Induction of Regulatory Properties in Dendritic Cells by Vα14 NKT Cells

Satoshi Kojo, Ken-ichiro Seino, Michishige Harada, Hiroshi Watarai, Hiroshi Wakao, Tetsuro Uchida, Toshinori Nakayama and Masaru Taniguchi

*J Immunol* 2005; 175:3648-3655; doi: 10.4049/jimmunol.175.6.3648
http://www.jimmunol.org/content/175/6/3648

---

**References**  This article **cites 47 articles**, 26 of which you can access for free at: http://www.jimmunol.org/content/175/6/3648.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2005 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Induction of Regulatory Properties in Dendritic Cells by \( \alpha \)14 NKT Cells

Satoshi Kojo, Ken-ichiro Seino, Michishige Harada, Hiroshi Watarai, Hiroshi Wakao, Tetsuro Uchida, Toshinori Nakayama, and Masaru Taniguchi

\( \alpha \)14 NKT cells exhibit various immune regulatory properties in vivo, but their precise mechanisms remain to be solved. In this study, we demonstrate the mechanisms of generation of regulatory dendritic cells (DCs) by stimulation of \( \alpha \)14 NKT cells in vivo. After repeated injection of \( \alpha \)-galactosylceramide (\( \alpha \)-GalCer) into mice, splenic DCs acquired properties of regulatory DCs in IL-10-dependent fashion, such as nonmatured phenotypes and increased IL-10 but reduced IL-12 production. The unique cytokine profile in these DCs appears to be regulated by ERK1/2 and I\( \kappa \)B\(_{\alpha} \). These DCs also showed an ability to suppress the development of experimental allergic encephalomyelitis by generating IL-10-producing regulatory DC4 T cells in vivo. These findings contribute to explaining how \( \alpha \)14 NKT cells regulate the immune responses in vivo.

in DCs are responsible for induction of CD4 regulatory T cells suppressing immune responses. Therefore, IL-10 from Vα14 NKT cells triggers a regulatory cascade by modulating other immune regulatory cells including DCs and CD4 T cells.

Materials and Methods

Mice

Six- to 10-wk-old female C57BL/6 (B6) mice were purchased from Japan CLEA. Ig28+/- (Vα14 NKT) knockout (KO) mice were generated previously (5) and backcrossed >10 times to B6 mice. IL-10 KO mice with a B6 background were purchased from The Jackson Laboratory. All mice were bred and maintained in the animal facilities in RACI, RIKEN (Kanagawa, Japan) under specific pathogen-free conditions. Animal care was in accordance with the guidelines of RIKEN.

Reagents

α-GalCer was synthesized in the Laboratory for Immune Regulation (RIKEN) and also provided by Kirin Brewery. CpG oligodeoxynucleotide (ODN 1668) was synthesized by Hokkaido System Science. LPS and peritesser toxin were purchased from Sigma-Aldrich. Myelin oligodendrocyte glycoprotein (MOG35–55, MEVGWYRSPFSRVVHLYRNGK, was synthesized by BEX. CFA and heat-killed Mycobacterium tuberculosis H37Ra were purchased from Difco. Recombinant murine CD40L was prepared in our laboratory (RIKEN). Monoclonal Abs and fluorescent reagents used for stimulation or blocking and FACS staining were obtained from BD Pharmingen. Recombinant IL-10 and IFN-γ were purchased from PeproTech. Abs for Western blot analyses were purchased from Cell Signaling Technology. A specific inhibitor for ERK1/2, U0126, was purchased from Promega.

Preparation of α-GalCer-loaded CD1d tetramers

A mouse CD1d and Ig fusion gene was created by fusing the cDNA of the extracellular domain of mouse CD1d in-frame to the CH2-CH3 portion of mutated human IgG1 to prevent binding to FeRγ. The resulting plasmid encoding the CD1d-Ig molecule along with β2-microglobulin constructed in baculoviral vector was transfected into Sf9 insect cells by Cellfectin (Invitrogen Life Technologies) according to the manufacturer’s instructions. The baculoviral stock obtained was used for viral amplification. The soluble CD1d-Ig fusion proteins were purified from the final large scale culture supernatant of baculoviral infected Sf9 cells grown in serum-free media by chromatography on a protein A-Sepharose column (Amersham Biosciences). Loading of CD1d-Ig dimers with α-GalCer was performed at neutral pH by overnight incubation at a molar ratio of 1:4 (CD1d-Ig dimer to α-GalCer) at ambient temperature. α-GalCer-loaded CD1d-Ig dimers were tetramerized with PE-conjugated polyclonal goat anti-human IgG F(ab')2 (Beckman Coulter).

In vivo administration of α-GalCer

Mice received i.p. injections of 2 μg of α-GalCer in 200 μl of PBS. For repeated Vα14 NKT cell stimulation, mice received three injections of α-GalCer at intervals of 3–4 days.

Isolation of DCs

DCs were purified from collagenase-treated mouse spleens by magnetic cell sorting (MACS) with PE-conjugated anti-CD11c mAb and anti-PE mAb-coupled magnetic beads (Miltenyi Biotec), according to the manufacturer’s instructions. In this report, DCs from mice receiving no α-GalCer treatment (GC0) are referred to as DC<sup>G0</sup>, DCs from mice injected once (GC1) or three (GC3) times with α-GalCer are referred to as DC<sup>G1</sup> or DC<sup>G3</sup>, respectively.

Flow cytometric analysis and sorting

Splenocytes were preincubated with 2.4G2 anti-FeRγ mAb to block non-specific binding. Then, the cells were stained with PE-conjugated anti-CD11c and a mixture of biotinylated mAbs to CD19, NK1.1, TER119, and CD3 for 30 min at 4°C. Biotinylated mAbs were detected with CyChrome-conjugated streptavidin. For surface phenotyping, cells were stained with FITC-labeled mAbs. Flow cytometric analysis was performed with a FACS-Calibur flow cytometer using CellQuest software (BD Biosciences). α-GalCer-loaded CD1d tetramer-positive Vα14 NKT cells and CD8α<sup>+</sup> DCs were sorted with MoFlo (DAKO).

Cytokine production by DCs

CD11c<sup>+</sup> DCs were isolated from naïve or α-GalCer-treated mice, and suspended in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 55 μM 2-ME. The cells were cultured in 96-well flat-bottom plates at 1 × 10<sup>6</sup> cells/well for 48 h in 200 μl of medium with or without LPS (10 μg/ml), CpG ODN (1 μM), or recombinant murine CD40L (2.5 μg/ml). In some experiments, a specific inhibitor of ERK1/2 (U0126) was added to some wells of the culture. In other experiments, purified DCs (1 × 10<sup>5</sup>) were cultured with Vα14 NKT cells (1 × 10<sup>5</sup>) in the presence or absence of α-GalCer (100 ng/ml), in 24-well plates with or without 50 μg/ml anti-IFN-γ (R4-6A2) or IL-10R (1B1) mAb. Three days later, DCs were isolated by depleting TCR-β-positive cells with MACS, and stimulated with CpG ODN. Concentrations of cytokines in the supernatants were measured by ELISA using OptEIA mouse cytokine detection kits (BD Pharmingen).

Western blotting, mobility shift assay, and real-time PCR

DC samples (2 × 10<sup>5</sup> cells) were suspended in 20 μl of 1× SDS sample buffer containing DTT, boiled for 15 min, and subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and then subjected to immunoblotting with Abs for MAPK and IκB kinase (IKK)αβ. Nuclear extracts from the DCs were subjected to NF-κB EMSA according to the manufacturer’s instructions. Expression levels of IκBα, in CDNA samples taken from DCs were measured by real-time PCR (Applied Biosystems) after normalization for the expression of hypoxanthine-guanine phosphoribosyltransferase.

EAE induction and clinical evaluation

Before EAE induction, the host mice were pretreated with DCs isolated from naïve or α-GalCer-treated mice. DCs were incubated with 100 μg/ml MOG<sub>35–55</sub> peptide in complete medium for 2 h at 37°C. After intensive washing, the MOG<sub>35–55</sub> peptide-pulsed DCs (5 × 10<sup>5</sup>) were injected i.v. into B6 mice on days −7, −5, and −3 before EAE induction (day 0). Then, the pretreated mice were injected s.c. with 200 μg of MOG<sub>35–55</sub> peptide in 100 μl of PBS emulsified with 100 μl of CFA and further enriched with 5 mg/ml M. tuberculosis (H37Ra). In addition, 500 ng of pertussis toxin was injected i.p. on day 0 and day 2. Clinical symptoms were monitored during daily after immunization. The clinical score was graded as follows: 0, no disease; 1, tail limpness; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness; 5, quadriplegia; 6, death. Cumulative disease scores were calculated by adding daily disease scores from the day after immunization until the end of the experiment.

In vitro culture of T cells

To identify Ag-specific T cells in the EAE-induced mice, splenic CD4 T cells were obtained 3 wk after EAE induction, were cultured for 4 days with MOG<sub>35–55</sub> peptide or OVA-pulsed DCs in the presence or absence of anti-IL-10R mAb (50 μg/ml), and were measured for their incorporation of [3H]thymidine. CD4 T cells thus cultured for 1 wk were restimulated with plate-bound anti-CD3 mAb and then assayed for their cytokine production by intracellular staining (29).

Results

Alteration of DC phenotype and cytokine profile by in vivo α-GalCer treatment

We first assessed the alteration of surface phenotypes of splenic DCs after repeated α-GalCer treatment by FACs. As reported (22), a single injection of α-GalCer induced full maturation in splenic DCs within only 6 h, as characterized by up-regulation of costimulatory (CD40, CD80, and CD86) and MHC class II (I-A<sup>+</sup>) molecules (DC<sup>G0</sup>) (Fig. 1). These characteristic phenotypic changes lasted for at least 36 h and gradually returned to normal levels by 4 days after the stimulation (Fig. 1B). Different from the findings in the case of the single α-GalCer stimulation, the maturation of DCs was not observed when α-GalCer was injected three times with intervals between injections of 3–4 days (DC<sup>G3</sup>). Rather, expression levels of surface molecules in these DCs were significantly lower and almost similar to those of unstimulated DCs (DC<sup>G0</sup>) (Fig. 1). These surface phenotypic alterations in DCs were not detected in Vα14 NKT cell-deficient (Vα14 NKT
whether the altered cytokine production in Vα14 NKT cells directly affected the high IL-10 production in DCs, we cocultured freshly isolated DCs and Vα14 NKT cells in the presence of α-GalCer, then assessed CpG ODN-induced IL-10 production by the DCs. After coculture with in vivo stimulated Vα14 NKT cells (three times), DCs obtained ability to produce approximately twice higher level of IL-10 than those cocultured with naive Vα14 NKT cells (Fig. 2C). This up-regulation of IL-10 production by DCs was completely inhibited by addition of mAb to IL-10R but not addition of mAb to IFN-γ (Fig. 2C) nor to IL-4 (data not shown). These results indicate that in vivo repeatedly stimulated Vα14 NKT cells can directly enhance IL-10 production of DCs in an IL-10-dependent manner.

**DC subpopulations after in vivo α-GalCer treatment**

To see the change of DC subpopulations after in vivo α-GalCer treatment, we examined the expression levels of CD8α/CD11b, B220/CD11c, and CD45RB/CD11c on DCs. However, there was no significant difference among DCGCC0, DCGCC1, and DCGCC3 in the proportion of these DC subpopulations (Fig. 3A and data not shown). We further examined the cytokine profiles in CD8α+ and CD8α− DC subpopulations. When CD8α+ DCGCC0 were stimulated, they produced a higher amount of IL-12 than did CD8α− DCGCC0, whereas CD8α− DCGCC0 produced a higher amount of IL-10 than did CD8α+ DCs (Fig. 3B). The IL-12 production was enhanced in CD8α+ DCGCC1, but that of DCGCC3 was significantly decreased (Fig. 3B). In contrast, IL-10 production of CD8α− DCGCC0 was significantly enhanced, but that of DCGCC3 was hardly changed (Fig. 3B). Taken together with FACS profiles shown in Fig. 2A, the results strongly suggest that the α-GalCer injections substantially changed the ability to produce IL-12 and IL-10 in CD8α+ and CD8α− DCs, respectively, without significant change in the proportion of DC subpopulations.

**Signal molecules responsible for changes in cytokine profiles in DCGCC3**

Next, we examined the intracellular signaling pathway responsible for the characteristic cytokine production in DCGCC3. By Western blot analysis, we found that phosphorylation of ERK1/2, which is known to be important for IL-10 production (32), but not of p38 was more rapidly and strongly induced in DCGCC3 upon CpG ODN stimulation as compared with induction in DCGCC0 and DCGCC1 (Fig. 4A). Consistently, IL-10 production from DCGCC3 was significantly suppressed by a specific inhibitor of ERK1/2 in a dose dependent fashion (Fig. 4B, left), indicating that a signal pathway through ERK1/2 is responsible for the elevated level of IL-10 production in DCGCC3.

We then analyzed the NF-κB signaling pathway, which is known to be important for IL-12 production (33), and found that it was also changed in DCGCC3. The EMSA demonstrated that the nuclear DNA binding of NF-κB after TLR stimulation was significantly inhibited in DCGCC3 (Fig. 4C, top). However, phosphorylation levels of IKKαβ were not significantly altered (Fig. 4C, bottom), suggesting that molecular components different from those in the authentic NF-κB signaling cascade are involved in DCGCC3. As shown in Fig. 4D, the expression of IκBNS, which directly binds NF-κB and inhibits its activity (34), was dramatically augmented in DCGCC3 compared with other DCs. These results strongly suggest that down-modulation of IL-12 production in DCGCC3 is due to the enhancement of IκBNS.

**Prevention of EAE development by DCGCC3**

We used EAE to investigate whether DCGCC3 have a capacity to prevent in vivo inflammatory immune responses. Syngeneic MOG35–55-pulsed DCGCC0, DCGCC1, or DCGCC3 were i.v. injected...
into naive B6 mice, and then EAE was induced (Fig. 5A). The onset and mean disease scores were not significantly different between mice pretreated with MOG35–55-pulsed DCGC0 or DCGC1 (Fig. 5B). In striking contrast, mice pretreated with MOG35–55-pulsed DCGC3 showed delayed onset and suppressed EAE development (Fig. 5B). These results clearly indicate that DCGC3 have the regulatory capacity to prevent EAE development. When IL-10 KO mice were used as the source of DCGC3, the suppression of EAE was abrogated (Fig. 5B), indicating that production of IL-10 from the transferred DCs is critical for the amelioration of EAE.

To investigate the involvement of cell types other than DCGC3 in the suppression of EAE development, we isolated CD4 T cells from EAE hosts, stimulated them in vitro with MOG35–55-pulsed DCs, and analyzed their cytokine production by FACS. The intracellular staining shown in Fig. 5C indicates that CD4 T cells from EAE-suppressed mice pretreated with DCGC3 generated more IL-10 and less IFN-γ producers compared with those from EAE-nonsuppressed mice. These results indicate that pretreatment with MOG-pulsed DCGC3 induces MOG-reactive IL-10-producing CD4 T cells. The proliferative response against MOG 35–55, but not OVA, of CD4 T cells from EAE-suppressed mice was significantly lower than that of those from EAE-nonsuppressed mice (Fig. 5D).

However, the reduced proliferative response was completely abrogated by the addition of an anti-IL-10R mAb (Fig. 5D). Therefore, IL-10 from CD4 T cells seems to act as a key molecule suppressing pathogenic CD4 T cells. Consistently, the transfer of MOG35–55-pulsed DCGC3 into IL-10 KO hosts had no suppressive effect (Fig. 5E), indicating a requirement for IL-10-producing host cells as effector cells for EAE suppression.

Stability of the regulatory properties induced in DCGC3

Finally, we examined the stability of the observed alterations in phenotypes and cytokine profiles in DCGC3. Mice were left 10–30 days after repeated α-GalCer injections, and rechallenged with α-GalCer in vivo before measuring their phenotypes and cytokine profiles. After 10 days, the expression level of CD86 on DCGC3 was almost the same as that of DCGC0 and, at 30 days, gradually increased, but was still found to be significantly lower than that of DCGC1 (Fig. 6A). When DCGC3 after a 30-day interval were stimulated with CpG ODN, they still showed very low IL-12 but high IL-10 production (Fig. 6B). Therefore, changes in surface phenotypes and cytokine profiles in DCGC3 are not transient but appear to persist for at least 1 mo.
Discussion

In the present study, we analyzed the cellular and molecular mechanisms of the induction of regulatory DCs by Vα14 NKT cell activation in vivo. Repeated stimulation of Vα14 NKT cells with α-GalCer led to a change in their cytokine profile, producing IL-10 but not IFN-γ despite their potential ability to produce large amounts of both IL-10 and IFN-γ under naive physiological conditions (Fig. 2B). Interestingly, these Vα14 NKT cells induced regulatory properties in DCs characterized by immature phenotypes of cell surface molecules (Fig. 1), high IL-10 and low IL-12 production (Fig. 2A), as well as an ability to suppress in vivo immune responses (Fig. 5). We believe that these findings contribute to explaining precise cellular and molecular mechanisms for the regulatory immune responses mediated by Vα14 NKT cells.

Although it has been shown that Vα14 NKT cells did not lose their IFN-γ production even after in vivo α-GalCer treatment (35), we detected significant loss of IFN-γ in purified Vα14 NKT cells isolated from mice that received three α-GalCer injections (Fig. 2B). We also found that in vivo-stimulated Vα14 NKT cells retained their ability to produce IL-10, which is also responsible for the protection of EAE has been reported (14, 15), IL-4 might play a role in the NKT cell-mediated immune regulatory cascade. However, we do not address this issue in the present experiments.

Several fractions of DC with regulatory properties expressing particular cell surface markers have been reported, such as CD8α− or CD8α+ DCs, B220+ plasmacytoid DCs, and CD45RBhigh IL-10-producing DCs (27, 36–41). Thus, we examined the cell surface markers and whether any particular DC subsets predominately expanded after repeated α-GalCer injections. However, in vivo α-GalCer treatment did not significantly change the proportion of subsets of DCs (Fig. 3A), arguing that the changes in cytokine profile of CD11c+ DCs simply mirrored the changes in their ability of cytokine production. In fact, the IL-12 production of CD8α− DC was decreased, whereas IL-10 production of CD8α− DC was intensified by in vivo α-GalCer treatments (Fig. 3B). This is partially in line with the finding by Naumov et al. (27) that CD8α− DCs were accumulated in the pancreatic lymph nodes of 2D-3652 REGULATORY INTERACTION BETWEEN NKT CELLS AND DCs

FIGURE 3. Proportion and cytokine production of DC subpopulations after α-GalCer treatment. A. Expression levels of CD8α/CD11b on DCs. Numbers shown represent the percentage of the subpopulations. No significant differences were detected. B. IL-10 and IL-12 production in CD8α+ and CD8α− DCs. Overnight after last α-GalCer injection, CD8α+ and CD8α− DCs were separated by sorting and stimulated with CpG ODN, and cytokine production was examined as in Fig. 2. Data are expressed as mean ± SD of triplicate cultures. Purity of DCs in this experiment was ≥98%. Representative data from more than two independent assays are shown.

FIGURE 4. Enhanced ERK1/2 phosphorylation and augmented IκBα production in DCs. A. Expression and phosphorylation of MAPK in DCs. DCs were stimulated with 1 μM CpG ODN, harvested at the indicated time points, and assayed for their phosphorylation and expression of ERK1/2 and p38 by Western blot analyses. B. Effects of a MAPK inhibitor, U0126, on the production of IL-10 and IL-12 in DCs. DCs stimulated with CpG ODN were measured for their IL-10 and IL-12 production by ELISA. C. EMSA for nuclear DNA binding of NF-κB (top), and phosphorylation and expression of IκBα in DCs (bottom). D. Quantification of IκBα mRNA levels by real-time PCR (left). PCR at 28 cycles with titrated cDNA templates were resolved in 2.5% agarose gel (right). Purity of DCs in this experiment was 93–95%. All data represent at least two independent experiments.
NOD mice after α-GalCer treatment. Therefore, although they have shown the down-regulation of IL-12 after α-GalCer stimulation but not the up-regulation of IL-10 production by the pancreatic DCs, it is possible that the in vivo α-GalCer treatment rendered the DCs to produce IL-10 and suppress the type 1 diabetes.

Concerning the changes in cytokine profiles in DCs after α-GalCer stimulation, it is possible that after maturation of DCs with a single injection of α-GalCer, most of mature DCs undergo apoptosis, and thus newly derived immature DCs are affected by the pre-existing activated Vα14 NKT cells producing high IL-10 and low IFN-γ. In fact DCs, which receive maturation stimuli, are expected to apoptosis as reported (42), although Vα14 NKT cells are rather resistant to apoptosis upon TCR-mediated stimulation by up-regulating antiapoptotic genes (43–45). Therefore, activated Vα14 NKT cells with IL-10-shifted cytokine production survive for a long time and may affect the function of DCs to be tolerogenic. In fact, the data shown in Fig. 2C support this notion that IL-10 from Vα14 NKT cells could change DCs to become IL-10-producing tolerogenic cells. The regulatory DCs (DCGC3) induced by Vα14 NKT cells have different patterns of cytokine production and phosphorylation of signal molecules from those of naive DCs, thus it is unlikely that DCGC3 are merely newly derived, naive DCs even if they show similar cell surface phenotypes. However, it is still possible that survivors from the apoptosis of mature DCs become DCGC3.

The molecular mechanisms important for the acquisition of regulatory function in DCs appear to involve two different signal cascades. Phosphorylated ERK1/2 in Vα14 NKT cell-induced regulatory DCs is up-regulated, indicating that the high IL-10 production is tightly regulated by a MAPK-dependent signal (Fig. 4). Furthermore, the nuclear DNA-binding activity of NF-κB molecules important for IL-12 production is significantly reduced, whereas the expression of IκBNS mRNA is greatly augmented (Fig. 4). Because IκBNS binds directly to NF-κB, resulting in the inhibition of the translocation of NF-κB into the nucleus, and suppresses NF-κB activity (34), the down-modulation of IL-12 and enhanced IL-10 production are likely to be due in part to the augmented expression of IκBNS.

As to the effector mechanisms in Vα14 NKT cell-mediated regulation, the Vα14 NKT cell-induced regulatory DCs prevented autoimmune disease development only by the generation of IL-10-producing CD4 regulatory T cells (Fig. 5, C–E). IL-10 derived from the regulatory DCs is essential for the generation of effector type CD4 regulatory T cells because the regulatory DCs from IL-10-deficient mice failed to suppress EAE development (Fig. 5B).
IL-10 derived from CD4 regulatory T cells seems to be a final effector molecule because an anti-IL-10R mAb abrogated the inhibitory activity mediated by CD4 regulatory T cells (Fig. 5D), and also because the regulatory DCs failed to generate functional CD4 regulatory T cells when they were transferred into IL-10 KO mice (Fig. 5E). Thus, IL-10-producing CD4 regulatory T cells generated by the regulatory DCs are T regulatory type 1-like T cells (46, 47).

In the Vα14 NKT cell-mediated regulatory cascade, cell interactions seem to occur in three steps: IL-10 produced by Vα14 NKT cells also because the regulatory DCs failed to generate functional CD4 regulatory T cells. Therefore, IL-10 from Ag-specific CD4 regulatory T cells take place. Therefore, IL-10 from Ag-specific CD4 regulatory T cells suppresses only specific T cell responses, although IL-10 itself mediates nonspecific suppression. These findings suggest a novel regulatory mechanism mediated by Vα14 NKT cells through the control of DC function in vivo, and may shed new light of the regulation of immune responses.

Disclosures

The authors have no financial conflict of interest.

References


