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CUTTING EDGE

Cutting Edge: Vα14-Jα281 NKT Cells Naturally Regulate Experimental Autoimmune Encephalomyelitis in Nonobese Diabetic Mice[1]

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Although deficiencies in the NKT cell population have been observed in multiple sclerosis and mouse strains susceptible to experimental autoimmune encephalomyelitis (EAE), little is known about the function of these cells in CNS autoimmunity. In this work we report that TCR Vα14-Jα281 transgenic nonobese diabetic mice, which are enriched in CD1d-restricted NKT cells, are protected from EAE. The protection is associated with a striking inhibition of Ag-specific IFN-γ production in the spleen, implying modulation of the encephalitogenic Th1 response. This modulation is independent of IL-4 because IL-4-deficient Vα14-Jα281 mice are still protected against EAE and independent of NKT cell-driven Th1 to Th2 deviation, because no increased autoantigen-specific Th2 response was observed in immunized Vα14-Jα281 transgenic mice. Our findings indicate that enrichment and/or stimulation of CD1d-dependent NKT cells may be used as a novel strategy to treat CNS autoimmunity. ***The Journal of Immunology,*** **2002, 168: 6007–6011.**

Experimental autoimmune encephalomyelitis (EAE)[3] is a T cell-driven autoimmune demyelinating disease affecting the CNS that serves as a model for multiple sclerosis (MS). EAE arises as a consequence of the breakdown of self-tolerance, induced by the immunization of susceptible animals with myelin Ags. Self-tolerance of T lymphocytes is instilled by recessive mechanisms as well as by T cell subsets able to dominantly suppress the activation, proliferation, and differentiation of autoreactive T cells (1). These regulatory T cells have recently been suggested to include NKT cells, a T cell subset that has been implicated in the regulation of tumor rejection and infectious immunity (2). This atypical T cell subset is characterized by the expression of classical NK cell markers together with a CD1d-restricted canonical $\alpha\beta$ TCR, Vα14-Jα281/Vβ8.2, -7, and -2 in mice and Vα24-JαQ/Vβ11 in humans (2).

The association of NKT cells with autoimmunity was originally suggested by selectively reduced numbers of NKT cells in mice and patients with systemic lupus erythematosus and insulin-dependent diabetes mellitus (3–6). More detailed studies correlated the spontaneous development of autoimmune diabetes in nonobese diabetic (NOD) mice to qualitative defects in their NKT cell population (4, 7, 8). Moreover, spontaneous autoimmune diabetes could be prevented by the overexpression, in vivo activation, or adoptive transfer of NKT cells, suggesting a regulatory function of NKT cells in organ-specific autoimmunity (7, 9, 10).

Indirect evidence suggests that NKT cells may also perform a regulatory role in CNS autoimmunity. In humans, quantitative deficiencies in NKT cells have been observed in MS (11). Similarly, NKT cell dysfunctions have been observed in mouse strains susceptible to EAE (4, 12). Passive EAE can be prevented in TCR knockout mice by the adoptive transfer of DX5$^+$TCR$\alpha\beta^+$ T cells (13). Moreover, active EAE can be controlled either by the prototypical NKT cell agonist α-galactosylceramide (α-GalCer) or by administration of an α-GalCer analog that enhances IL-4 production of NKT cells (14, 15). Although these studies demonstrate the potential of NKT cells to control EAE, it is unclear whether Vα14-Jα281 NKT cells are natural regulators of EAE. Our present data strongly argue that NKT cells naturally inhibit CNS autoimmunity and prevent the accumulation of autoreactive T cells in the spleen and CNS.

Materials and Methods

Mice and induction of EAE

Vα14-Jα281 (9) and Vα8 (A. Lehuen, unpublished observation) transgenic (Tg) NOD mice were generated by microinjecting a TCR α-chain Vα14-Jα281 or a Vα8-Jα37 construct into fertilized NOD eggs. TCR C$\alpha^{-/-}$ NOD and Vα14-Jα281 Tg C$\alpha^{-/-}$ NOD were described previously (9). Vα14-Jα281 IL-4$^{-/-}$ NOD mice and Vα8 Tg C$\alpha^{-/-}$ NOD were obtained by two backcrosses onto IL-4$^{-/-}$ NOD and C$\alpha^{-/-}$ NOD, respectively (16). CD1d-deficient mice were backcrossed at least seven times onto the NOD strain (17). Mice were housed under specific pathogen-free conditions.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; α-GalCer, α-galactosylceramide; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NOD, nonobese diabetic; Tg, transgenic.

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Eight- to 12-wk-old male mice were immunized s.c. with 100 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (MEVGWYR SPFSRVVHLYRNGK; Mimotopes, Clayton Victoria, Australia) emulsified in CFA (Difco, Detroit, MI) and supplemented with 5 mg/ml *Mycobacterium tuberculosis* (strain H37RA; Difco). Pertussis toxin (List Biological Laboratories, Campbell, CA) was injected i.v. at day 0 (200 ng) and day 2 (400 ng) postimmunization. Clinical scores were recorded daily.

Histological analysis

Animals were anesthetized and perfused with 2% paraformaldehyde in PBS. Tissues were embedded in paraffin, and tissue sections were stained with H&E or Luxol fast blue.

T cell purification, cell transfer, proliferation assays, and ELISA

For negative selection of T cells (80–85% pure) by MACS, single-cell suspensions were incubated with F4/80 mAb (Caltag Laboratories, Burlingame, CA) and B220 mAb (BD PharMingen, San Diego, CA), followed by microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) coated with anti-rat IgG mAb. For cell transfer 50×10^6 purified T cells were injected i.v. 48 h preimmunization. Splenic single-cell suspensions (10^7 cells/ml) or cocultures of draining lymph node (LN)-derived purified T cells (7.5×10^5/ml) and irradiated syngeneic splenocytes (37.5×10^5/ml) were stimulated with anti-CD3 mAb (BD PharMingen), α-GalCer (a generous gift from Kirin Brewery, Gunma, Japan), or $MOG_{35–55}$. Proliferation was assessed after 48 h by incorporation of [^{3}H]thymidine (1 μCi/well). Cytokines were detected by ELISA after 24 and 48 h (9).

Statistical analysis

Proliferation and cytokine production were compared using the unpaired Student *t* test, whereas EAE day of onset, cumulative severity, and maximal severity were compared using the log rank test, Mann-Whitney *U* test, and relative to an identified distribution (RIDIT) analysis (18), respectively.

Results and Discussion

Vα14-Jα281 NKT cells regulate EAE

To address whether a regulatory function of CD1d-dependent NKT cells is naturally exploited to curb the encephalitogenic process in NOD mice, we undertook two simultaneous approaches. First, using Vα14-Jα281 Tg mice, we evaluated whether an increase in the number of NKT cells could reduce the severity of EAE. Second, we tested whether CD1d-deficient mice, which lack Vα14-Jα281 NKT cells (17), develop an exacerbated EAE.

NOD mice develop, 11 days (on average) after immunization with $MOG_{35–55}$, a relapsing-remitting EAE resulting in limb paralysis (Fig. 1*A* and Table I). In stark contrast, the EAE observed in Vα14-Jα281 Tg NOD mice is significantly delayed and milder (Fig. 1*A* and Table I). This difference in clinical severity is corroborated by the histological analysis, as a clear inflammatory infiltrate is observed in the CNS of NOD mice (Fig. 1, *C* and *E*), while only a minimal, but definite, infiltrate is noted in the Vα14-Jα281 Tg NOD mice (Fig. 1, *D* and *G*). Moreover, perivascular demyelination is observed only in the CNS of non-Tg mice, but not Vα14-Jα281 Tg mice (Fig. 1*F*). These observations were reaffirmed by experiments in which 50×10^6 purified T cells from normal NOD were transferred into T cell-deficient $C\alpha^{-/-}$ NOD, Vα8 Tg $C\alpha^{-/-}$ NOD lacking NKT cells, and Vα14-Jα281 Tg $C\alpha^{-/-}$ NOD enriched in NKT cells (Fig. 2*A*). Following MOG immunization 48 h posttransfer, the severity of EAE was indistinguishable in the two control groups ($C\alpha^{-/-}$ and Vα8 Tg $C\alpha^{-/-}$ recipients) but was significantly reduced in Vα14-Jα281 Tg $C\alpha^{-/-}$ recipients. Taken together, these data indicate that a quantitative enhancement of NKT cells conveys protection against EAE.

We did not observe any difference in the severity of EAE in $CD1d^{-/-}$ mice as compared with NOD littermates (Fig. 1*B* and Table I). This was not unexpected, as NOD mice are known to harbor a quantitatively and qualitatively defective NKT cell population. Moreover, the regulatory function of the already compromised NKT cell population might be overwhelmed by the ability of the bacterial adjuvants, used to induce EAE, to strongly activate the innate and adaptive immune systems. The spontaneous occurrence of autoimmune diabetes (no requirement for adjuvants) in NOD mice might explain why NOD $CD1d^{-/-}$ mice do develop an exacerbated diabetes (19).

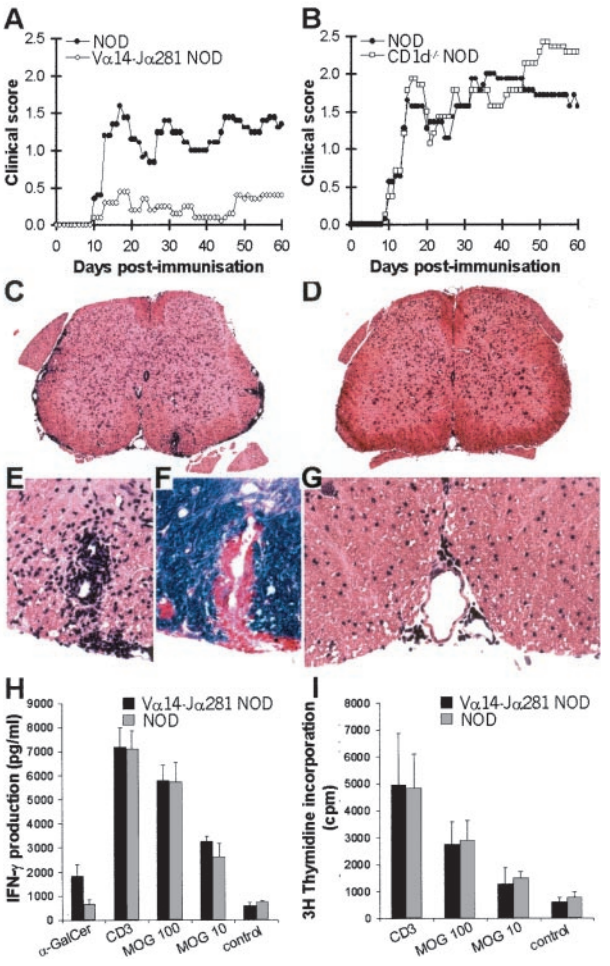

FIGURE 1. Vα14-Jα281 NKT cells control EAE. *A* and *B*, Wild-type NOD (●; *A*, $n = 10$; *B*, $n = 7$), Vα14-Jα281 Tg NOD (*A*, ◇; $n = 10$) and $CD1^{-/-}$ NOD (*B*, □; $n = 7$) male mice were immunized with 100 μg of $MOG_{35–55}$ emulsified in CFA. The clinical severity of EAE was monitored daily. *C–G*, Histological severity of EAE. The spinal cord of NOD mice (*C*, *E*, and *F*) and Vα14-Jα281 Tg NOD mice (*D* and *G*) was analyzed 20 days after immunization. Pronounced inflammation is observed in the spinal cord of NOD mice (*C* and *E*), associated with some perivascular demyelination (*F*). Vα14-Jα281 Tg NOD mice develop a low-level inflammation in the meninges (*D* and *G*). Samples were stained with 30× H&E (*C* and *D*), 150× H&E (*E* and *G*), and 150× Luxol fast blue (*F*). *H* and *I*, NKT cells do not prevent T cell priming in the draining LN. T cells were purified from the draining LN of Vα14-Jα281 Tg NOD mice (filled bars) and NOD littermates (shaded bars) 10 days after immunization with 100 μg of $MOG_{35–55}$ emulsified in CFA. IFN-γ production (*H*) and the proliferation (*I*) of T cells cocultured with irradiated APCs and α-GalCer (0.05 μg/ml), anti-CD3 mAb (0.5 μg/ml), or $MOG_{35–55}$ (100 and 10 μg/ml) were measured for each individual mouse. The data represent mean ± SEM of eight individual mice per group. Similar results were obtained in three independent experiments.

Overexpression of NKT cells does not prevent efficient priming of MOG-specific T cells in Vα14-Jα281 Tg animals

The introduction of a rearranged TCR transgene in Vα14-Jα281 Tg mice may have resulted in limited T cell repertoire diversity

Table I. *Clinical severity of EAE*[a]

Mice	*n*	Day of Onset (SEM)	Mean Maximum Severity (SEM)	Mean Cumulative Severity (SEM)	Relapses (SEM)	Mortality (%)
NOD	23	10.9 (±0.6)	2.7 (±0.2)	83.1 (±9.2)	0.7 (±0.2)	1/23 (4.3)
NOD Vα14-Jα281	22	21.9 (±3.3)[b]	1.4 (±0.3)[c]	26.1 (±6.7)[d]	0.2 (±0.2)	1/22 (4.5)
NOD	7	12.9 (±1.0)	2.6 (±0.5)	81.1 (±26.0)	0.6 (±0.2)	1/7 (14.3)
NOD CD1d$^{-/-}$	7	11.2 (±0.9)	2.9 (±0.7)	89.1 (±23.3)	0.4 (±0.3)	2/7 (28.5)

[a] Shown are the pooled data from three independent experiments comparing Vα14-Jα281 Tg vs non-Tg NOD and one experiment involving the CD1d$^{-/-}$ and CD1d$^{+/+}$ NOD mice.
[b] Value of $p < 0.0003$ (log rank test).
[c] Value of $p < 0.001$ (RIDIT analysis).
[d] Value of $p < 0.0001$ (Mann-Whitney *U* test).

due to the preferential expression of the rearranged Vα14-Jα281 transgene, resulting in reduced Ag priming. Although this has carefully been excluded by previous studies (9, 20), we confirmed that both the intensity and the quality (MOG-specific T cell proliferation and IFN-γ production, IL-4 being undetectable) of the autoreactive T cell response induced in the draining LN upon MOG immunization was comparable in Vα14-Jα281 Tg and non-Tg NOD mice (Fig. 1, *H* and *I*). These data in combination with the adoptive transfer experiments (Fig. 2*A*) indicate that the striking inhibition of actively induced EAE observed in the Vα14-Jα281 Tg mice is not due to an infringement of the MOG-specific T cell repertoire but rather due to the immune regulation mediated by the enriched NKT cell population.

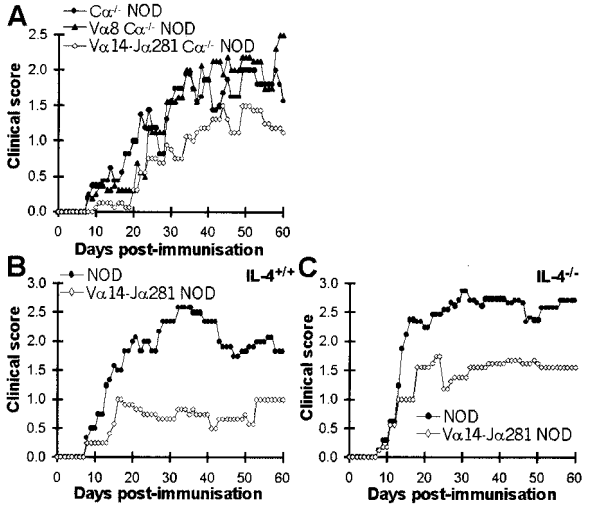

FIGURE 2. Vα14-Jα281 NKT cells control EAE induced by an identical donor-derived T cell repertoire. *A*, The severity of MOG-induced EAE was assessed in male Cα$^{-/-}$ (●), Vα8 Tg Cα$^{-/-}$ (▲), and Vα14-Jα281 Tg Cα$^{-/-}$ NOD (◇) mice following i.v. transfer of 50×10^6 purified T cells from normal NOD males. Data represent the mean of eight mice per group. The day of onset ($p = 0.01$, log rank test) and the mean cumulative score ($p = 0.02$, Mann-Whitney *U* test) were significantly delayed or reduced in Vα14-Jα281 Tg Cα$^{-/-}$ NOD recipients as compared with the pooled control groups. *B* and *C*, The regulatory function of Vα14-Jα281 NKT cells does not require IL-4. The severity of MOG_{35-55}-induced EAE was compared in IL-4-sufficient (*B*) and IL-4-deficient (*C*) Vα14-Jα281 Tg (*B* and *C*, ◇) and non-Tg NOD littermates (*B* and *C*, ●). Pooled data of two independent experiments including 8–12 mice per group are presented. The day of onset (log rank test), cumulative severity (Mann-Whitney *U* test), and maximum severity of EAE (RIDIT analysis) were all significantly delayed or decreased in IL-4$^{-/-}$ Vα14-Jα281 Tg as compared with IL-4$^{-/-}$ NOD mice ($p < 0.05$, one-tailed analysis).

IL-4 is not required for the regulatory function of Vα14-Jα281 NKT cells in EAE

To address the mechanism by which NKT cells control EAE we focused on the ability of NKT cells to explosively release IL-4 and IFN-γ upon TCR ligation. By analogy to conventional T cells, the cytokine profile of NKT cells can be polarized, with those NKT cells polarized to produce IL-4 being suggested to control autoimmune diabetes and EAE (7, 10, 14, 15, 20, 21).

To address the role of IL-4 in our model we crossed Vα14-Jα281 Tg and non-Tg littermates with IL-4-deficient NOD mice. The disruption of IL-4 expression by itself did not significantly affect EAE severity (Fig. 2, *B* and *C*). Importantly, as shown in Fig. 2*C*, the protection mediated by the overexpression of NKT cells is preserved in mice lacking functional IL-4 expression. These data indicate that the efficacy of NKT cells to regulate EAE is not affected by the lack of IL-4. Moreover, no evidence of a MOG-specific Th2 bias was obtained when assessing the MOG-specific IL-4 release in splenocyte cultures (Fig. 3*C*) and the

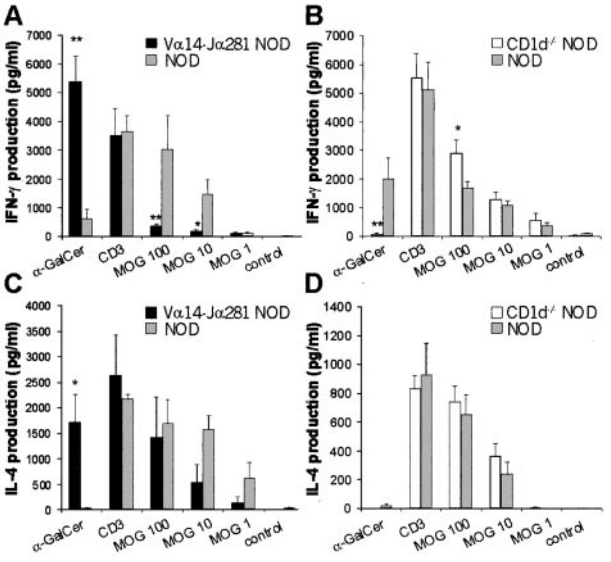

FIGURE 3. NKT cells inhibit the MOG-induced IFN-γ response in the spleen. The MOG_{35-55}-specific IFN-γ (*A* and *B*) and IL-4 (*C* and *D*) response of individual splenocyte cultures obtained from NOD Vα14-Jα281 Tg (*A* and *C*, filled bars) and NOD CD1d$^{-/-}$ mice (*B* and *D*, open bars) was compared with wild-type NOD mice (shaded bars). Individual spleen cultures were generated 10 days after immunization and stimulated in vitro for 24 (IL-4) and 48 (IFN-γ) h with α-GalCer (0.05 μg/ml), anti-CD3 mAb (0.5 μg/ml), or MOG_{35-55} (100 and 10 μg/ml). The results represent the mean ± SEM of eight mice per group. The experiment was performed three times with similar results. *, $p < 0.05$; **, $p < 0.001$ (Student *t* test).

MOG-specific serum IgG isotypes of immunized Vα14-Jα281 Tg mice (data not shown). Consequently, our data reveal that the natural regulatory function of NKT cells in EAE is independent of IL-4 and an autoantigen-specific Th2 switch; this in contrast to the control of EAE induced by exogenous NKT cell activation (14, 15).

Vα14-Jα281 NKT cells inhibit the encephalitogenic autoantigen-specific IFN-γ response

Because activated encephalitogenic T cells migrate from the LN to the spleen before their infiltration into the CNS, we contrasted the MOG-specific T cell response in the LN with that of the spleen to address whether the MOG-specific T cell population is exposed to NKT cell-mediated regulation. As compared with wild-type NOD, splenic cultures of CD1d-deficient NOD mice exhibited an unaltered IL-4 and IFN-γ response following $MOG_{35–55}$ stimulation, confirming that the impaired NKT cell population in NOD mice does not alter the generation of encephalitogenic T cells (Figs. 1*B* and 3, *B* and *D*). Strikingly, stimulation of T cells with $MOG_{35–55}$ resulted in a significantly reduced production of IFN-γ in spleen cultures from Vα14-Jα281 Tg mice, compared with those of non-Tg littermates (Fig. 3*A*). As no such reduction in the autoantigen-specific IFN-γ response was observed in the draining LN (Fig. 1*H*), these data indicate that the autoreactive T cell response is exposed to NKT cell-mediated regulation once they have migrated away from the draining LN, where the relative number of NKT cells is low (20). Indeed, splenocytes from MOG-immunized Vα14-Jα281 Tg mice, but not from non-Tg littermates, produced large amounts of IFN-γ and IL-4 in response to α-GalCer (Fig. 3, *A* and *C*), confirming the overrepresentation of functional CD1d-dependent NKT cells in the spleen.

Having excluded the induction of an autoantigen-specific Th2 bias, alternative mechanisms able to account for a reduced MOG-induced IFN-γ response in the spleen could include an altered migratory behavior of autoreactive T cells, or exposure to NKT cell-produced immunoregulatory cytokines other than IL-4. Indeed, NKT cell-produced IL-10, IL-13, and TGF-β have been implicated in the down-regulation of tumor surveillance, the prevention of autoimmunity, and the preservation of immune privilege in the eye (2, 22). In addition, as activated T cells up-regulate CD1d (23), a direct cytotoxic NKT cell response might be induced against activated autoreactive T cells, as has been reported for thymocytes (24).

It is interesting to note that the regulatory function of NKT cells can be enhanced by their ability to recruit cell populations with the capacity to control autoimmune/inflammatory reactions. Indeed, NKT cells rapidly activate components of the innate immune response, including NK cells, which in turn are able to regulate EAE (2, 25). Alternatively, NKT cells might stimulate the generation of Ag-specific regulatory T cell populations able to suppress autoimmune processes (22).

Whether the regulatory function of NKT cells is used locally in the CNS during episodes of inflammation remains to be defined. However, NKT cells are not only detectable in the CNS of MS patients (11); they might be triggered in situ as the expression of CD1d is up-regulated on murine glial cells during CNS inflammation (26).

In conclusion, this study shows that Vα14-Jα281 NKT cells naturally regulate EAE in NOD mice. Preliminary results suggest that this property also applies to other mouse strains, as EAE severity is moderately reduced in Vα14-Jα281 Tg C57BL/6 mice (our unpublished observation). Although the precise regulatory mechanisms of Vα14-Jα281 NKT cells remain to be defined, our data indicate that they include preventing the accumulation of autoreactive T cells in the spleen. This study further strengthens the concept of developing pharmacological stimuli for CD1d-dependent NKT cells as a novel strategy to treat CNS autoimmunity (15, 27, 28).

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