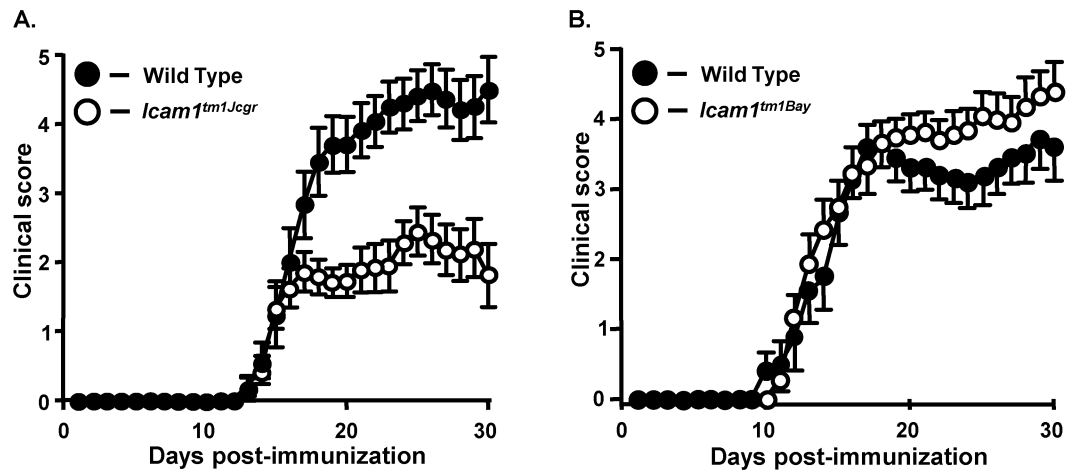


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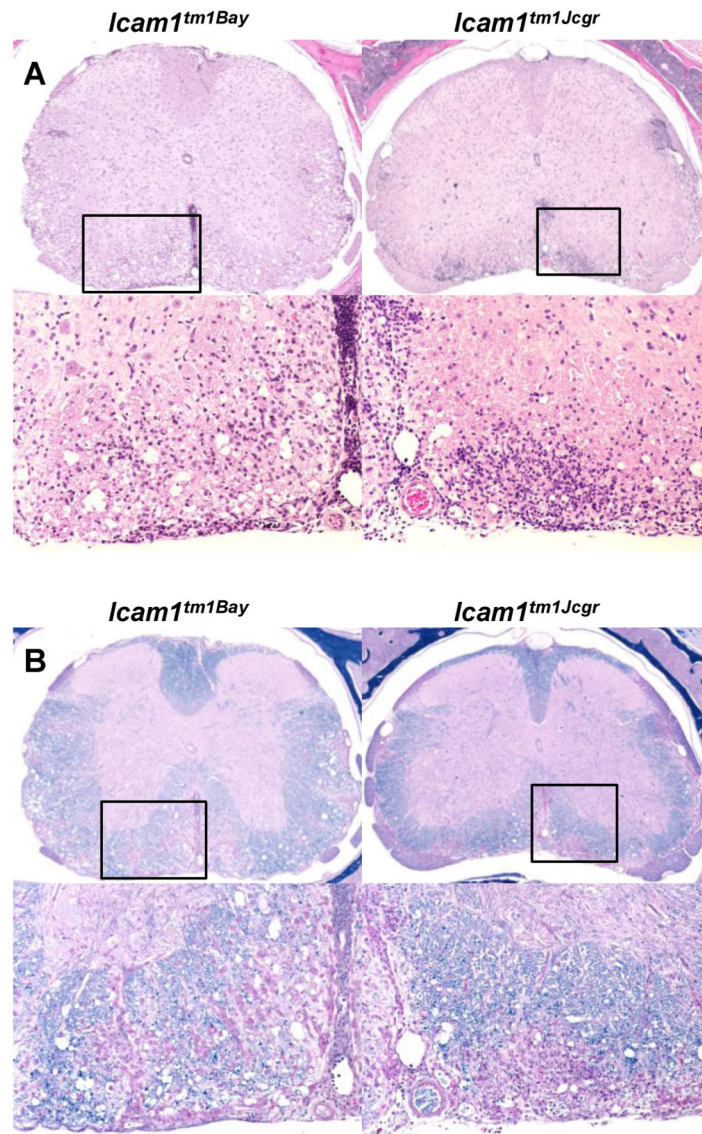


**Fig. 1.** Schematic representation of ICAM-1 isoform structure and expression in wild type and ICAM-1 mutant mice. (A) Wild type, (B) *Icam1<sup>tm1Bay</sup>*, and (C) *Icam1<sup>tm1Jcgr</sup>* mice. Each oval represents an Ig-like domain. The numbering of each is based on the full-length isoform containing five Ig-like domains.

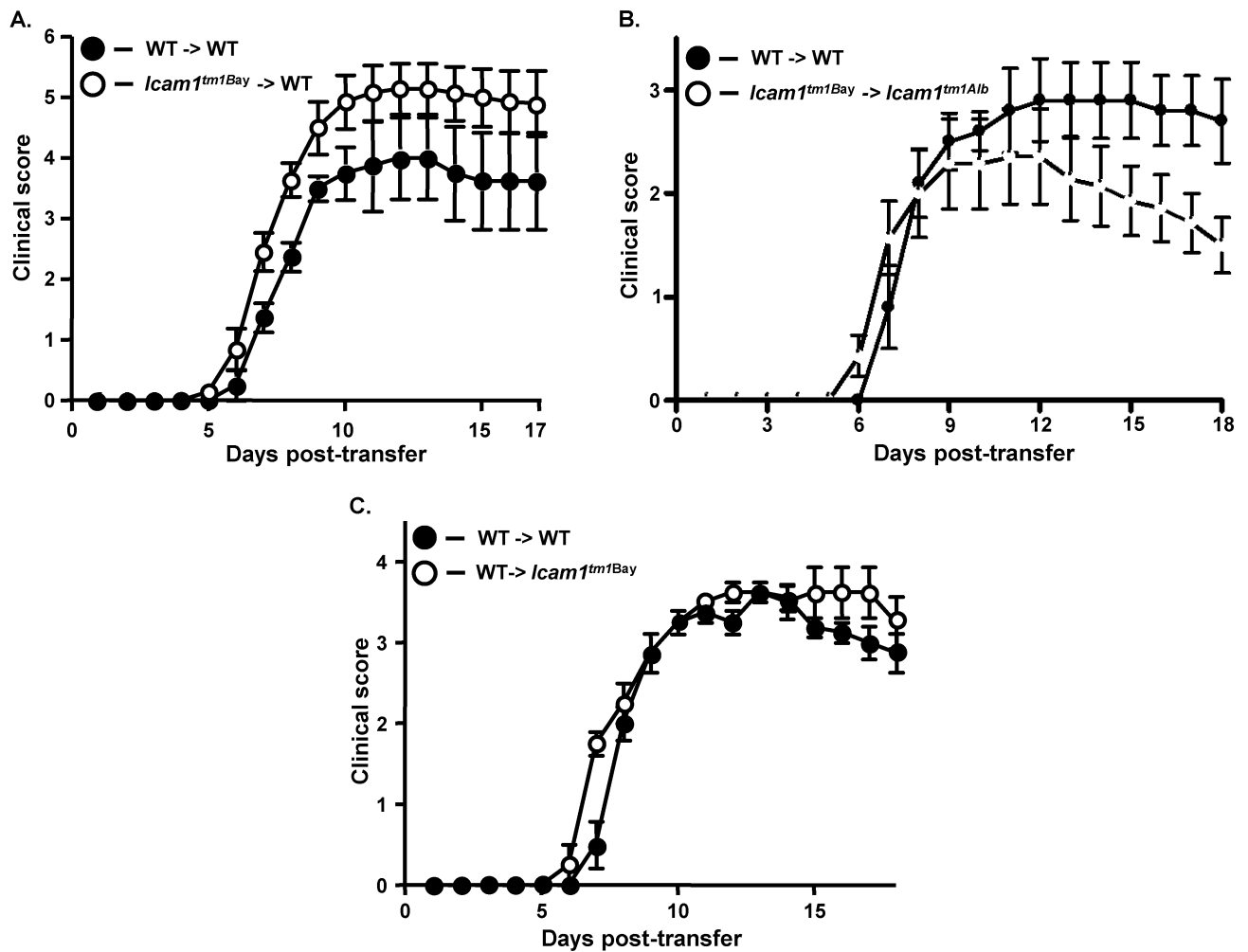


**Fig. 2.**

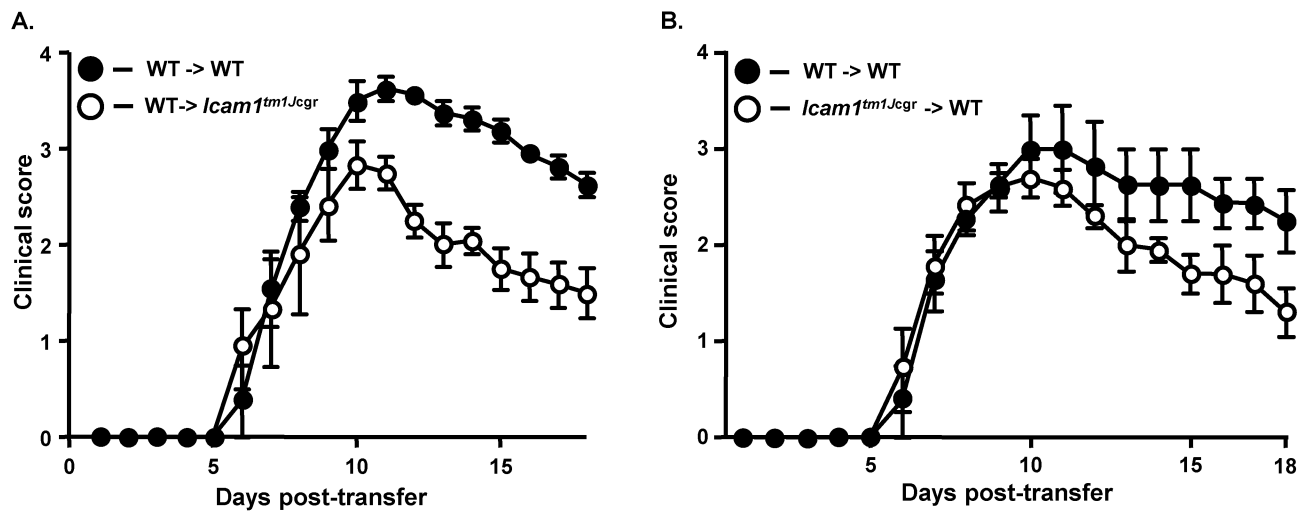
The clinical course of active EAE is attenuated in *Icam1<sup>tm1Jcgr</sup>* mice, but exacerbated in *Icam1<sup>tm1Bay</sup>* mice compared to wild type mice. Active EAE was induced with MOG<sub>35-55</sub> peptide and clinical signs scored for 30 days as described in Materials and Methods. Results shown are the daily mean clinical score for wild type and ICAM-1 mutant mice from two to three experiments. A) Wild type (n=12) and *Icam1<sup>tm1Jcgr</sup>* mice (n=19). B) Wild type (n=10) and *Icam1<sup>tm1Bay</sup>* mice (n=16).



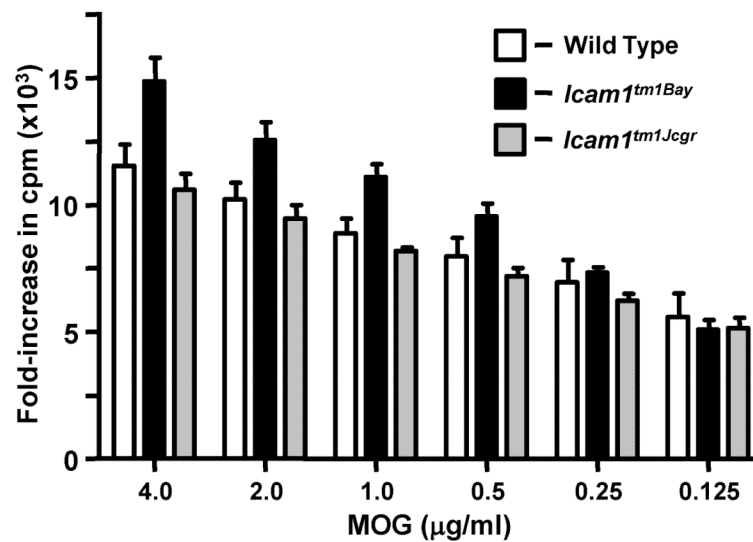
**Fig. 3.** Leukocyte infiltration and demyelination in *Icam1<sup>tm1Jcgr</sup>* mice and *Icam1<sup>tm1Bay</sup>* mice during EAE. Spinal cords from wild type and *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* mice (n=3 for each group) were obtained at 30 days post-immunization, fixed in 10% buffered formalin and paraffin-embedded. Sections from the cervical, thoracic and lumbar regions (5 $\mu$ m) were stained with H&E (A) or Luxol fast blue (B) and scored as described in the Materials and Methods. Representative sections from *Icam1<sup>tm1Jcgr</sup>* mice and *Icam1<sup>tm1Bay</sup>* stained with H&E are shown in A. In *Icam1<sup>tm1Bay</sup>* mice there was moderate parenchymal and meningeal infiltration while in *Icam1<sup>tm1Jcgr</sup>* mice infiltration was more parenchymal and widespread. The boxed region in the upper panel shows the region of magnification in the lower panel. Representative adjacent sections were stained with LFB (B). *Icam1<sup>tm1Bay</sup>* mice had extensive demyelination and inflammation compared to *Icam1<sup>tm1Jcgr</sup>* mice. The boxed region in the upper panel shows the region of magnification in the lower panel. Original magnification 4 $\times$ .



**Fig. 4.** Encephalitogenic T cells from *Icam1<sup>tm1Bay</sup>* mice drive the development of EAE in wild type and *Icam1<sup>tm1alb</sup>* mice. **A.** Transferred EAE was induced in wild-type (n=7) mice by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from *Icam1<sup>tm1Bay</sup>* mice with active EAE. As a control transferred EAE was induced in wild type mice (n=4) by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from wild-type mice with active EAE. Results shown are the daily mean clinical score from three separate experiments. **B.** Transferred EAE was induced in *Icam1<sup>tm1alb</sup>* (n=7) mice by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from *Icam1<sup>tm1Bay</sup>* mice with active EAE. Control transferred EAE in wild type mice (n=5) was run in parallel. Results shown are the daily mean clinical score from two separate experiments. **C.** Transferred EAE was induced in wild type (n=4) and *Icam1<sup>tm1Bay</sup>* mice (n=4) mice by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from wild-type mice with active EAE. Results shown are the daily mean clinical score from two separate experiments.

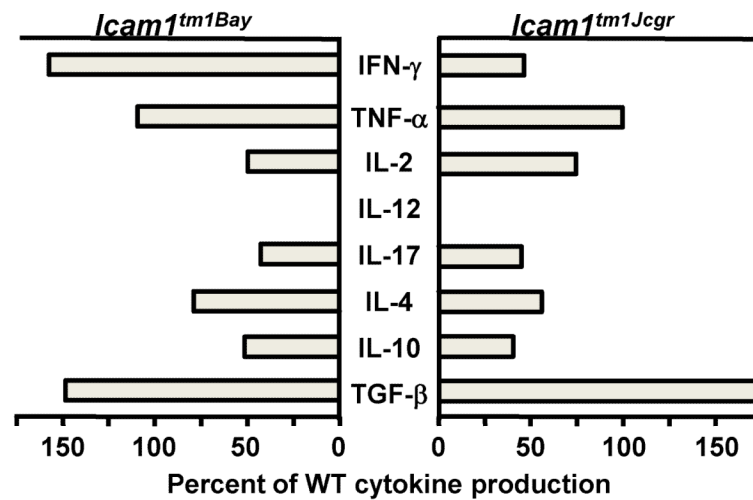


**Fig. 5.** Adoptive transfer of wild type encephalitogenic T cells to *Icam1<sup>tm1Jcgr</sup>* mice or *Icam1<sup>tm1Jcgr</sup>* encephalitogenic T cells to wild type mice produces attenuated EAE. A, Transferred EAE was induced in wild type (n=4) and *Icam1<sup>tm1Jcgr</sup>* mice (n=6) mice by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from wild-type mice with active EAE. Results shown are the daily mean clinical score from two separate experiments. B, Transferred EAE was induced in wild-type (n=5) mice by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from *Icam1<sup>tm1Jcgr</sup>* mice with active EAE. As a control transferred EAE was induced in wild type mice (n=4) by injecting encephalitogenic T cells ( $\sim 5 \times 10^6$ ) derived from wild-type mice with active EAE. Results shown are the daily mean clinical score from three separate experiments.

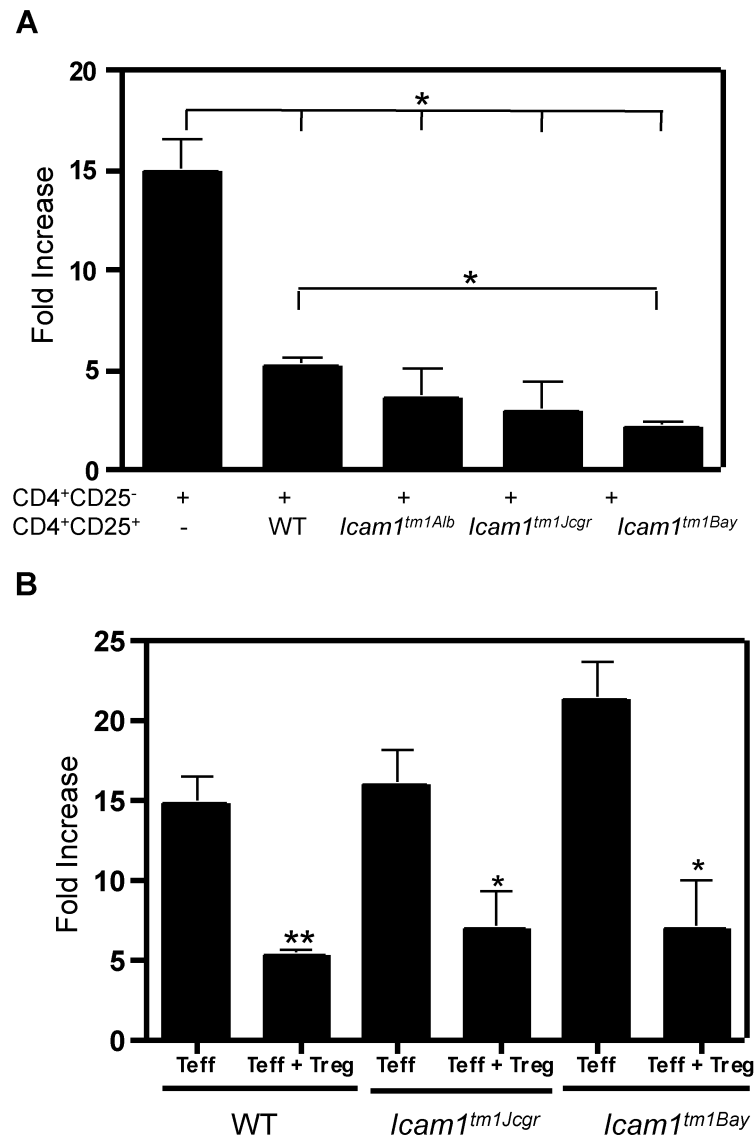


**Fig. 6.** *Icam1<sup>tm1Bay</sup>* T cells proliferate comparably compared to wild type T cells. Encephalitogenic T cells enriched by nylon-wool adherence from the spleens of wild type, *Icam1<sup>tm1Bay</sup>* mice and *Icam1<sup>tm1Jcgr</sup>* mice (n=4, all groups) undergoing active EAE, or T cells from healthy controls (naïve cells), were co-cultured with irradiated splenic APCs plus MOG peptide (0.125 - 4 µg/ml). The cells were pulsed with <sup>3</sup>H-thymidine and harvested at 18 h for determination of radioisotope incorporation. The results shown are expressed as the mean + SEM of fold-induction of wild type, *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Jcgr</sup>* T cell proliferation relative to background proliferation.





**Fig. 7.** Splenic *Icam1<sup>tm1Bay</sup>* T cells produce elevated levels of IFN- $\gamma$  compared to wild type and *Icam1<sup>tm1Jcgr</sup>* mice during EAE. Encephalitogenic T cells enriched by nylon-wool adherence from the spleens of wild type, *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Jcgr</sup>* mice (n=4 all groups) undergoing active EAE (day 15), were co-cultured with irradiated splenic APCs from naïve donors and stimulated with MOG peptide (1 $\mu$ g/well). Supernatants were collected 48 hours after stimulation and assayed by ELISA to quantitate production of each cytokine. Cytokine production is shown as percent of wild type cytokine production.



**Fig. 8.** Alterations in ICAM-1 isoform expression do not affect regulatory T cell function. *In vitro* suppression assays were performed as described in the Materials and Methods using wild type, *Icam1<sup>tm1Alb</sup>*, *Icam1<sup>tm1Jcgr</sup>*, or *Icam1<sup>tm1Bay</sup>* CD4<sup>+</sup>CD25<sup>-</sup> responder or CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Cultures were stimulated with soluble anti-CD3 and anti-CD28 antibodies for 72 hours and were pulsed with <sup>3</sup>H-thymidine for the last 18 hours. A, The results are shown as fold-increase over baseline controls for WT CD4<sup>+</sup>CD25<sup>-</sup> cells alone (n=6), control CD4<sup>+</sup>CD25<sup>-</sup> cells with wild type CD4<sup>+</sup>CD25<sup>+</sup> cells (n=7), wild type CD4<sup>+</sup>CD25<sup>-</sup> cells with *Icam1<sup>tm1Alb</sup>* CD4<sup>+</sup>CD25<sup>+</sup> cells (n=5), wild type CD4<sup>+</sup>CD25<sup>-</sup> cells with *Icam1<sup>tm1Jcgr</sup>* CD4<sup>+</sup>CD25<sup>+</sup> cells (n=4), and wild type CD4<sup>+</sup>CD25<sup>-</sup> cells with *Icam1<sup>tm1Bay</sup>* CD4<sup>+</sup>CD25<sup>+</sup> cells (n=4). B, wild type Treg cells are able to suppress proliferation of *Icam1<sup>tm1Jcgr</sup>* and *Icam1<sup>tm1Bay</sup>* CD4<sup>+</sup>CD25<sup>-</sup> T cells. The results shown are fold-increase over baseline controls for wild type CD4<sup>+</sup>CD25<sup>-</sup> cells alone (n=6), wild type CD4<sup>+</sup>CD25<sup>-</sup> cells with wild type CD4<sup>+</sup>CD25<sup>+</sup> cells (n=7), *Icam1<sup>tm1Jcgr</sup>* CD4<sup>+</sup>CD25<sup>-</sup> cells alone (n=3), *Icam1<sup>tm1Jcgr</sup>* CD4<sup>+</sup>CD25<sup>-</sup> cells with wild type CD4<sup>+</sup>CD25<sup>+</sup> cells (n=4), *Icam1<sup>tm1Bay</sup>* CD4<sup>+</sup>CD25<sup>-</sup> cells alone (n=4), *Icam1<sup>tm1Bay</sup>* CD4<sup>+</sup>CD25<sup>-</sup> cells with wild type

CD4<sup>+</sup>CD25<sup>+</sup> cells (n=4). Statistical analysis was performed using Anova (\*, p 0.1 and \*\*\*, p 0.001).

**Table I**

EAE signs in wild-type mice, *Icam1<sup>tm1Bay</sup>* and *Icam1<sup>tm1Jcgr</sup>* mice.

	CDI <sup>A</sup>	Disease Onset <sup>B</sup>	Disease Incidence <sup>C</sup>	Mortality
Wild type (n=12)	58.3	16.3d	100	33%
<i>Icam1<sup>tm1Jcgr</sup></i> (n=19)	31	17.3d	95	16%
Wild type (n=10)	56.4	13.4d	100	20%
<i>Icam1<sup>tm1Bay</sup></i> (n=16)	64	13.7d	100	44%

<sup>A</sup> Cumulative Disease Index is the mean of the sum of daily clinical scores observed between days 7 and 30.

<sup>B</sup> Disease onset is defined as the first day of two consecutive days with a clinical score of two or more.

<sup>C</sup> Disease incidence is defined as the percent of mice that displayed any clinical signs of disease.

**Table 2**Transferred EAE signs in wild-type mice and *Icam1<sup>tm1Bay</sup>* mice.

	CDI <sup>A</sup>	Disease Onset <sup>B</sup>	Disease Incidence <sup>C</sup>
WT > WT (n=4)	35.9	7.5d	100
<i>Icam1<sup>tm1Bay</sup></i> > WT (n=7)	49.3	7d	100
WT > WT (n=4)	29.5	8.2d	100
<i>Icam1<sup>tm1Bay</sup></i> > <i>Icam1<sup>tm1Alb</sup></i> (n=7)	23.8	7.7d	86
WT > WT (n=4)	33.2	8d	100
WT > <i>Icam1<sup>tm1Bay</sup></i> (n=4)	37.1	8d	100

<sup>A</sup> Cumulative Disease Index is the mean of the sum of daily clinical scores observed between days 7 and 30.

<sup>B</sup> Disease onset is defined as the first day of two consecutive days with a clinical score of two or more.

<sup>C</sup> Disease incidence is defined as the percent of mice that displayed any clinical signs of disease.

**Table 3**Transferred EAE signs in wild-type mice and *Icam1<sup>tm1Jcgr</sup>* mice.

	CDI <sup>A</sup>	Disease Onset <sup>B</sup>	Disease Incidence <sup>C</sup>
WT > WT (n=4)	34.9	8d	100
WT > <i>Icam1<sup>tm1Jcgr</sup></i> (n=6)	24.2	8d	100
WT > WT (n=4)	29.2	7.8d	100
<i>Icam1<sup>tm1Jcgr</sup></i> > WT (n=5)	26.4	7d	100

<sup>A</sup> Cumulative Disease Index is the mean of the sum of daily clinical scores observed between days 7 and 30.

<sup>B</sup> Disease onset is defined as the first day of two consecutive days with a clinical score of two or more.

<sup>C</sup> Disease incidence is defined as the percent of mice that displayed any clinical signs of disease.