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IL-21 receptor expression determines the temporal phases of experimental autoimmune encephalomyelitis

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Abstract

The IL-21 receptor (IL-21R) consists of a unique subunit and a common γ chain (γ_c) that is shared with other cytokines including IL-2, IL-4, IL-7, and IL-15. The interaction between IL-21 and IL-21R results in significant effects on both innate and adaptive immune responses. In this study we examined the influence of IL-21R deficiency (IL-21R$^{-/-}$) on the development of experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (MS). IL-21R$^{-/-}$ mice developed EAE earlier and more severe neurological impairment than control mice, yet those mice could effectively recover from neurological deficits. The impact on EAE initiation by IL-21R deficiency was associated with a defect of CD4$^+$CD25$^+$ T regulatory (Treg) cells and a down-regulated expression of Foxp3. The recovery from IL-21R$^{-/-}$ EAE was correlated with an expansion of Treg cells as well as an organ-specific redistribution of NK cells. These results suggest that a temporal influence of IL-21 on the activity of immunoregulatory circuits can be important in the modulation of the course of the autoimmune disease.

Keywords: IL-21 receptor; EAE; Autoimmunity; CD4$^+$CD25$^+$ regulatory T cells; NK cells

Introduction

IL-21 is a cytokine produced mainly by activated CD4$^+$ T and by NKT cells, and regulates the functions of several immune cell subsets of the adaptive and innate immune system depending on their activation status (Coquet et al., 2007; Leonard and Spolski, 2005; Mehta et al., 2004). IL-21 can promote Th2 cell function (Pesce et al., 2006; Strengell et al., 2002; Wurster et al., 2002), it can cooperate with IL-15 in enhancing the expansion and activity of CD8$^+$ T (Zeng et al., 2005) and NK cells (Strengell et al., 2003; Zeng et al., 2005), and it can enhance the survival and proliferation of NKT cells (Coquet et al., 2007). IL-21 can induce B cell apoptosis or, with IL-4, stimulate B cell proliferation to promote differentiation toward memory and/or plasma cells (Ettinger et al., 2005; Mehta et al., 2003).

More recently, IL-21 has been shown to counteract the regulatory effects of CD4$^+$CD25$^+$ T cells (Peluso et al., 2007). Additionally, dendritic cells (DCs) expanded in the presence of IL-21 have an immature phenotype and inhibit T cell responses, in contrast to IL-15-generated DCs that preferentially stimulate T-cell responses (Brandt et al., 2003; Leonard and Spolski, 2005).

Given the broad effects of IL-21 on diverse lymphocyte populations, the interaction of IL-21 with its receptor, IL-21

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receptor (IL-21R), may likely play a prominent role in inflammation and immune responses.

The IL-21R is a newly identified type I-cytokine receptor that shares the common γ chain (γ_c) with the receptors of IL-2, IL-4, IL-7, IL-9, and IL-15. IL-21R is expressed on lymphoid cells including T, B, NK, dendritic cells and macrophages (Leonard, 2001; Leonard and Spolski, 2005; Mehta et al., 2004). Despite its wide expression on immune cells, IL-21R-deficient (IL-21R$^{-/-}$) mice display normal lymphoid development and a dysfunction of IgG1 production together with increased IgE response (Ozaki et al., 2002).

To investigate the impact of IL-21R deficiency on the development of EAE, we induced EAE using myelin oligodendrocyte glycoprotein (MOG_{35-55}) for immunization of IL-21R$^{-/-}$ mice and control C57BL/6 (B6) mice. We found that IL-21R$^{-/-}$ mice developed EAE with earlier onset and more severe neurological defects than controls. The recovery phase of EAE did not differ from that in control B6 mice and associated with a dynamic change of IL-21R$^{-/-}$ CD4$^+$CD25$^+$ T regulatory (Treg) cells in response to IL-2, as well as organ-specific redistribution of NK cells.

Materials and methods

Mice

Female B6 mice, 7 to 8weeks of age, were purchased from Taconic (Germantown, NY). IL-21R$^{-/-}$ mice were provided by Dr. Deborah Young and Mary Collins (Collins et al., 2003) and backcrossed to those of a B6 background for 10 generations. All mice were maintained in specific pathogen-free condition in accordance with the guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at the animal facilities of Barrow Neurological Institute, St. Joseph's Hospital and Medical Center.

Antigens and antibodies

The murine MOG_{35-55} peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) was synthesized (purity > 95%) by Bio Synthesis Inc (Lewisville, TX). Anti-NK1.1 monoclonal antibody (mAb) (PK136) (Koo and Peppard, 1984) was obtained from hybridomas from the American Type Culture Collection (Manassas, VA). Mouse IgG2a (Sigma, St. Louis, MO) was used as the isotype control antibody for anti-NK1.1$^+$ mAb.

EAE induction and assessment

EAE was induced in mice by subcutaneous (s.c.) injections into the flank and tail base with 100 μg of MOG_{35-55} peptide emulsified in complete Freund's adjuvant (CFA) containing 500μg of heat inactivated *Mycobacterium tuberculosis* (Difco, Detroit, MI). Supplemental injections of 200 ng pertussis toxin were given intravenously (i.v.) on the same day and two days later (List Biologic, Campbell, CA). The mice were monitored daily for clinical signs of EAE and scored blind on a scale of 0 to 5 with graduations of 0.5 for intermediate scores (Mendel et al., 1995): 0, normal; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind-limb weakness or hind-limb paralysis; 3, complete hind-limb paralysis; 4, complete hind-limb paralysis with forelimb weakness or paralysis; 5, moribund or deceased state.

In vivo depletion of NK1.1$^+$ cells

For depletion of NK1.1$^+$ cells in vivo, 100μg anti-NK1.1 mAb was injected intraperitoneally (i.p.) into each mouse at day-2 post immunization (p.i.). Control mice received isotype control IgG2a. Every five days thereafter, 50 μg anti-NK1.1 mAb was injected i.p. until the termination of experiments (Vollmer et al., 2005). Depletion of NK1.1$^+$ cells was confirmed by flow cytometry with anti-mouse NK1.1-PE (PK136) (BD PharMingen, San Diego, CA), and was always > 90% NK1.1$^+$ cell depletion.

Histopathology

Mice were euthanized via inhalation of halothane (Sigma, St. Louis, MO) and fixed by cardiac perfusion with 4% paraformaldehyde. Spinal cords and brains of mice were removed, cryoprotected in 20% sucrose solution in PBS, embedded in OCT (Fisher Scientific, Waltham, MA), and then frozen for cryostat sectioning of 10 μM. Lymphocytic infiltration and demyelination were evaluated by Hematoxylin & Eosin (H&E) and Luxol Fast Blue (LFB) staining, respectively. Histological findings were graded into 5 categories: 0, no inflammatory cells; 1, leptomeningeal infiltration; 2, mild perivascular cuffing; 3, extensive perivascular cuffing; 4, extensive perivascular cuffing and severe parenchymal cell infiltration (Jee and Matsumoto, 2001).

Cell isolation and proliferation assays

The culture medium was complete RPMI 1640 containing 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10mM HEPES, 10% FBS, 10μM β-mercaptoethanol, and 1% nonessential amino acid (Mediatech, Inc., Herndon, VA). Mononuclear cells were obtained from spleens and prepared in single-cell suspensions as previously described (Vollmer et al., 2005). Briefly, splenocytes were isolated mechanically by gentle scraping of the fresh spleen tissues through a 70 μM strainer into RPIM 1640 with 1% FBS. After red blood cell lysis with BD Pharm Lyse™ buffer (BD Bioscience, San Jose, CA), cells were washed in culture medium and filtered through a 40 μM strainer. Mononuclear cells (4×10^5/well) were cultured in 96-well round-bottom plates with 200 μl culture medium in the presence of MOG_{35-55} (10 μg/ml), or Con A (5 μg/ml), or with the indicated antigens for 72 h. Cell proliferation was assayed by incubation with 1 μCi [^{3}H] thymidine/well (specific activity 42 Ci/mmol; Amersham, Arlington Heights, IL, USA) during the last 18 h. Cells were harvested by an automatic cell harvester (TOMTEC) and the incorporated radioactivity was evaluated by using a Wallac MicroBeta Counter (PerkinElmer, Waltham, MA). Each treatment was assayed in triplicate and the results are expressed as mean ± SD.

Flow cytometry and CFSE labelling

Aliquots of ~ 30×10^6 splenocytes were labeled with 0.5 μM CFSE at 37 °C in water bath for 10min. Cells with or without CFSE labeling were cultured at 37 °C for 3 days in 24-well plates (2×10^6cells/well) with or without antigens (MOG_{35-55} 10 μg/ml or Con A 2.5 μg/ml) and then stimulated with PMA/ionomycin/BFA (PMA 20 ng/ml, ionomycin 1 μg/ml, 5 μg/ml BFA) for 5 h at 37 °C. Cells were then stained for surface markers with fluorochrome-conjugated mAbs: anti-CD3-PeCy5 (17A2), anti-CD4-ApcCy7 (GK1.5), anti-CD8α-PeCy7 (53-6.7), and anti-NK1.1-PE (PK136) (BD PharMingen) for 15 min at 4 °C. After fixation and permeabilization, intracellular cytokines were stained using Alexa 647/PE-conjugated anti-IFN-γ/anti-IL-2/anti-IL-4/anti-IL-10/anti-IL-17 mAb for 30 min at 4 °C. For intracellular Foxp3 expression, fresh cells were stained with surface markers anti-CD4-FITC (H129.19) and anti-CD25-APC (PC61.5) for 15min at 4 °C, fixed, permeabilized, and then stained with anti-Foxp3-PE (FJK-16s) (eBioscience, San Diego, CA). Flow cytometric data were collected on a FACSAria™ flow cytometer (Becton Dickinson, Mountain View, MD) and analyzed with Diva™ software. Isotype-matched negative control mAbs were used for all stains. To determine the percentage of cells producing the selected cytokines, values obtained with isotype controls were subtracted from those with specific mAbs.

RT-PCR for cytokine gene expression

Total RNA was extracted from cell suspensions of spinal cords using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). First strand cDNA of each sample was synthesized using a reverse transcription kit (Invitrogen, Carlsbad, CA). RT-PCR was performed as previously described (Liu et al., 2007), using the following primers: IL17.F: 5′-CCT CCA GAA TGT GAA GGT CA-3′; IL17.R: 5′-CTA TCA GGG TCT TCA TTG CG-3′; IFNγ.F: 5′-AGC TCT TCC TCA TGG CTG TT-3′; IFNγ.R: 5′-TTT GCC AGT TCC TCC AGA TA-3′; HPRT.F: 5′-AGC CTA AGA TGA GCG CAA GT-3′ HPRT.R: 5′-TTA CTA GGC AGA TGG CCA CA-3′. The HPRT gene was amplified and served as an endogenous control. 1μl of first strand cDNA product was amplified with platinum Taq polymerase (Invitrogen) and gene-specific primer pairs. Each sample was assayed in triplicate and experiments were repeated twice. The relative amounts of mRNA were calculated by plotting the Ct (cycle number), and average relative expression was determined by the $2^{-\Delta\Delta Ct}$ comparative method.

Isolation of mononuclear cells from the CNS

The CNS-infiltrating mononuclear cells were isolated as previously described (Liu et al., 2007). Briefly, fresh brain and/or spinal cords were removed from mice, cut into small pieces, and digested in 10 mM Hepes/NaOH buffer containing 1 mg/ml of collagenease for 1h at 37 °C. Tissues were homogenized with a syringe, filtered through a 70 μM cell strainer to obtain single cell suspension, and centrifuged. Cell pellets were resuspended in 30% Percoll and centrifuged against 70% Percoll. The cell monolayer between the 30%–70% Percoll interface was collected for staining.

ELISA

IL-21 and IL-2 ELISA were performed using the DuoSet ELISA kit from R&D Systems (Minneapolis, MN) (Coquet et al., 2007). Briefly, mononuclear cells (4×10^5/well) from the spleens of $IL21R^{-/-}$ and B6 mice after removal at indicated time points were cultured with anti-CD3 and anti-CD28 mAbs for 72 h. Supernatants were harvested and analyzed for the presence of IL-21 or IL-2 by ELISA. 96-well microtiter plates were coated with IL-21 or IL-2 capture Ab at 4 °C overnight. After blocking with 10% FBS, samples or standards were added and incubated for 2 h at room temperature. Plates were incubated with detection Ab for 2 h and then with Streptavidin–HRP for 30min, followed by color development with TMB substrate reagent. Results were expressed as OD at 450 nm.

In vitro IL-2 and IL-21 cultures

Spleen mononuclear cells (4×10^5/well) from $IL21R^{-/-}$ and B6 mice were cultured with IL-2 (10 ng/ml) or/and IL-21 (1, 10, 100 μg/ml) for 4 days at 37 °C before harvest for surface and intracellular staining.

Treg cell suppression assays

Spleen $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were isolated using the Miltenyi Mouse $CD4^+CD25^+$ Regulatory T cell Isolation Kit™ (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instruction. $CD4^+$ T cells were first isolated through negative selection by removing all other cell types. Pre-isolated $CD4^+$ T cells were incubated with magnetic beads conjugated with anti-CD25 antibody to separate $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were then sorted using a FACSAria™. Isolated T cells were > 95% pure upon re-analysis by flow cytometry.

$CD4^+CD25^-$ cells were used as responders. $CD4^+CD25^-$ T cells (1×10^5) were cultured in U-bottom 96-well plates with 10^4 spleen DCs, MOG_{35-55} (10μg/ml), and anti-CD3 (0.1 μg/ml). $CD11c^+$ cells were isolated from splenocytes of EAE mice at the indicated time point and further loaded with MOG_{35-55} peptide ex vivo. These DCs were more potent than DCs from naïve mice in generating measurable T cell response (Liu et al., 2005). Proliferation was determined by incorporation of ^3H thymidine for the last 18h of the culture.

Statistics

Clinical scores were analyzed with the nonparametric Mann–Whitney *U* test. Comparisons between groups were evaluated by the one-tailed unpaired Student's *t* test for two groups, and ANOVA for multiple comparisons. Values of $P < 0.05$ were considered significant.

Results

IL-21R$^{-/-}$ mice develop EAE earlier and with more neurological impairment than controls

We induced EAE with MOG_{35-55} peptide in IL-21R$^{-/-}$ mice as well as in control wild-type B6 mice, and monitored the clinical course of disease for more than 60 days. Compared with B6 mice, IL-21R$^{-/-}$ mice developed EAE with earlier onset (12.5 days for IL-21$^{-/-}$ mice vs. 16.5 days for B6 mice, $P < 0.05$; Fig. 1A and Table 1) and with more severe disease symptoms. The mean maximal disease score of IL-21R$^{-/-}$ mice was 3.3 vs. 2.6 of B6 controls (Fig. 1A and Table 1). Surprisingly, IL-21R$^{-/-}$ mice recovered from disease faster than B6 mice (Fig. 1A). However, by day 60 p.i., there was no difference in severity of EAE between the IL-21R$^{-/-}$ mice and control mice (Fig. 1A).

The extent of neurological damage in the two groups of mice was analyzed by histopathological analyses on mice spinal cords and brains. IL-21R$^{-/-}$ mice had massive cellular infiltration and inflammation associated with demyelination, particularly at the peak phase of EAE (mean histological scores: 3.2 ± 0.9 for IL-21$^{-/-}$ mice vs. 2.2 ± 0.5 for B6 mice). Tissue inflammation was higher also during EAE recovery (mean histological scores: 1.5 ± 0.8 for IL-21$^{-/-}$ mice vs. 0.5 ± 0.1 for B6 mice) (Fig. 1B, Table 1). Collectively, these data suggest that in the absence of IL-21R, severity of EAE increases in the early phases of disease, and associates with more rapid recovery.

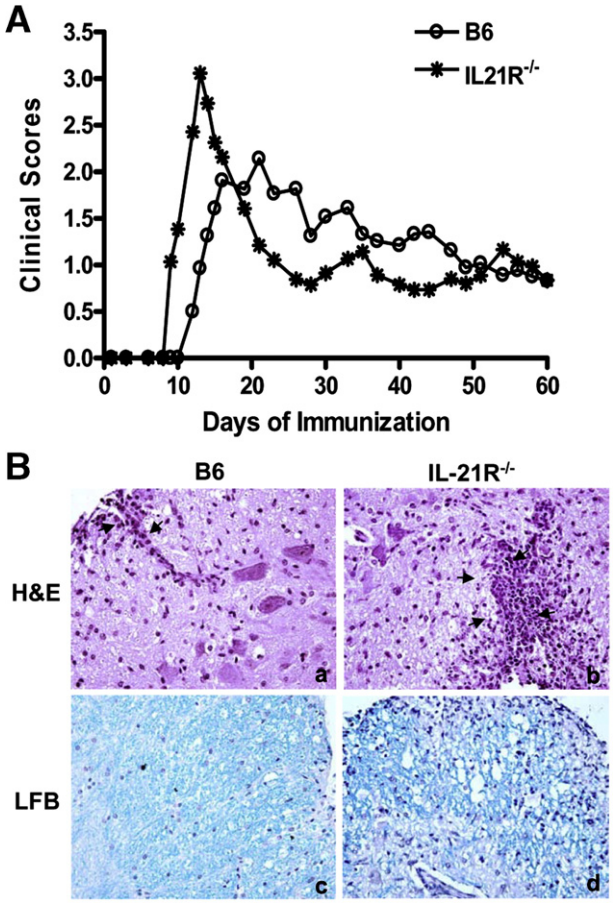

Fig. 1. Influence of IL-21R deficiency on the development of EAE. (A) Clinical course of active EAE. Groups of B6 (n=24) and IL-21R$^{-/-}$ (n=22) mice were immunized with MOG_{35-55} peptide and monitored for clinical symptoms of EAE as described in the Materials and methods. Results are pooled from 4 independent experiments and are expressed as daily mean clinical scores. (B) Mononuclear cell infiltration and demyelination in the CNS. Brains and spinal cords of B6 and IL-21R$^{-/-}$ mice were obtained at the peak phase of EAE, and representative sections stained with H&E (a: B6; b: IL-21R$^{-/-}$) and LFB (c: B6; d: IL-21R$^{-/-}$). Arrows indicate the regions of cellular infiltration and inflammation.

Table 1
MOG_{35-55}-induced EAE in IL-21R$^{-/-}$ mice

Mouse group	Clinical status		Histological Score	
	Day of onset (mean±SD)	Maximum disease score (mean±SD)	Peak	Recovery
B6	16.5±6.2	2.6±1.7	2.2±0.5	0.5±0.1
IL-21R$^{-/-}$	12.5±1.9*	3.3±0.7*	3.2±0.9†	1.5±0.5

Groups of mice were immunized with MOG_{35-55} as described in Materials and methods, and observed for 60–90 days after immunization. Sections of spinal cords and brains from representative EAE mice were stained with H&E for histological examination. *P<0.05, $^{\dagger}P$<0.01, as compared to corresponding wild-type B6 controls.

IL-21R deficiency in EAE associates with increased autoreactive T cell responses

Autoreactive T cell responses in the absence of IL-21R were evaluated by means of T cell proliferation using CFSE dilution. Fig. 2 shows that CD4^{+} T cell response to MOG_{35-55} in IL-21R$^{-/-}$ mice were higher than in B6 controls in the early phases of diseases and waned by day 60 p.i. These data suggested that lack of IL-21R affected autoreactive T cell proliferation during the initial course of EAE.

Production of cytokines was also measured. Compared to B6 controls, peripheral CD4^{+} and CD8^{+} T cells of IL-21R$^{-/-}$ mice had increased expression of IFN-γ on day 3 and 12 p.i. (Fig. 3A). Interestingly IL-21R$^{-/-}$ CD8^{+} cells secreted more IFN-γ than CD4^{+} cells (Fig. 3A). As reported previously (Liu et al., 2006), the numbers and percentages of CD4^{+} and CD8^{+} cells were comparable in IL-21R$^{-/-}$ and control mice (data not shown). However, on day 60 p.i. (recovery phase), the IFN-γ levels in IL-21R$^{-/-}$ CD4^{+} and CD8^{+} cells were decreased and comparable to those of B6 mice (Fig. 3B).

During the peak of EAE (day 12 p.i.), IL-21R$^{-/-}$ mice also produced more IL-4/IL-10 than B6 EAE controls (Fig. 3C). The increase of IL-4 and IL-10 in IL-21R$^{-/-}$ mice were markedly lower than that of IFN-γ (Fig. 3C). These results suggest that IL-21R-deficiency in EAE associates with a temporary expansion of Th1 and Th2 responses, particularly Th1, an aspect wanes over the course of the disease.

Recently, IL-17-producing T cells (Th17) have been implicated to play a prominent role in the pathogenesis of EAE (Bailey et al., 2007; Komiyama et al., 2006). However, we did not detect significant differences of IL-17 production in IL-21R$^{-/-}$ mice as compared to the B6 mice with EAE, although IL-21R$^{-/-}$ EAE mice had a trend of lower IL-17 production (Supplementary data), and cytokine mRNA expression in spinal

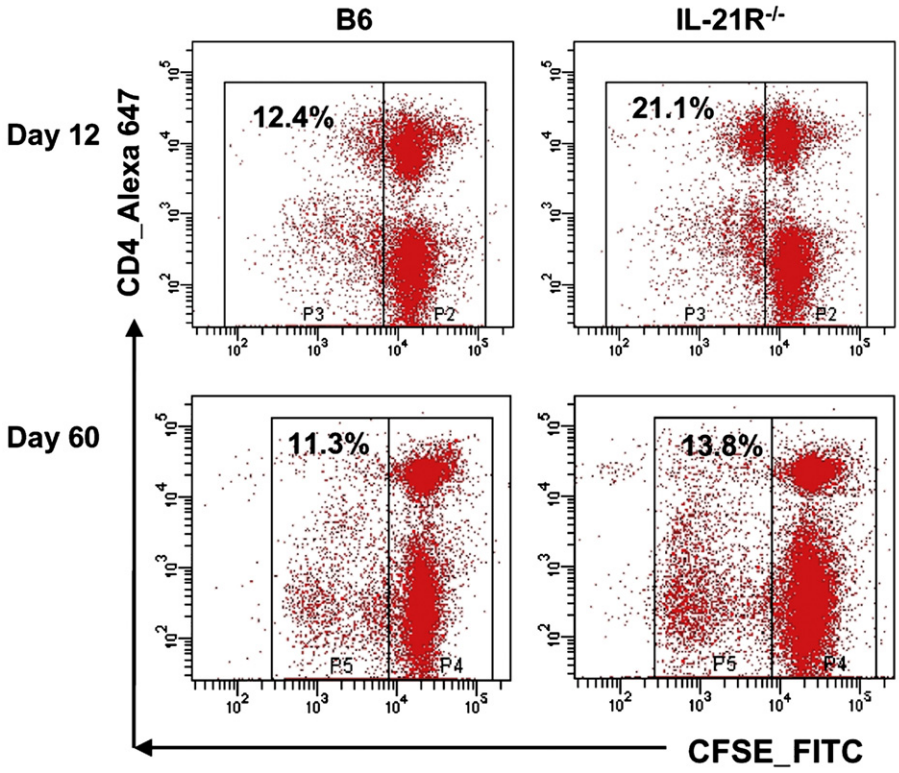

Fig. 2. Effects of IL-21R deficiency on T cell proliferation in EAE. Splenocytes from $MOG_{35–55}$-immunized IL-21R$^{-/-}$ and B6 mice were harvested on the indicated days post-immunization and analyzed ex vivo for proliferative responses to MOG_{35-55} (10 μg/ml) by CFSE dilution. Dot plots gated on lymphocytes were evaluated for percentages of proliferating cells. Results are representative of 3 independent experiments with similar results.

cords in IL-21R$^{-/-}$ and B6 EAE mice were not statistically different (Fig. 3D).

Recovery from EAE associates with the insurgence of T regulatory (Treg) cells in IL-21R$^{-/-}$ mice

CD4$^+$CD25$^+$ Treg cells are involved in the maintenance of immune tolerance. Since IL-21R and IL-2R share a common γ chain, the structure of IL-21R has homologies with that of IL-2R (Leonard and Spolski, 2005). To test whether Treg cells might be affected by IL-21R deficiency, we quantified the numbers of Treg cells and assessed their Foxp3 expression - a marker of a suppressive phenotype for these cells. Compared with EAE B6 control mice, IL-21R$^{-/-}$ EAE mice had decreased numbers of CD4$^+$CD25$^+$ Treg cells (Figs. 4A, B; $P < 0.05$) during EAE induction (day 3 p.i.) and at the peak stage of EAE (day 12 p.i.). This finding correlated with corresponding decreased percentages of Foxp3$^+$ cells (Figs. 4C, D; $P < 0.05$). In in vitro suppression assays, Treg cells isolated from IL-21R$^{-/-}$ EAE mice at day 12 p.i. had a reduced capacity in inhibiting proliferation of T effector cells, in comparison with Treg cells isolated from IL-21R$^{-/-}$ EAE mice at day 60 p.i. (Fig. 5). As EAE progressed, the differences in the numbers of Treg cells as well as their expression of Foxp3 between IL-21R$^{-/-}$ mice and control mice disappeared (Fig. 4). Consistent with these observations, flow cytometric analysis of mononuclear cell infiltration into the brain and spinal cords showed that, on day 12 p.i., IL-21R$^{-/-}$ mice had decreased numbers of CD4$^+$CD25$^+$ cells compared with B6 mice (data not shown). Yet, on day 60 p.i. of their recovery from EAE, IL-21R$^{-/-}$ and B6 mice had comparable numbers of CD4$^+$CD25$^+$ cells (data not shown). Thus, similar to the effect on autoreactive T cells, IL-21R deficiency had a transitory influence on Treg cells in EAE.

Treg cells of IL-21R$^{-/-}$ mice are highly sensitive to IL-2

When we quantified the production of IL-2 and IL-21 in IL-21R$^{-/-}$ and B6 mice, we found that IL-21 was detectable as early as day 3 p.i., whereas IL-2 was undetectable at this stage (Figs. 6A, B). IL-21 increased further by day 12, a time when IL-2 was also detected (Figs. 6A, B). Since similar kinetics of IL-21 and IL-2 production were observed in both IL-21R$^{-/-}$ and B6 mice, the data might suggest the possibility that lack of IL-21R negatively impacted the numbers of Treg cells during early EAE and, subsequently, production of IL-2 could positively impact Treg cell numbers.

To assess whether IL-21R$^{-/-}$ Treg cells could respond to IL-2, we isolated splenocytes of B6 and IL-21R$^{-/-}$ mice and cultured them in the presence of IL-2. Flow cytometry studies indicated that IL-21R$^{-/-}$ Treg cells expanded in the presence of IL-2 (Figs. 6C, D).

Effects of EAE on NK cells and NKT cells in IL-21R$^{-/-}$ mice

NK cells have an important role in EAE, as they can suppress disease (Huang et al., 2006; Zhang et al., 1997).

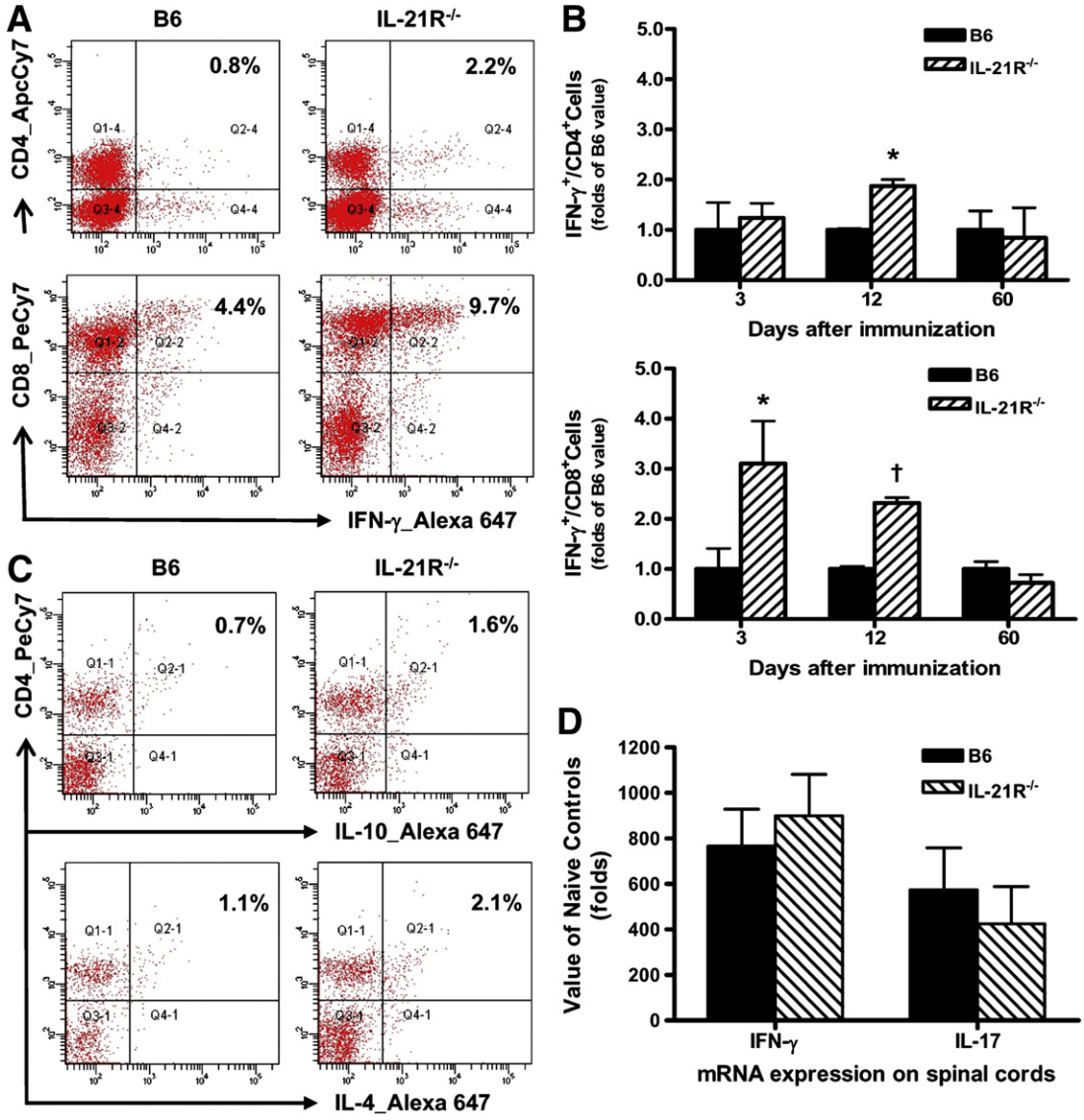

Fig. 3. Cytokines in IL-21R$^{-/-}$ mice during EAE. Splenocytes from MOG_{35-55}-immunized IL-21R$^{-/-}$ and B6 mice were harvested on the indicated day p.i. and cultured in the presence of MOG_{35-55} (10 μg/ml) for 72 h. Intracellular cytokines were examined by flow cytometry. (A) MOG_{35-55}-induced IFN-γ production by IL-21R$^{-/-}$ CD4$^+$ and CD8$^+$ T cells on day 12 p.i. as shown in representative plots from 3 separate experiments (n=2 to 3 mice/group). (B) MOG_{35-55}-induced IFN-γ production by IL-21R$^{-/-}$ CD4$^+$ and CD8$^+$ T cells on days 3, 12, and 60 p.i. Bars represent percentages of IFN-γ-producing cells. Results pooled from 3 independent experiments (n=2 to 3 mice/group). (C) MOG_{35-55}-induced IL-4 and IL-10- production by IL-21R$^{-/-}$ CD4 T cells on day 12 p.i. as shown in representative plots from 3 separate experiments (n=2 to 3 mice/group). (D) Cytokine mRNA expression in the CNS of IL-21R$^{-/-}$ and B6 EAE mice. Total RNA was isolated from spinal cords of naïve mice and mice on day 25 day p.i. and analyzed by RT-PCR. The results represent fold increase as compared to naïve controls and are expressed as means ± SD (n=4). *P<0.05, $^{\dagger}P$<0.01.

Activated NKT cells are also important regulatory cells in this disease (Singh et al., 2001; Zajonc et al., 2005). Therefore, we examined NK and NKT cells in IL-21R$^{-/-}$ mice during EAE. Compared to B6 EAE mice, in early disease (day 3 p.i) IL-21R$^{-/-}$ mice had reduced numbers of NK cells (3.4% for IL-21R$^{-/-}$ mice vs. 7.3% for B6 mice, Fig. 7A). No difference was found at other time points (Fig. 7B), indicating that NK cells by day 3 p.i. only expanded in B6 mice but not in IL-21R$^{-/-}$ mice (Fig. 7B).

While an expansion of NKT cells was observed both in IL-21R$^{-/-}$ mice and controls (Fig. 7A), the number of infiltrating NKT cells in the CNS was unaffected.

In the CNS, IL-21R$^{-/-}$ mice but not control mice had an accumulation of NK cells at later stages of EAE (Fig. 7C). Importantly, depletion of NK cells worsened EAE in IL-21R$^{-/-}$ mice (Fig. 7D). Thus, NK cell accumulation in the CNS during late stages of EAE in IL-21R$^{-/-}$ mice associated with protection from EAE.

Discussion

We show that a deficiency of IL-21R influences the course of MOG_{35-55}-induced EAE significantly. IL-21R$^{-/-}$ mice developed severe EAE with early onset, and quickly recovered from

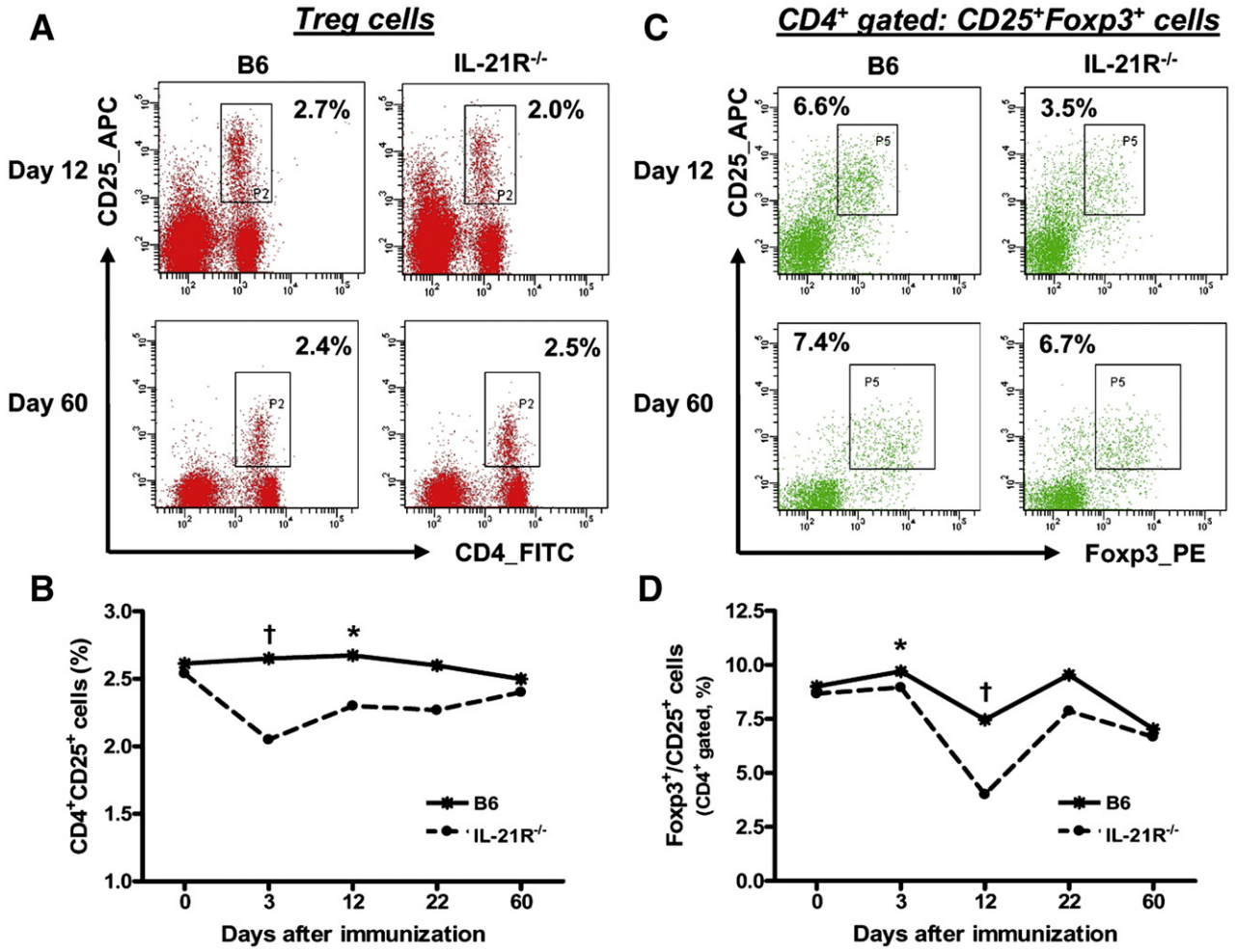

Fig. 4. Peripheral Treg cells of IL-21R$^{-/-}$ mice in EAE. Splenocytes from MOG$_{35-55}$-immunized IL-21R$^{-/-}$ and B6 mice were prepared on the indicated days after immunization, stained with anti-CD4 and anti-CD25 mAb, and then stained with intracellular anti-Foxp3 mAb as described in the Materials and methods. Dot plots were gated on lymphocytes in 2 to 4 representative independent experiments (n=3 mice/group). Results of frequencies of individual cell populations were pooled from 3 independent experiments. *P<0.05, $^{\dagger}P$<0.01. (A and B) CD4^{+}CD25^{+} Treg cells; (C and D) CD25^{+}Foxp3^{+} cells on CD4^{+} gated subpopulation.

disease. A contribution of IL-21 to the kinetics of immune regulation in EAE was associated with temporal changes in the number and localization of both Treg cells and NK cells compartments.

The effector function of T cells largely depends on cytokines. IL-21 is a cytokine that shares properties with both Th1 and Th2 responses. Some groups have indicated that IL-21 is a proinflammatory cytokine with a capacity for up-regulating the expression of genes associated with the Th1 response (Strengell et al., 2002), and that the over-expression of IL-21 or IL-21R associated with accumulation of mononuclear cells (Parrish-Novak et al., 2002; Pelletier et al., 2004; Vollmer et al., 2005). Other groups showed that IL-21 is a cytokine that can inhibit the differentiation of Th cells into IFN-γ-producing Th1 cells (Wurster et al., 2002), and that IL-21R augments Th2 cells (Pesce et al., 2006) and/or Th2 responses, i.e. in allergic airway inflammation (Frohlich et al., 2007).

Here, we find that in EAE the lack of IL-21R leads to transient increase of IFN-γ as well as IL-4 and IL-10 expression

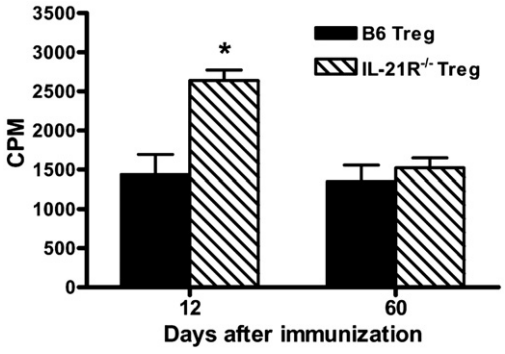

Fig. 5. Comparison of suppressor activity of IL-21R$^{-/-}$ Treg cells during EAE. Comparison of suppressor activity of IL-21R$^{-/-}$ Treg cells during EAE. Purified CD4^{+}CD25^{+} Treg cells and responder CD4^{+}CD25^{-} cells (1×10^5) were cultured at 1:4 ratio for 60 h with 10 μg/ml MOG $_{35-55}$-primed 10^4 APC. Baseline proliferation of responder cells was 2800±233 CPM. The data represent two separate experiments with similar outcomes, n=4 mice per group each, *P<0.05.

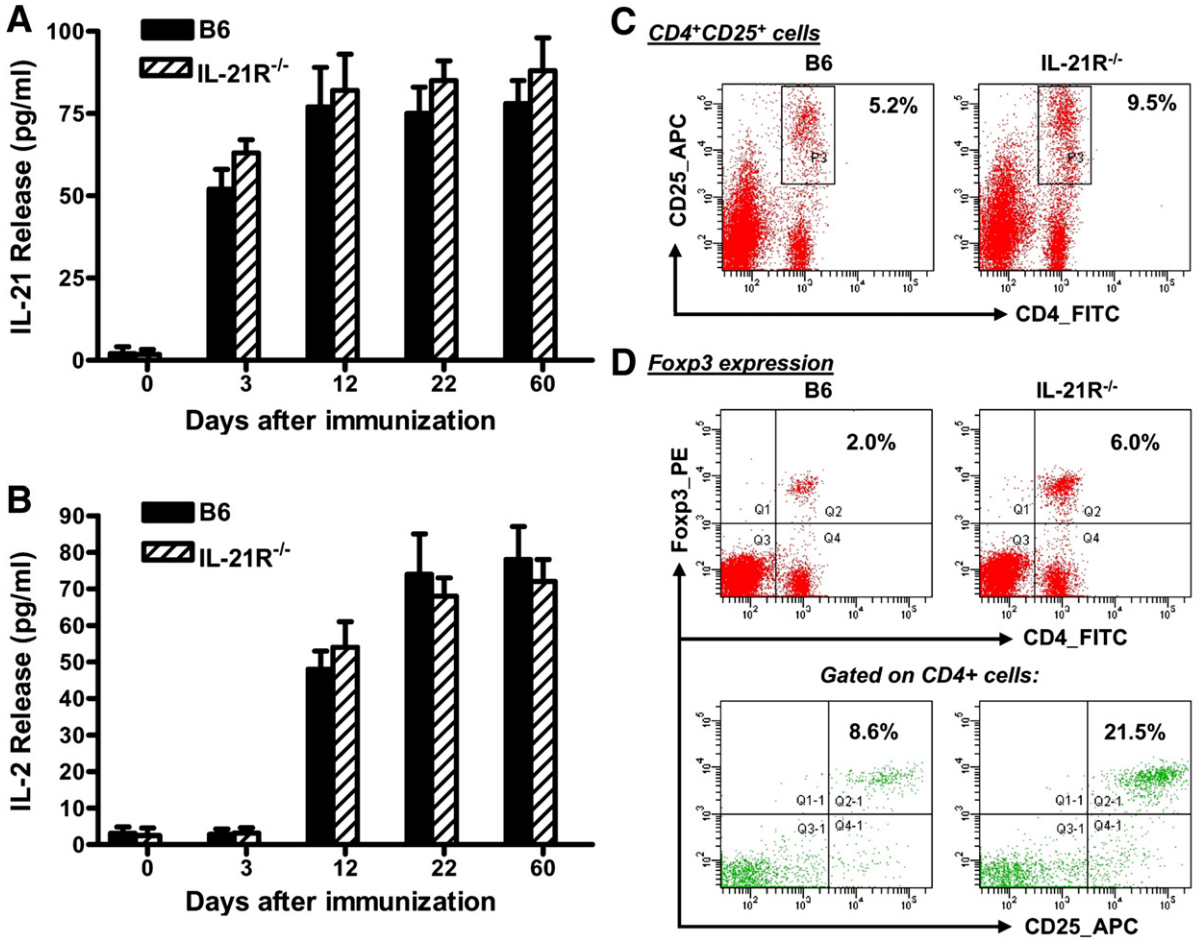

Fig. 6. IL-21R$^{-/-}$ Treg cells to respond to IL-2. (A) Kinetics of IL-21 production in EAE; (B) Kinetics of IL-2 production in EAE. Data shown are from one of 3 independent experiments with similar results. (C) CD4^{+}CD25^{+} cells of IL-2 cultures. (D) Foxp3 expression of IL-2 cultures by CD4^{+} cells (upper panel) and in relation to CD25^{+} cells gated on CD4^{+} cells (lower panel). Splenocytes of B6 and IL-21R$^{-/-}$ mice were cultured with IL-2 (10 μg/ml) for 4 days and plots shown are representative from 4 independent experiments.

but, as EAE progresses, the effects of IL-21R deficiency disappear. However, timing of anti IL-21R blockade might account for different effects in other diseases, i.e. in rodent models of systemic autoimmunity, administration of IL-21 R Fc ameliorated disease in rheumatoid arthritis (Young et al., 2007), and reduced progression of lupus-like disease (Herber et al., 2007).

The dynamic impact of IL-21R on Th1 or Th2 cells has not been recognized previously, and its underlying mechanism can be important for a better understanding of the biology of IL-21 in vivo. Th17 cells, a helper T cell subset, is involved in the pathogenesis of several autoimmune diseases such as MS/EAE (Bailey et al., 2007; Komiyama et al., 2006). In the present study, we did not detect any significant differences of IL-17-producing Th17 cells between IL-21R$^{-/-}$ and B6 mice with EAE, but IL-21R$^{-/-}$ EAE T cells showed a trend of lower levels of IL-17 expression, which is consistent with one recent report that IL-21 may initiate an alternative pathway to amplify IL-17 induction by Th17 cells, and that IL-21R$^{-/-}$ T cells are defective in generating a Th17 response (Korn et al., 2007). Our results suggest that Th17 cells did not significantly impact the phenotype of IL-21R$^{-/-}$ mice.

It is widely accepted that the pathogenic effects of autoreactive T effectors cells in EAE are highly influenced by regulatory cells. One of the such regulatory cell population is CD4^{+}CD25^{+} cells, which express the forkhead transcription factor Foxp3 and contribute to maintenance of peripheral immune tolerance (Sakaguchi, 2004, Ziegler, 2006). The maturation of these regulatory cells is highly dependent on IL-2 (Thornton et al., 2004). IL-21R has structure homology with IL-2R, as it shares a common γ-chain and similar to IL-2Rβ (Leonard and Spolski, 2005; Ozaki et al., 2000).

This study shows that the early onset and severe neurological defects of IL-21R$^{-/-}$ EAE during the priming phase associates with a defect of CD4^{+}CD25^{+} cells and down-regulated expression of Foxp3. Significantly, the production of IL-2 can compensate for impaired IL-21 in that it rescued defective Treg cells (which contribute to the recovery from EAE in IL-21R$^{-/-}$ mice).

Incidentally, in our cultures we found that IL-21 in the presence of IL-2 was suppressive for CD4^{+}CD25^{+} Treg cells in vitro (unpublished data). This finding is consistent with that of Peluso et al., who showed that IL-21 counteracts CD4^{+}CD25^{+} Treg cell-mediated suppression of human CD4^{+} T lymphocytes

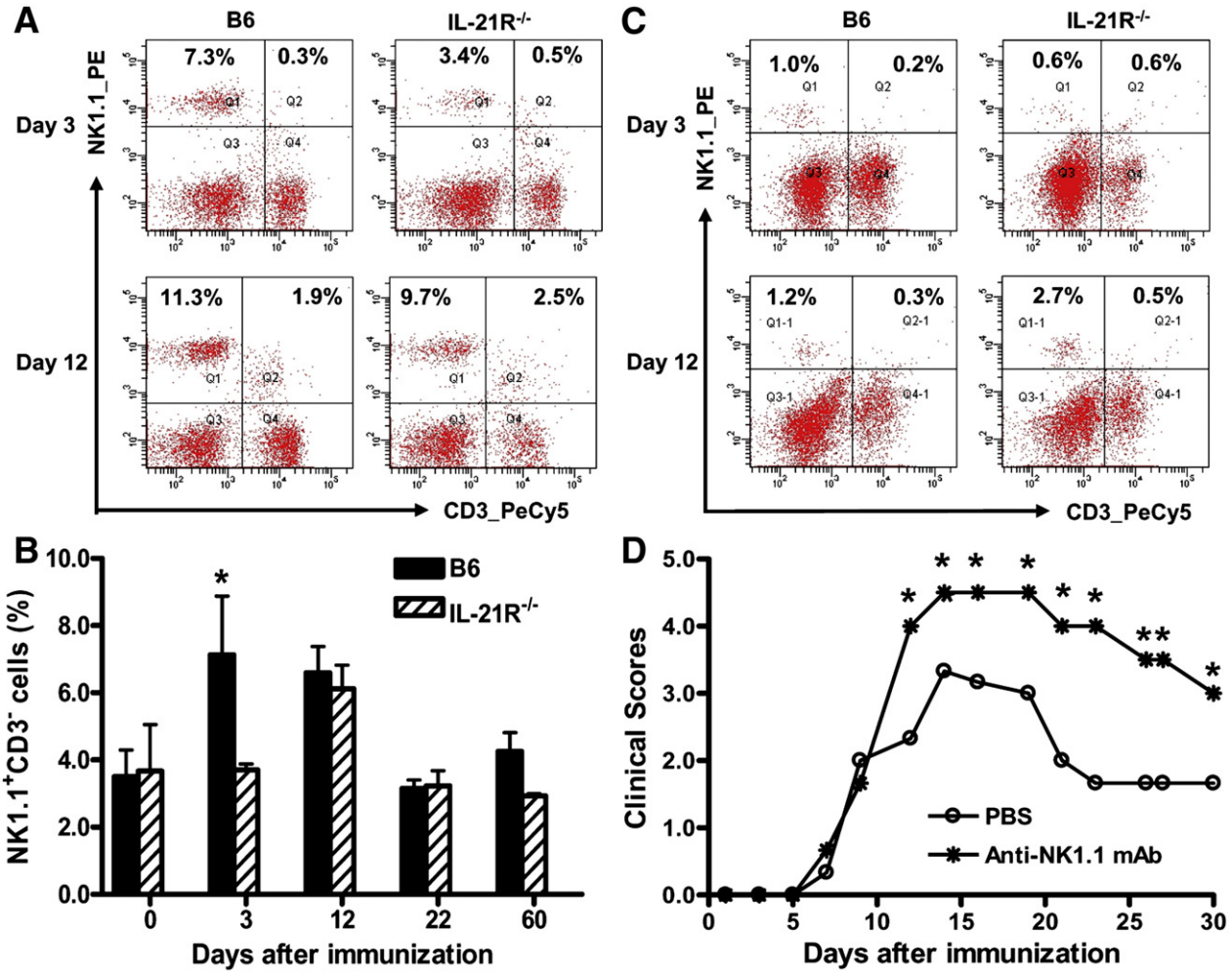

Fig. 7. IL-21R$^{-/-}$ NK cells do not expand early in EAE but have suppressive activity on disease. Splenocytes or CNS inflammatory cells from groups of MOG_{35-55}-immunized IL-21R$^{-/-}$ and B6 mice were prepared on the indicated days after immunization, and stained with anti-NK1.1 and anti-CD3 mAb. NK1.1^{+}CD3^{-} and NK1.1^{+}CD3^{+} cells were defined as NK and NKT cells, respectively. Dot plots are representative from 2 to 4 independent experiments (n=2 to 3 mice/group). Results of frequencies of individual cell population were pooled from 2 to 4 independent experiments and statistical evaluation compared between B6 and IL-21R$^{-/-}$ EAE groups. *P<0.05. (A and B) Spleen cells; (C) CNS infiltrating cells; and (D) Clinical course of EAE in IL-21R$^{-/-}$ mice (receiving mouse IgG2a) and IL-21R$^{-/-}$ mice treated with anti-NK1.1 mAb. Results are representative of 3 independent experiments (n=4 to 5 mice/group) and expressed as daily mean clinical scores. *P<0.05.

without affecting numbers of Foxp3 cells or survival of Treg cells (Peluso et al., 2007). Also, Comes et al. indicated that IL-21 partially reverts the immunosuppressive activity of CD4^{+}CD25^{+} Treg cells isolated from tumor-draining lymph nodes (Comeset al., 2006). Both reports were based on in vitro studies - one in cell culture from healthy human CD4^{+} T lymphocytes, and another in a tumor cell line. Thus, as in our experience, it appears that in vitro vs. in vivo effects may have significantly discernable modalities which are currently under investigation in our laboratory.

A reciprocal relationship between Th17 cells and Foxp3^{+} Treg cells has recently been proposed (Bettelli et al., 2006). According to that, the development of Th17 and Treg cells both require the presence of transforming growth factor-β (TGF-β), but the addition of IL-6 would preferentially skew the response towards Th17. The Abbas' group reported that the role of IL-17 and Treg cells in autoimmunity is critically dependent on the timing and nature of the disease, as regulatory T cells favored IL-17 production in an animal model of systemic autoimmunity but prevented the disease when administrated early in the course of the disease — via suppression of the expansion of T cells (Lohr et al., 2006). In our model, we did not found dramatic reduction of IL-17 production in IL-21R$^{-/-}$ EAE mice when compared with B6 mice. Thus, in our model the influence of Th17/IL-17 on Treg lymphocytes and EAE phenotype might not be substantial.

In a previous study, we showed the administration of IL-21 exacerbated EAE in B6 mice (Vollmer et al., 2005), but we missed to investigate the effects of exogenous IL-21 on Treg cells. In regard to NK cells, depletion of this immune cell subset increases severity of EAE (Zhang et al., 1997), and a failure of NK cell to home to the CNS (because of the lack of fractalkine receptor) leads to fatal EAE (Huang et al., 2006). The development of NK cells depends on the action of γ_c-dependent cytokines. IL-21 had a role in the proliferation and maturation of NK cells (Parrish-Novak et al., 2000), and promotes their cytotoxic activity and IFN-γ production (Kasaian et al., 2002). In our EAE model, we find an initial reduction of peripheral

numbers of NK cells and then an accumulation of NK cells in the CNS during the recovery phase. Since IL-21R$^{-/-}$ NK cells maintained their suppressive activity in vivo (Fig. 7D), re-distribution of NK cells to a different anatomical location could help to explain the clinical expression of EAE in the IL-21R$^{-/-}$ mice.

In conclusion, our findings reveal temporal but important impact of IL-21 signaling in the development of EAE. The dynamic changes of Treg cells, as well as an organ redistribution of NK cells may contribute to the finding of early development and severe neurological impairment associated with rapid recover from neurological deficit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2007.11.004.

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