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Notch Ligand Delta-Like 4 Blockade Alleviates Experimental Autoimmune Encephalomyelitis by Promoting Regulatory T Cell Development

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Abstract

Notch signaling pathway plays an important role in T cell differentiation. Delta-like ligand (Dll)4, one of five known Notch ligands, has been implicated in regulating Th2 cell differentiation in animal models of human diseases. However, the role of Dll4 in Th1/Th17-mediated autoimmune diseases remains largely unknown. Using an anti-Dll4 blocking mAb, we show that neutralizing Dll4 during the induction phase of experimental autoimmune encephalomyelitis in C57BL/6 mice significantly increased the pool of $CD4^+Foxp3^+$ regulatory T cells (Treg) in the periphery and in the CNS, and decreased the severity of clinical disease and CNS inflammation. Dll4 blockade promoted induction of myelin-specific Th2/Treg immune responses and impaired Th1/Th17 responses compared with IgG-treated mice. In vitro, we show that signaling with recombinant Dll4 inhibits the TGF-β–induced Treg development, and inhibits Janus kinase 3-induced STAT5 phosphorylation, a transcription factor known to play a key role in Foxp3 expression and maintenance. Depletion of natural Treg using anti-CD25 Ab reversed the protective effects of anti-Dll4 Ab. These findings outline a novel role for Dll4–Notch signaling in regulating Treg development in EAE, making it an encouraging target for Treg-mediated immunotherapy in autoimmune diseases, such as multiple sclerosis.

Experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease of the CNS, is widely used as an animal model for multiple sclerosis (1). EAE can be induced in mice by immunization with myelin Ags or by passive transfer of autoreactive T cell lines or clones (2, 3). IFN-γ–producing (Th1) and IL-17–producing (Th17) $CD4^+$ Th cells play a critical role in EAE pathogenesis. Th1 and Th17 cells could be detected in inflammatory CNS lesions and induce EAE upon adoptive transfer (4), whereas regulatory T

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cells (Treg) mediate immunological tolerance and limit inflammation and tissue damage (5, 6).

The activation, proliferation, and differentiation of naive T cells require Ag-induced signals by MHC/Ag complex engaging the TCR, growth, and survival factors in the form of cytokines, and signals provided by molecules expressed on APCs, called costimulators (7). Whereas some costimulators are essential to initiate a T cell response, others, such as Notch signaling molecules, play a role in fine-tuning the immune response. In mammals, cells express four Notch receptors, Notch1, Notch2, Notch3, and Notch4, and five Notch ligands, Jagged1, Jagged2, Delta-like ligand (Dll)1, Dll3, and Dll4 (8). The engagement of a Notch receptor expressed on T cells by a Notch ligand expressed mainly on APCs initiates a series of enzymatic reactions leading to the cleavage of the Notch receptor intracellular domain (NICD) that translocates to the nucleus, binds the transcription factor recombining binding protein (RBP)-J, and recruits coactivators, including mastermind-like proteins. The newly formed NICD/RBP-J/mastermind-like complex acts as a transcriptional activator for downstream target genes (9, 10).

A growing body of evidence supports a role for Notch signaling in regulating T cell differentiation. APCs encountering pathogens that induce a Th1 cell response show upregulation of the Dll ligands, whereas exposure to Th2 cell-inducing products upregulates Jagged ligands (11–13). Moreover, ectopic expression of Dll ligands on DCs promotes Th1 cell differentiation and inhibits Th2 cell differentiation (11, 14, 15), whereas expression of Jagged ligands on APCs was shown to induce Th2 cell differentiation (11). Blocking Dll4-mediated Notch signaling in a context of Th2-mediated animal model disease increases the disease severity by enhancing Th2 cytokine production (16, 17). We have previously reported that Dll1 blockade suppressed EAE and was associated with decreased frequencies of Th1 and Th2 effector cells while having no effect on frequencies of Th17 and Treg (18). Although Dll4 blockade has been described to have a protective role in a model of virus-induced demyelinating disease that was attributed to a decrease in the total number of CNS-infiltrating Th1 and Th17 cells (19), the cellular and molecular mechanisms involved in mediating protection remain unclear.

Using an anti-Dll4 blocking mAb, we show that blocking Dll4–Notch signaling in EAE decreases the severity of clinical disease and CNS inflammation by increasing the pool of $CD4^+Foxp3^+$ Treg in the peripheral compartment and the target organ, causing an elevation in the Th2/Th1–Th17 ratio. Furthermore, Dll4 appears to have a unique role in suppressing Treg induction and expansion by inhibiting the JAK3/STAT5 activation pathway necessary for Foxp3 expression and maintenance.

Materials and Methods

Reagents and Abs

The anti-mouse Dll4 blocking mAb (HMD4-1) was generated, as previously described (20). Rat IgG was obtained from Sigma-Aldrich and used as control. Recombinant mouse Dll4-Fc fusion protein, IL-2, and TGF-β1 were purchased from R&D Systems. Fluorochrome-conjugated Notch ligands mAb and isotype control were purchased from Biolegend. All other FACS Abs were purchased from BD Pharmingen or eBioscience. β-actin mouse mAb was purchased from Sigma-Aldrich. All other Western blot Abs were purchased from Cell Signaling Technology. Collagenase and DNaseI were purchased from Sigma-Aldrich. Anti-CD25 (PC61) mAb were purchased from BioXCell.

Mice and EAE induction with myelin oligodendrocyte glycoprotein(35–55)

Six- to 8-wk-old female wild-type C57BL/6 mice were purchased from The Jackson Laboratory. Foxp3.GFP knock-in mice were provided by V. Kuchroo (Center for Neurologic Diseases, Harvard Medical School, Boston, MA). Mice were immunized s.c. in the flanks with 100 μg myelin oligodendrocyte glycoprotein (MOG)(35–55) peptide (New England Peptide) in 50 μl PBS and 50 μl CFA containing 250 ng *Myobacterium tuberculosis* (Fisher Scientific) and injected i.p. with 200 ng pertussis toxin (List Biological Laboratories) on the day of immunization and 2 d later. The mice were injected with anti-Dll4 or control IgG i.p. at 500 μg on the day of the immunization and then 250 μg on days postimmunization (DPI) 2, 4, 6, and 8. Animals were kept for at least 23 d, and EAE clinical disease was scored as follows: grade 1, limp tail or isolated weakness of gait without limp tail; grade 2, weakness of gait or partial hind and/or front leg paralysis; grade 3, total hind leg paralysis; grade 4, total hind leg and partial front leg paralysis; grade 5, moribund or dead animal. Mice were housed in specific pathogen-free conditions in the New Research Building Animal Facility at Harvard Medical School. All animal experiments were done in compliance and approval of the Harvard Medical Area Standing Committee on Animals.

Preparation of CNS mononuclear cells

Mice were sacrificed and perfused through the left cardiac ventricle with cold PBS. The spinal cords were flushed out with PBS by hydrostatic pressure. Spinal cords were incubated in DMEM with collagenase and DNaseI for 45 min at 37°C, in a humidified 10% CO_2 atmosphere, and then dissociated by passing the tissues through a 70-μm cell strainer. Cells were washed and resuspended in a 30% Percoll solution and loaded onto a 70% Percoll solution. After centrifugation, the supernatant containing floating myelin was removed and mononuclear cells were gently collected from the interface, washed, and resuspended in culture medium for further analysis.

Histological analysis

Mice were scarified on day 14 after immunization, and spinal cords were fixed in Bouin's fixative and embedded in paraffin. Slides were stained for H&E stains, and inflammatory foci (>10 mononuclear cells) were counted in a blinded fashion.

Flow cytometry staining and analysis

Cells were isolated from lymph nodes, spleen, or spinal cords of naive control or MOG(35–55)-immunized mice, and were stained with fluorochrome-labeled mAbs against CD4, CD8, CD11b, CD11c, CD69, CD44, and CD62L for ex vivo cell population frequency determination. For intracellular cytokine staining, cells were stimulated in culture medium with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (0.3 μg/ml; Sigma-Aldrich) in the presence of monensin (GolgiStop; BD Biosciences) for 4 h at 37°C, in a humidified 10% CO_2 atmosphere, and then washed and stained for 20 min with fluorochrome-labeled mAbs against surface cell markers, fixed, and permeabilized using Cytofix/Cytoperm and perm/wash buffer (BD Biosciences). Following permeabilization, intracellular fluorochrome-labeled mAbs against IL-4, IL-10, IL-17, IFN-γ, TNF-α, and Foxp3 were added for 30 min at 4°C, and the cells were washed, acquired on LSR II (BD Biosciences), and analyzed using FlowJo software.

ELISPOT assay

Cells were cultured in DMEM supplemented with FBS (10%), 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, and 100 U penicillin/100 g streptomycin/ml. Cells were seeded in triplicate at 5×10^5 cells/well with different concentrations of MOG(35–55) peptide. After 48 h of culture, ELISPOT assay was used to

assess the frequency of MOG(35–55)-specific IL-4–, IFN-γ (BD Pharmingen)–, and IL-17 (eBiosciences)–producing cells, according to manufacturer instructions. The resulting spots were counted on a computer-assisted ELISPOT image analyzer (Cellular Technology, Shaker Heights, OH), and frequencies were expressed as the number of cytokine-producing spots/well.

In vitro Treg induction and expansion assays

$CD4^+GFP^-$ (induction assay) and total $CD4^+$ T (expansion assay) cells were FACS sorted from splenocytes of naive Foxp3.GFP.KI mice and stimulated in vitro with plate-bound anti-CD3 and anti-CD28 (1 μg/ml) and plate-bound recombinant Dll (rDll)4 protein or control IgG (2 μg/ml) in the presence or absence of TGF-β (3 ng/ml) and IL-2 (20 ng/ml). IL-2 was supplemented on day 2 of incubation in IL-2–containing conditions. After 4 d of incubation, the cells were washed and Foxp3 expression was assessed by FACS staining.

Western blotting

Cells were lysed in RIPA buffer (Thermo Scientific) with a protease inhibitor mixture (Roche Diagnostics) and a phosphatase inhibitor mixture (Sigma-Aldrich); 20 μg total protein was loaded into each well of a SDS-PAGE gel for separation by electrophoresis and then transferred on nitrocellulose membrane. The resulting blots were blocked for 1 h with TBS-Tween 20 containing 5% powder skim milk and then probed overnight at 4°C with primary Abs: phospho-STAT5 rabbit mAb (Tyr694), phospho-JAK3 rabbit mAb (Tyr980/981), and β-actin mouse mAb were used as the loading control. Blots were then washed five times and probed for 1 h with the appropriate HRP-conjugated secondary Ab. Membranes were developed with Immobilon Western Chemiluminescent HRP substrate (Millipore).

In vivo Treg depletion

Mice were injected i.p. with 250 μg anti-CD25 mAbs (PC61) or control PBS on days −3 and −1 before immunization.

Statistical analysis

The Mann–Whitney *U* test was used for clinical disease analysis. Statistical evaluations of frequency measurements were performed using the unpaired Student *t* test. The *p* values <0.05 were considered statistically significant.

Results

Dll4 blockade during the priming phase ameliorates EAE

To examine the role of Dll4 during the course of EAE, we immunized C57BL/6 mice with MOG(35–55)/CFA and administered anti-Dll4 mAb of proven blocking properties (20) or control IgG, starting on the day of immunization for five consecutive doses every second day (200 mg × 5/mouse), and monitored disease progression. Treatment with anti-Dll4 during the induction phase of EAE significantly decreased clinical severity compared with control IgG-treated mice (mean maximal clinical score of anti-Dll4–treated mice 1.7 ± 0.3 versus 3.0 ± 0.2 in control IgG recipients; $p < 0.05$ by two-tailed Mann–Whitney *U* test; $n = 5$/group). Data are representative of three independent experiments (Fig. 1*A*). In addition, histological examination revealed marked decrease in inflammation in spinal cord sections of anti-Dll4–treated mice compared with IgG-treated mice (Fig. 1*B*).

Dll4 blockade promotes T cell differentiation into an anti-inflammatory phenotype

We investigated the effect of Dll4 blockade on $CD4^+$ T cell cytokine production in peripheral and target organ. Splenocytes were isolated on day 10, and spinal cord-infiltrating cells were isolated on day 14 from anti-Dll4– or IgG-treated mice. We observed a decrease in the IFN-γ– and IL-17–producing T cells and increase in IL-4– and IL-10–producing T cells in both splenocytes (Fig. 2*A*) and CNS cells (Fig. 2*B*) of the anti-Dll4–treated mice compared with IgG-treated mice, as measured by flow cytometry. This was confirmed by ELISPOT analysis in which splenocytes from mice treated with anti-Dll4 showed less MOG(35–55)-specific production of IFN-γ and IL-17 and more IL-4 than did splenocytes from mice treated with IgG (Fig. 2*C*). It should be noted that Dll4 blockade had no significant effect on $CD4^+$ T cell activation and survival or on cytokine production by $CD8^+$, $CD11b^+$, or $CD11c^+$ cells (data not shown).

Dll4 suppresses Treg expansion in vivo and in vitro

Next, we investigated the frequency of $CD4^+Foxp3^+$ T cell in spleen, draining lymph nodes, and spinal cords of MOG(35–55)-immunized mice treated with anti-Dll4 or control IgG during EAE. We observed a significantly increased frequency of $CD4^+Foxp3^+$ Treg in the anti-Dll4 blocking Ab-treated mice compared with IgG controls (Fig. 3*A*, 3*B*). This observation suggested a possible role for Dll4 in regulation of $CD4^+Foxp3^+$ cells' expansion and/or induction.

rDll4 was previously shown to promote the activation of Notch signaling by increasing the accumulation of the cleaved active form of Notch1 and increasing the expression of its downstream target genes HES1 and HES5 (21–24). To address a possible role for Dll4 in $CD4^+Foxp3^+$ Treg development, we sorted total $CD4^+$ or $CD4^+$ $Foxp3^-$ (GFP-negative) T cells from splenocytes of naive Foxp3.GFP.KI mice and polyclonally stimulated the cells in vitro with anti-CD3/anti-CD28 and plate-bound rDll4 or control IgG in the presence or absence of TGF-β. rIL-2 was added in some conditions when indicated. After 4 d of incubation, we measured Treg frequency by staining for CD4 and Foxp3. As expected, TGF-β treatment induced the conversion of $CD4^+Foxp3^-$ cells into $CD4^+Foxp3^+$ cells (Fig. 3*C*) and promoted the expansion of the Treg pool in the total $CD4^+$-treated cells (Fig. 3*D*). IL-2 supplementation further enhanced Treg expansion. However, the presence of rDll4 resulted in a complete blockade of both Treg induction and expansion even when an optimal concentration of rIL-2 was added (Fig. 3*C*, 3*D*).

Thus, the in vivo and in vitro results suggest a direct involvement of Dll4 in regulating the $CD4^+Foxp3^+$ T cell development.

Induction of Dll4–Notch signaling inhibits JAK3 and STAT5 phosphorylation

Previous studies have indentified the transcription factor STAT5 as a key regulator of Foxp3 expression and Treg development and maintenance (25, 26). We reasoned that Dll4-mediated Notch signaling might regulate STAT5 phosphorylation and thus suppress Foxp3 expression. To test this hypothesis, we stimulated naive $CD4^+$ T cells (0–60 min) with plate-bound anti-CD3/anti-CD28 in the presence of rDll4 or control IgG, and cells were lysed and used for Western blotting analysis of STAT5 phosphorylation and its upstream kinase, JAK3. We found that rDll4 treatment decreased both JAK3 and STAT5 phosphorylation (Fig. 4). These data suggest that Dll4 suppresses the development of $CD4^+Foxp3^+$ regulatory cells by regulating JAK3/STAT5 signaling cascade upstream of Foxp3.

Depletion of Treg before EAE induction abolishes anti-Dll4 mAb-mediated EAE suppression

To further confirm the implication of Treg in the anti-Dll4 mAb-mediated suppression of EAE, we depleted mice of natural Treg population by administering anti-CD25 mAb (PC61) on days −3 and −1 before immunization, a technique that has been widely used and documented (27–32). Treg depletion was confirmed by flow cytometry staining prior to immunization, showing a 69% decrease in frequency of $CD4^+Foxp3^+$ cells (Fig. 5*B*). Depletion of Treg abolished the protective effect of anti-Dll4, and mice developed EAE of similar severity as the IgG control group (3.6 ± 0.2 versus 3.7 ± 0.1 in control IgG recipients; $p = 0.9$, value nonsignificant by two-tailed Mann–Whitney *U* test; $n = 5$/group). Data are representative of two independent experiments (Fig. 5*A*). This result confirms that the protective effect of anti-Dll4 Ab is Treg dependent.

Discussion

Treg have received much attention during the last decade for their unique role in regulating the immune response and maintaining homeostasis and self-tolerance. A decreased frequency of Treg or genetic deficiency or dysfunction of the Treg signature transcription factor, Foxp3, causes a wide spectrum of autoimmune diseases in mice as well as in humans (33–35). Treg play a critical role in modulating the immune response in multiple sclerosis (36) as well as in its animal model, EAE. Adoptive transfer of Treg reduced EAE severity by decreasing CNS inflammation (5), whereas Treg depletion using anti-CD25 mAb exacerbated EAE (27–29, 37).

In the current study, we describe a novel mechanism for Dll4-mediated immune regulation. rDll4 protein treatment suppressed TGF-β–mediated Treg induction and expansion in vitro, whereas Dll4 blockade during the induction phase of EAE significantly expanded the $CD4^+Foxp3^+$ Treg population in the spleen, peripheral lymph nodes, and spinal cords, leading to a reduction in the clinical disease severity and CNS inflammation. EAE reduction was associated with decreased frequencies of autoreactive Th1 and Th17 cells and increased frequencies of Th2 cells.

Dll4-mediated Notch signaling is thought to play an important role in regulating Th cell differentiation (38–40). Ectopic expression of Dll4 on APCs enhances their ability to promote Th1 cell differentiation while inhibiting Th2 cell differentiation. Furthermore, APCs encountering pathogens that would regularly promote a Th1 immune response upregulate their expression of Dll4 (11–13). Ito et al. (41) have found that Dll4 expression on DCs specifically upregulated Th17 cytokine expression in a granuloma model induced by mycobacteria-associated Ag. Mukherjee et al. (21) have reported that in vitro, and under Th17 skewing conditions, rDll4 enhanced Th17 differentiation and RORc expression. In agreement with these studies, we found that Dll4 blockade during EAE decreased Th1 and Th17 frequencies while expanding Th2 cells. However, we believe that although the above-mentioned mechanisms could play a role in the shift in the immune response described, we attribute it mainly to an effect of the Dll4 on Treg, as discussed below.

The signaling cross-talk between the Notch and Smads, the intracellular mediators of TGF-β, has been previously reported. Smads bind the intracellular domains of both Notch1 and Notch4 (42–44). NICD complexes with RBP-J and Smad3, facilitating the translocation of phosphorylated Smad3 to the nucleus and the binding to the Foxp3 promoter (42). Blocking Notch signaling by using the γ-secretase inhibitor (GSI), anti-Notch1 blocking mAb, or by using cells that express reduced levels of Notch1 led to inhibition of TGF-β–induced Foxp3 expression and decreased peripheral Treg (45). However, GSI are general Notch pathway inhibitors and cannot be used to dissect the role of individual Notch receptors, or individual

Notch ligands. GSI could also target other substrates in addition to Notch (46). Other reports showed that blockade of Notch1 signaling with an anti-Jagged1 or a blocking anti-Notch1 Ab inhibits Treg suppressor function (44). In this article, we show that Notch signaling pathway has the opposite outcome on Treg when mediated through the Dll4 ligand; signaling through Dll4 inhibits Treg generation.

IL-2 plays a critical role in TGF-β–mediated Treg induction and expansion, and this has been demonstrated by using IL-2–deficient T cells or by IL-2 neutralization under Treg-polarizing conditions (25, 47–49). A recent study shows that Dll4 suppresses IL-2 production by T cells (17). These observations may suggest that Dll4 could suppress TGF-β–mediated Treg induction and expansion by downregulating IL-2 signaling. However, using rDll4 protein, we show that Dll4 suppressed TGF-β–mediated $CD4^+Foxp3^+$ Treg induction and expansion in vitro even under conditions supplemented with optimal IL-2 concentrations, suggesting that Dll4 inhibitory effect is downstream of the TGF-β or IL-2 signaling cascades. DAPT, a γ-secretase inhibitor that is also used to block Notch signaling, abrogates the described effect, showing that rDll4 inhibition of Treg development is Notch dependent (Supplemental Fig. 1). Furthermore, we confirmed Dll4 suppression of Treg using Ag-specific system by coculturing naive OVA-specific $CD4^+$ T cells with irradiated A20 B cells over-expressing Dll4 (Supplemental Fig. 2*a*) in the presence of OVA (323–339) peptide that resulted in suppression of $CD4^+Foxp3^+$ T cells (Supplemental Fig. 2*b*) and expansion of IFN-γ–producing T cells (Supplemental Fig. 2*c*).

STAT5 is essential for IL-2–dependent Foxp3 expression, and is critical for both Treg development and maintenance (25, 26). STAT5 phosphorylation has been shown to be regulated by the upstream kinase JAK3 (50). Our data clearly demonstrate that rDll4 treatment suppresses JAK3 and STAT5 phosphorylation, and hence, explain the mechanisms behind Dll4-mediated inhibition of Foxp3 expression. STAT5 phosphorylation has also been described to be critical for promoting Th2 cell differentiation while suppressing Th1 and Th17 cell differentiation (51), which could explain the shift in the immune response observed when blocking Dll4 during EAE. To address whether Dll4 blockade is suppressing the clinical disease by mainly regulating Treg development, we opted to induce EAE and block Dll4 in Treg-depleted mice. The use of anti-CD25 mAb for the depletion of a small $CD4^+$ T cell population was first described by Sakaguchi et al. (52) and was shown to result in the breakdown of self-tolerance, causing various autoimmune diseases. CD25 has been accepted since as a marker for Treg, and there is strong evidence confirming that the anti-CD25 mAb clone we used in this study (PC61 rat IgG1) leads to a rapid loss of $CD4^+Foxp3^+$ T cells (27–32). Treg depletion using anti-CD25 mAb prior to induction of EAE completely abrogated the protective effect of the anti-Dll4 mAb, showing that Dll4 blockade effect is mainly attributed to an enhanced Treg development.

Although the data presented in this manuscript provide strong evidence for the Dll4-mediated Notch signaling effect on $CD4^+$ T cell differentiation, we found no significant differences in $CD8^+$, $CD11b^+$, and $CD11c^+$ cells isolated from IgG- or anti-Dll4–treated EAE mice (data not shown). However, we cannot exclude an effect of anti-Dll4 on CNS cells or other tissues.

In summary, our present study reveals a novel and unique role for Dll4 signaling in regulating the immune responses by suppressing STAT5 activation and Foxp3 expression in $CD4^+$ cells. These findings, combined with the published literature showing a role of Dll4-mediated Notch signaling in promoting Th1 and Th17 differentiation while suppressing Th2 differentiation, make targeting this pathway in autoimmune diseases such as multiple sclerosis worth further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

Dll	Delta-like ligand
DPI	days postimmunization
EAE	experimental autoimmune encephalomyelitis
GSI	γ-secretase inhibitor
MOG	myelin oligodendrocyte glycoprotein
NICD	notch receptor intracellular domain
RBP	recombining binding protein
rDll	recombinant Dll
Treg	regulatory T cell

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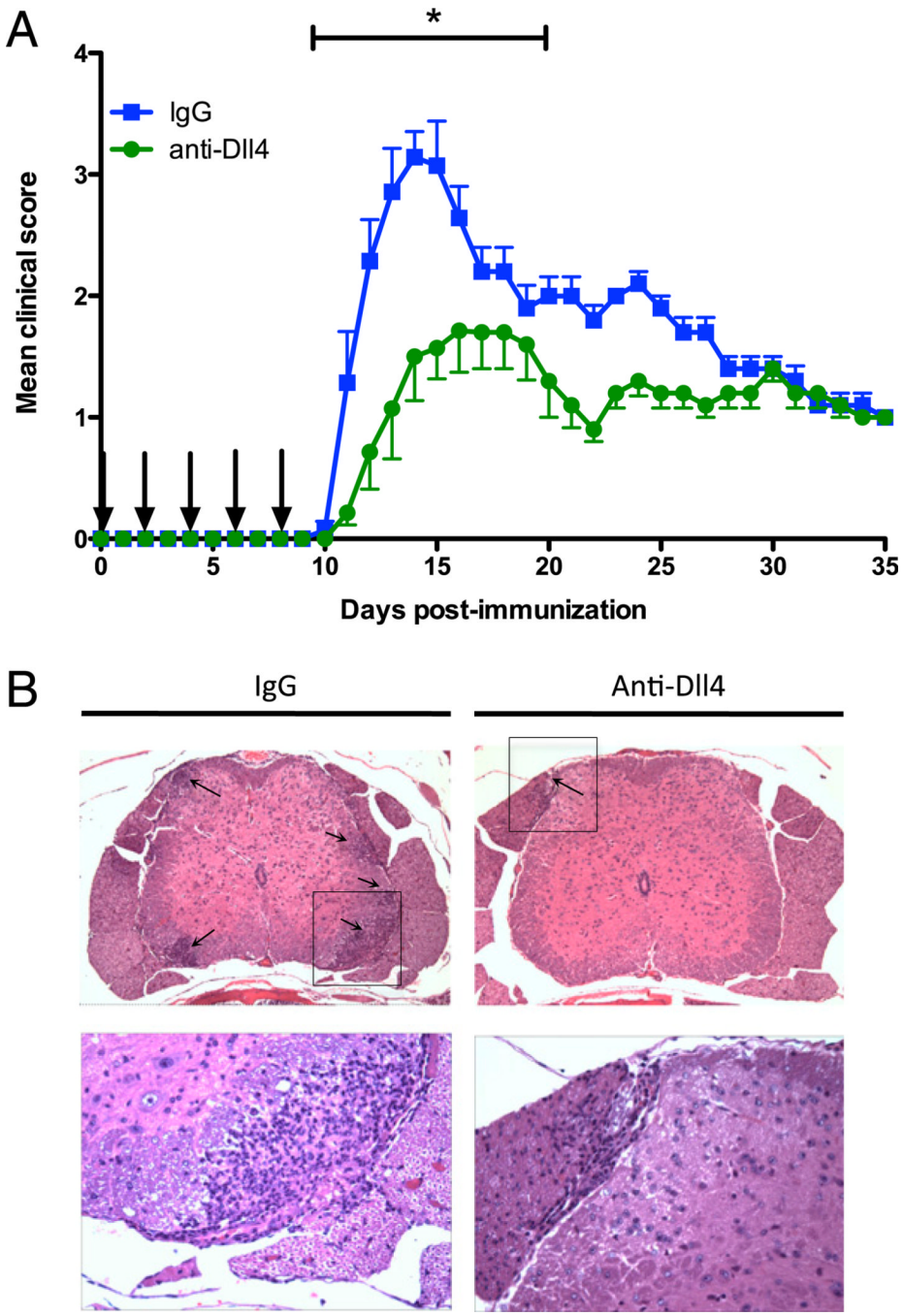

FIGURE 1.

Dll4 blockade during EAE induction phase decreases disease severity and CNS inflammation. *A*, Clinical scores of C57BL/6 mice immunized with MOG(35–55)/CFA and treated with anti-Dll4 blocking Ab or control IgG every other day and for a total of five injections (arrows) starting on the day of the immunization. Results are representative of three independent experiments with five mice per group. *$p < 0.05$. *B*, Histopathology of H&E-stained spinal cord sections of mice described in *A* on DPI 14. Boxed area in *top row* is enlarged below. Original magnification ×10 (*top row*) and ×40 (*bottom row*). Results are representative of two independent experiments with two mice per group.

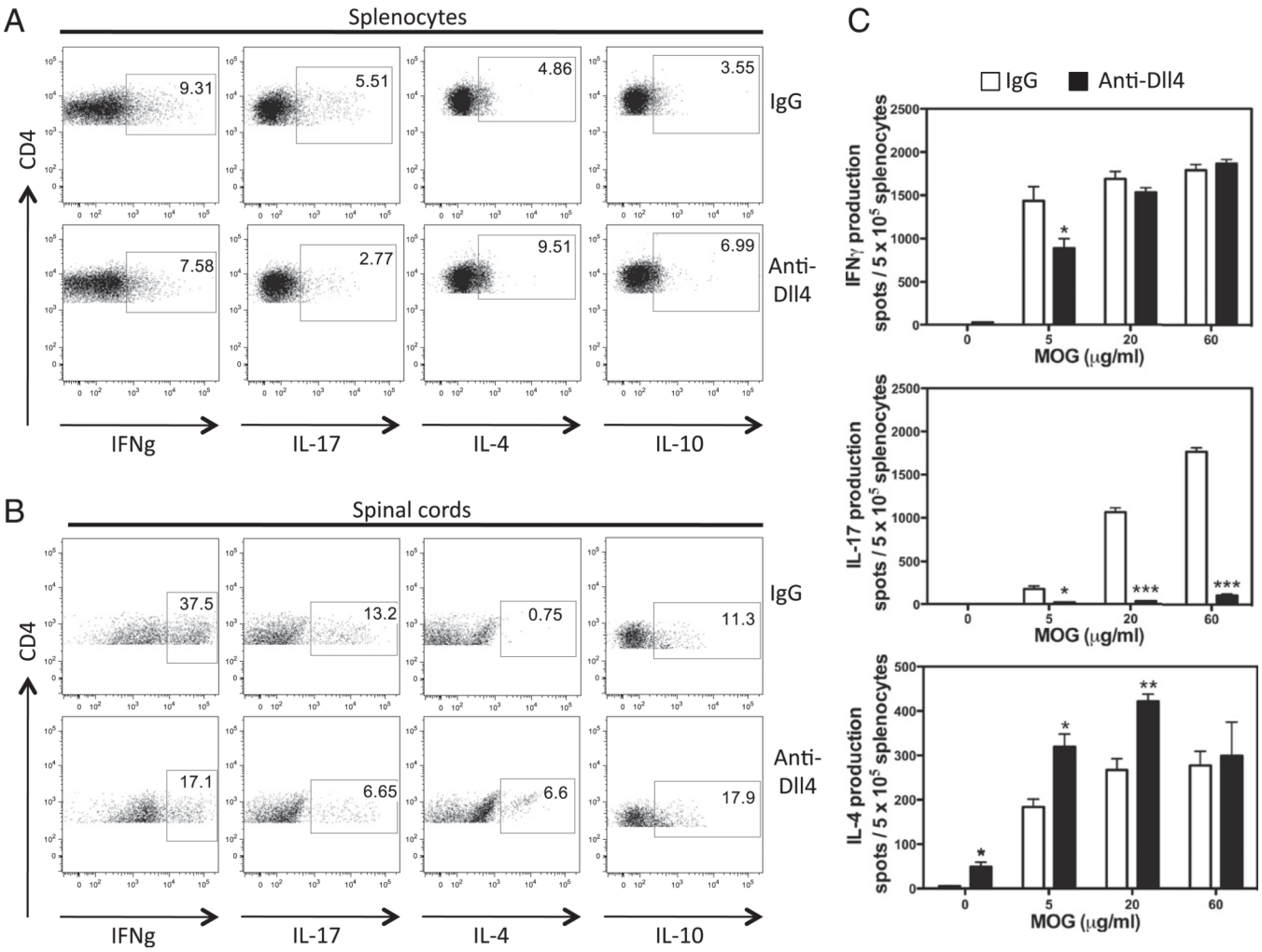

FIGURE 2.

Dll4 blockade in EAE regulates T cell differentiation. EAE was induced in C57BL/6 mice immunized with MOG(35–55)/CFA and treated with anti-Dll4 blocking Ab or control IgG every other day and for a total of five injections starting on the day of the immunization. Flow cytometry plots from individual mice from each group of splenocytes (*A*) on day 10 (preclinical disease) and spinal cord-infiltrating cells (*B*) on day 14 (after disease onset). Results are representative of three independent experiments with five mice per group. *C*, Enumeration of cytokine-producing cells by ELISPOT collected on day 10 postimmunization and then cultured in triplicates in the presence of various MOG(35–55) concentrations (0–60 mg/ml). Results are representative of two independent experiments with five mice per group (means ± SEM). **p* < 0.05, ***p* < 0.005, ****p* < 0.0005 by Student *t* test.

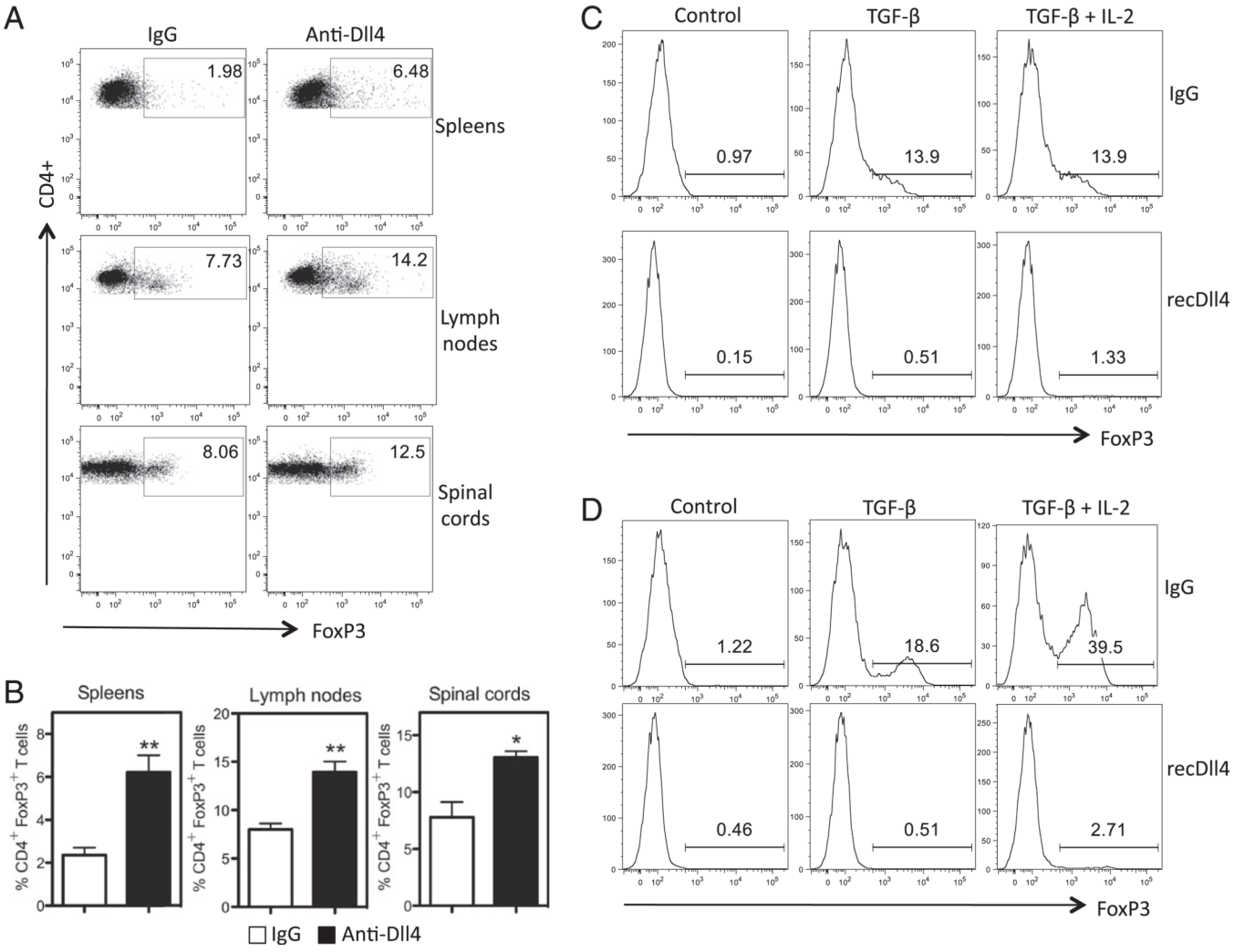

FIGURE 3.

Dll4-mediated signaling regulates Treg expansion in vivo and in vitro. EAE was induced in C57BL/6 mice immunized with MOG(35–55)/CFA and treated with anti-DLL4 blocking Ab or control IgG every other day and for a total of five injections (arrows) starting on the day of the immunization. *A*, Flow cytometry plots from individual mice from each group for CD4 and Foxp3 staining of splenocytes or lymph node cells on DPI 10 (preclinical disease) and spinal cord cells on DPI 14 (peak of disease). Results are representative of three independent experiments with five mice per group. *B*, Statistical analysis of staining described in *A*. $*p < 0.05$, $**p < 0.005$ by unpaired *t* test. $CD4^+Foxp3^-$ T cells (*C*) or total $CD4^+$ T cells (*D*) were isolated from naive Foxp3.GFP.KI mice and stimulated in vitro with plate-bound anti-CD3 and anti-CD28 (1 μg/ml) in the presence of plate-bound rDll4 or control IgG (2 ug/ml). Optimal dose TGF-β (3 ng/ml) or IL-2 (20 ng/ml) was added to the culture medium when indicated and then supplemented to the culture medium on day 2 of culture. Cells were washed and stained for CD4 and Foxp3 on day 4 of culture. Results are representative of three independent experiments.

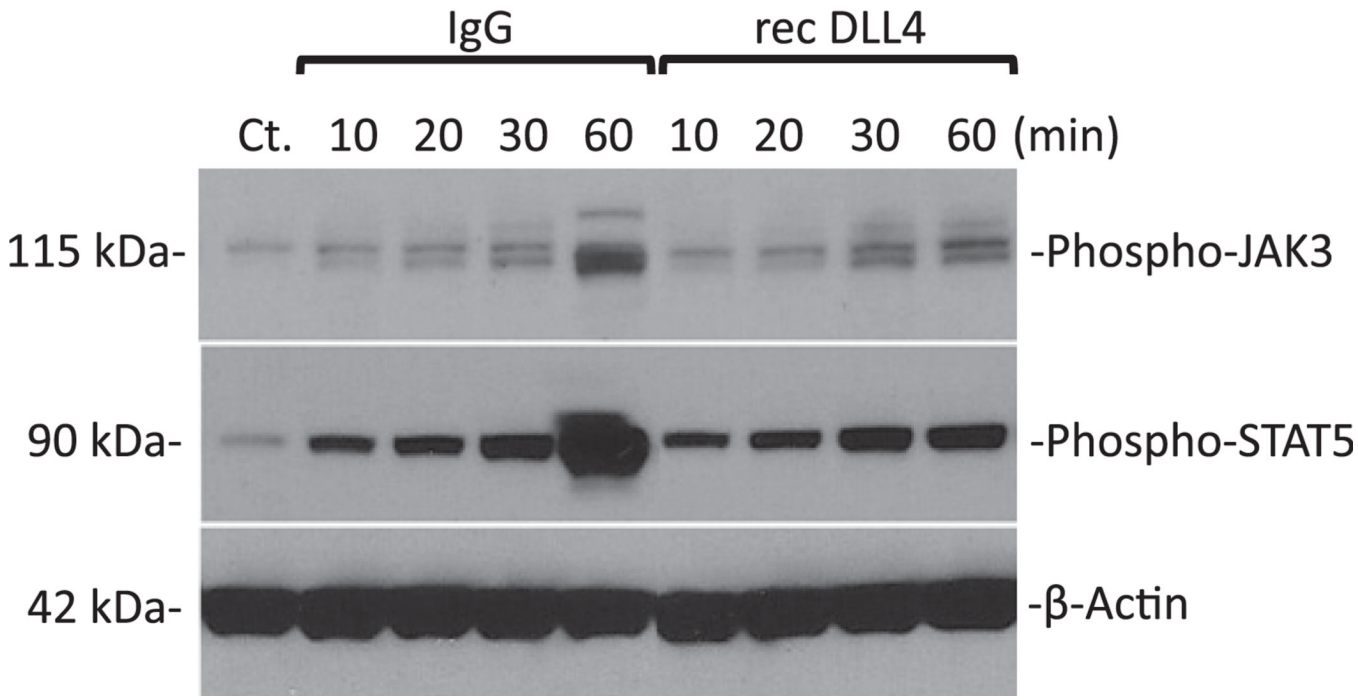

FIGURE 4.

Dll4 signaling inhibits JAK3/STAT5 activation. Naive $CD4^+$ cells were stimulated with anti-CD3 and anti-CD28 (1 μg/ml) in the presence of rDll4 or control IgG (2 μg/ml) for 10, 20, 30, and 60 min. Nonstimulated cells were used as control (Ct). Cell lysates were prepared, and Western blotting with anti–phospho-STAT5 was performed. The same blots were stripped and reprobed with anti–phospho-JAK3 or anti-mouse β-actin (used as the protein-loading control). Results are representative of three independent experiments.

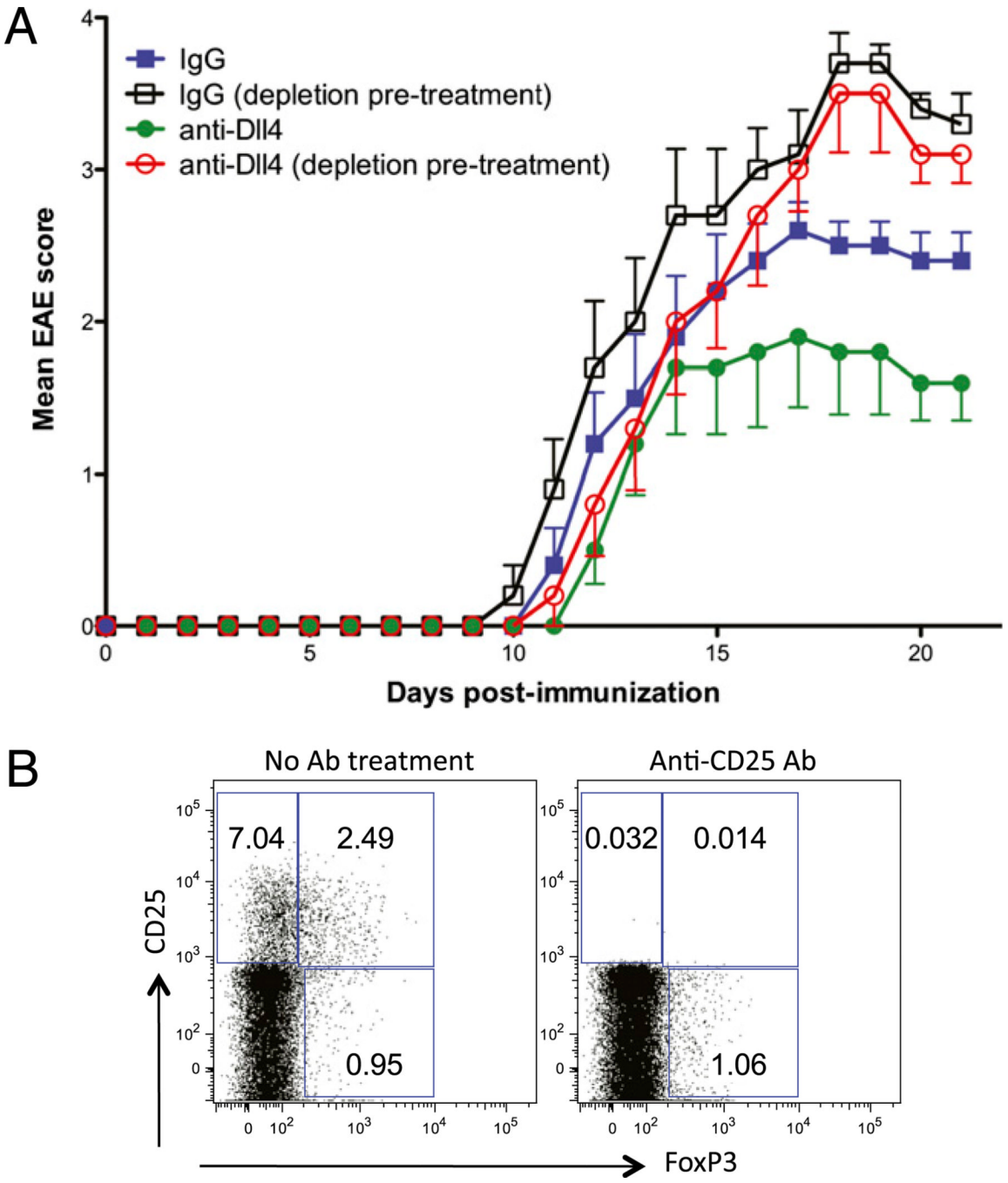

FIGURE 5.

Anti-Dll4–mediated suppression of EAE is Treg dependent. *A*, Naive C57BL/6 mice received 250 μg anti-CD25 mAb (open symbols) or not (filled symbols) i.p. on days −3 and −1 before EAE induction. Mice were immunized with MOG(35–55)/CFA and treated with anti-Dll4 mAb (circles) or control IgG (squares) on the day of the immunization and on days 2, 4, 6, and 8 after immunization. Clinical EAE scores are representative of two independent experiments with five mice per group. *B*, Flow cytometric profile of $CD4^+$ cells from splenocytes of mice described in *A* prior to immunization. Results are representative of two independent experiments with three mice per group.