Activated Invariant NKT Cells Control Central Nervous System Autoimmunity in a Mechanism That Involves Myeloid-Derived Suppressor Cells

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Activated Invariant NKT Cells Control Central Nervous System Autoimmunity in a Mechanism That Involves Myeloid-Derived Suppressor Cells

Vrajesh V. Parekh,* Lan Wu,* Danyvid Olivares-Villagómez,* Keith T. Wilson,*†‡ and Luc Van Kaer*†

Invariant NKT (iNKT) cells are a subset of T lymphocytes that recognize glycolipid Ags presented by the MHC class I–related protein CD1d. Activation of iNKT cells with glycolipid Ags, such as the marine sponge–derived reagent α-galactosylceramide (α-GalCer), results in the rapid production of a variety of cytokines and activation of many other immune cell types. These immunomodulatory properties of iNKT cells have been exploited for the development of immunotherapies against a variety of autoimmune and inflammatory diseases, but mechanisms by which activated iNKT cells confer disease protection have remained incompletely understood. In this study, we demonstrate that glycolipid-activated iNKT cells cooperate with myeloid-derived suppressor cells (MDSCs) in protecting mice against the development of experimental autoimmune encephalomyelitis (EAE) in mice, an animal model for multiple sclerosis. We show that α-GalCer induced the expansion and immunosuppressive activities of MDSCs in the spleen of mice induced for development of EAE. Disease protection in these animals also correlated with recruitment of MDSCs to the CNS. Depletion of MDSCs abrogated the protective effects of α-GalCer against EAE and, conversely, adoptive transfer of MDSCs from α-GalCer-treated mice ameliorated passive EAE induced in recipient animals. The cytokines GM-CSF, IL-4, and IFN-γ, produced by activated iNKT cells, and inducible NO synthase, arginase-1, and IL-10 produced by MDSCs, contributed to these effects. Our findings have revealed cooperative immunosuppressive interactions between iNKT cells and MDSCs that might be exploited for the development of improved immunotherapies for multiple sclerosis and other autoimmune and inflammatory diseases. The Journal of Immunology, 2013, 190: 000–000.

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Abbreviations used in this article: aEAE, active EAE; Arg1, arginase-1; B6, C57BL/6; BEC, S(2-boronoethyl)-L-cysteine; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; α-GalCer, α-galactosylceramide; G-MDSC, granulocytic MDSC; iNKT, invariant NKT; iNOS, inducible NO synthase; LN, lymph node; MDSC, myeloid-derived suppressor cell; MOG, myelin oligodendrocyte glycoprotein; M-MDSC, monocytic MDSC; MOGp, MOG peptide; pEAE, passive EAE; (5, 6). During this activation process, iNKT cells also interact with other cells of the immune system, such as NK cells, dendritic cells (DCs), B cells, and conventional T cells, resulting in their activation (7, 8). Activation of iNKT cells can also influence the differentiation of Th cells, typically skewing the response toward TH2 cytokine production (9). Owing to their capacity to produce a mixture of cytokines and to interact with a variety of other cell types of the immune system, iNKT cells can either promote or suppress immune responses in different diseases (10). They confer natural immunity against cancer, provide protective immunity to various infectious agents, generally play a suppressive role during autoimmune responses and graft-versus-host disease, and contribute to the development of allergic airway disease, contact hypersensitivity, hepatitis, ischemia-reperfusion injury, atherosclerosis, and obesity-associated disease (9, 11, 12). Because iNKT cells display such a wide variety of versatile functions, they are often referred to as the Swiss Army knife of the immune system (6).

Multiple sclerosis is a chronic inflammatory disease that causes demyelination of the neurons in the CNS, resulting in muscular weakness, loss of coordination, and speech and visual disturbances, ultimately leading to paralysis. Experimental autoimmune encephalomyelitis (EAE) in mice is an experimental model that is used frequently to study multiple sclerosis. Our laboratory has previously shown that iNKT cell activation by the prototypical agonist, α-galactosylceramide (α-GalCer), prevents the development of EAE (13, 14). Similar results have been obtained by other groups (15–17); however, the mechanism of this protection remains poorly defined (7, 9). The available evidence suggests that the cytokines IL-4 and IL-10, which are secreted by iNKT cells and are critical for the protective effects of α-GalCer against EAE (13), influence Th cell differentiation, leading to an overall deviation toward TH2 cytokine production and suppression of patho-
genic T cell responses. Surprisingly, recent studies have also provided evidence of a critical role of IFN-γ in these activities of α-GalCer (18, 19), suggesting that Th2 cell deviation is not the dominant mechanism of protection involved. These paradoxical findings led us to search for additional cellular targets for the cytokines released by iNKT cells that might have a critical role in protection against EAE. We focused on myeloid-derived suppressor cells (MDSCs), a regulatory cell population that exhibits strong T cell suppressive functions in vitro and in vivo (20–24).

MDSCs are a major population of cells that regulate immune responses during inflammation. These cells accumulate in various organs such as the spleen and liver, bone marrow and peripheral blood in response to inflammatory conditions such as cancer, autoimmunity, sepsis, trauma, and chronic infections (20–24). They have a strong capacity to suppress T cell responses in vitro and in vivo, a property that has been exploited to prevent the generation of autoimmunity. MDSCs are a heterogeneous population of cells that consist of myeloid progenitor cells and immature myeloid cells. In normal mice, these cells compose 1–2% of the spleen and 20–30% of the bone marrow (21, 22). Under steady state conditions, these cells lack immune-suppressive properties and quickly differentiate into various myeloid cells such as macrophages, DCs, and granulocytes. Only under pathologic conditions do these cells expand vigorously, retain their immature phenotype, and acquire immunosuppressive functions (21, 22, 25). MDSCs in mice are mainly defined as cells that coexpress CD11b and Gr1 markers. The immunosuppressive functions of these cells have been associated with high expression of enzymes involved in arginine metabolism, including inducible NO synthase (iNOS or NOS2) and arginase-1 (Arg1) (23, 26).

In this study, we demonstrate that α-GalCer–mediated protection against EAE involves the immunosuppressive functions of MDSCs. Our findings provide evidence for cooperative interactions between iNKT cells and MDSCs that could be exploited for the development of immunotherapies for multiple sclerosis and other autoimmune diseases.

Materials and Methods

Mice

B6.C57BL/6 (B6), B6.129P2-Nos2tm1Lau (iNOS–/–) mice were purchased from The Jackson Laboratory, and B6.129P2–H2–d1i (IL-10–/–) mice were obtained from Dr. Fang Yan (Vanderbilt University School of Medicine, Nashville, TN), who originally obtained these animals from The Jackson Laboratory, and B6.129P2–H2–d1i (IL-10–/–) mice were obtained from Dr. Fang Yan (Vanderbilt University School of Medicine, Nashville, TN), who originally obtained these animals from The Jackson Laboratory. B6.CD1d−/− mice were generated in our laboratory (27). All breeders and experimental mice were housed in specific pathogen-free conditions in compliance with guidelines from the Institutional Animal Care and Use Committee at Vanderbilt University.

Reagents

α-GalCer was purchased from Funakoshi Co. Mouse CD1d monomers were obtained from the National Institutes of Health Tetramer Facility (Emory University, Atlanta, GA) and were used to prepare α-GalCer/CD1d-tetramers. Fluorescently labeled Abs against TCR-β, B220, CD11b, iNOS, CD11c, F4/80, MHC class II, CD80, CD4, Ly6G, Ly6C, Gr1, CD68, CD25, CD45.1, CD45.2, NK1.1, IFN-γ, IL-4, TNF-α, IL-17A, IL-1d, and BrdU were obtained from BD Biosciences or eBioscience, and single-cell suspensions were prepared. As indicated in some experiments, splenocytes depleted of CD11b+ cells were used. The cells were stimulated with 50 μg/ml MOGp at 1 × 10^7 cells/ml in 10 ml RPMI 1640 medium containing 10% FBS (complete medium), in a 25cm2 flask. After 72 h, dead cells were removed by Histopaque-1077 (Sigma), and the live cells were washed and resuspended in PBS for adoptive transfer. Recipient mice (5–6 wk old) were irradiated sublethally at 400 rad to generate a lymphopenic environment 6 h prior to injection of MOGp-specific T cells. Live cells from the restimulation cultures were injected i.v. at 5 × 10^6–10^7 cells per mouse. The clinical scores were graded similarly to aEAE.

Induction of passive EAE

Passive EAE (pEAE) was induced in B6 mice as described previously (28). Active EAE (aEAE) was induced in B6 mice as described above. At day 11, draining lymph nodes (LNs) were harvested, and single-cell suspensions were prepared. As indicated in some experiments, splenocytes depleted of CD11b+ cells were used. The cells were stimulated with 50 μg/ml MOGp at 1 × 10^7 cells/ml in 10 ml RPMI 1640 medium containing 10% FBS (complete medium), in a 25cm2 flask. After 72 h, dead cells were removed by Histopaque-1077 (Sigma), and the live cells were washed and resuspended in PBS for adoptive transfer. Recipient mice (5–6 wk old) were irradiated sublethally at 400 rad to generate a lymphopenic environment 6 h prior to injection of MOGp-specific T cells. Live cells from the restimulation cultures were injected i.v. at 5 × 10^6–10^7 cells per mouse. The clinical scores were graded similarly to aEAE.

Adoptive transfer of MDSCs to modulate EAE

MDSCs generated during aEAE were used to prevent pEAE in an adoptive transfer experiment as described before in a different model (29). Mice induced with α-GAE were treated with vehicle or α-GalCer and sacrificed 11 d later. MDSCs were enriched from the spleen on the basis of CD11b and Gr1 marker expression using magnetic sorting. Sequential purification of Gr1+ and CD11b+ cells was performed by positive sorting using an Anti-PE MultiSort Kit (Miltenyi Biotec) according to the manufacturer’s protocol. MDSCs thus obtained were pulsed with MOGp at 100 μg/ml for 1 h before adoptive transfer. pEAE was then induced as described above by transfer of MOGp-specific T cells in irradiated mice and peptide-pulsed MDSCs (5 × 10^6) were transferred on days 1, 4, and 9 after transfer of MOGp-specific T cells.

Gemcitabine-mediated depletion of MDSCs during EAE

MDSCs were depleted (or functionally inactivated) from mice as described above in a cancer model (30, 31). B6 mice were induced with αEAE as above, and gemcitabine was administered on days 6 and 9 at 20 mg/kg body weight. Such treatment routinely depleted >80% of MDSCs in the spleen (Fig. 4A).

Generation of bone marrow chimeras

B6 (CD45.2) mice were lethally irradiated (1000 cGy) and 6 h later wild-type (CD45.1) and CD1d−/− (CD45.2) bone marrow cells (10^7) cells were mixed at a 1:1 ratio were injected into these animals. The mice were housed in nude housing conditions for 6–8 wk before use in experiments. Transfer of control wild-type (CD45.1) bone marrow cells alone was used to confirm >96% chimerism in the recipient mice (data not shown).

Enrichment of MDSC subsets and evaluation of nuclear morphology

Female B6 mice were induced with aEAE as described above and at day 11, CD11b+ cells were positively sorted by magnetic sorting (Miltenyi Biotec). The cells were stained with anti-Ly6G and -Ly6C Abs, and Ly6G<sup>−</sup>Ly6C<sup>−</sup> granulocytic (G)-MDSCs and Ly6G<sup>−</sup>Ly6C<sup>−</sup> monocytic (M)-MDSCs were sorted by preparative grade flow cytometry using a FACSaria III cell sorter (BD Biosciences) after doublets were excluded from the analyses. More than 96% of pure cells were routinely obtained. The G-MDSCs (<10^7) cells were subjected to cytospin for analysis of nuclear morphology. The cytospun cells were air dried and stained with HEMAIR staining kit (Fisher Diagnostics) according to the manufacturer’s protocol.

T cell suppression assay

We used an in vitro assay in which MOGp-specific, IL-17A–producing T cells were used to determine the immunosuppressive activity of MDSCs. aEAE was induced in B6 mice, and 11 d later splenic CD4<sup>+</sup> T cells were purified with anti-CD4 microbeads, and DCs were purified with anti-CD11c microbeads using positive sorting (Miltenyi Biotec). CD4<sup>+</sup> T cells (2 × 10^6) were activated with 2 × 10^6 DCs in the presence or absence of 50 μg/ml MOGp for 3 d in complete medium in a U-bottom, 96-well plate. MDSCs purified from mice treated with vehicle or α-GalCer during induction of EAE were added to these cultures at varying ratios to prevent the activation of MOGp-specific T cells. IL-17A production was measured in the culture supernant by ELISA as a readout of Th17 cell
activation. In some experiments, neutralizing anti-cytokine Abs or their respective isotype controls were used to restore the activity of MOGp-specific T cells. A chemical inhibitor of arginase activity, BEC, was used in cultures of MDSCs and Th cells in HL-1 medium containing 1% FBS (BioWhittaker).

Flow cytometry

Single-cell suspensions of the spleen and LNs were prepared and stained with fluorescently labeled mAbs as described previously (11). In all experiments, dead cells were excluded from the analysis by electronic gating. The iNKT cell population was identified as B220− tetramer+ TCR− β cells. For intracellular staining, cells were stimulated with 1 μg/ml ionomycin and 50 ng/ml PMA in the presence of GolgiPlug (BD Phar- mingen) for 5 h. Intracellular cytokine staining was performed with Cytofix/Cytoperm reagents (BD Pharmingen) according to the manufacturer’s protocol. MDSCs were identified based on simultaneous expression of CD11b and Gr1 markers. CD11b+Ly6G− cells were considered M-MDSCs (32). Data acquisition was performed on a FACSCalibur instrument (BD Bio- sciences), and data were analyzed using FlowJo software (Tree Star).

ELISA

A standard sandwich ELISA was performed to measure mouse IL-17A, IFN-γ, and IL-4, using OptEIA kit (BD Biosciences). For detection, streptavidin-horseradish peroxidase conjugate (Zymed Laboratories) was used, and the color was developed with the substrate 3,3′,5,5′-tetramethylbenzidine (Dako) in the presence of H2O2.

ELISPOT assay

ELISPOT assays were performed to determine the frequency of MOGp-specific Th17 cells in the spleen and LN. EAE was induced in B6 mice, and these animals were treated with vehicle or α-GalCer. Mice were sacrificed at time points indicated in the figure legends, and CD4+ T cells and CD11c+ DCs were purified. ELISPOT plates were coated with IL-17A cytokine capture Abs (eBioscience) under sterile conditions. CD4+ T cells (5 × 103) were added to 3 replicate wells coated with cytokine capture Abs for 24 h. The plates were washed and developed according to the manufacturer’s protocol (eBio- science). The spots were counted with an immunospot image analyzer (Cellular Technology).

Isolation of CNS infiltrates

Spinal cords were dissected from the vertebral column by perfusion of PBS, cut into small pieces, and digested with collagenase D (Roche) at 0.2 mg/ml with 0.05% DNase I (Sigma) for 45 min at 37°C. The tissue was me- chanically disrupted with the help of a 21-gauge needle and spun down. The cells were resuspended in 40% Percoll (GE Healthcare) and underlaid with 70% Percoll. After centrifugation at 700 × g for 15 min, the cells at the interface were collected, washed, and stained with Abs against CD4 or surface markers of MDSCs.

In vivo BrdU labeling

BrdU (1 mg/dose) was injected into B6 mice twice a day for 2 d starting from day 7 after EAE induction. Five hours after the last BrdU injection, the mice were sacrificed and splenic cells were stained with markers of MDSCs as described above. The cells were fixed with 2% paraformaldehyde for 2 h at room temperature and permeabilized with Cytoperm reagents (BD Pharmingen). The cells were treated with DNase I solution in PBS (2.5 kU/ml) for 30 min at 37°C and washed again with Cytoperm buffer. The cells were then stained with isotype control or anti-BrdU Abs for 45 min at 4°C. The cells were analyzed using flow cytometry as described above.

Quantitative real-time PCR

RNA was purified using Trizol reagent (Invitrogen) according to the manufacturer’s instructions, including a DNAse I digestion step. cDNA was prepared from 1–2 μg RNA using ThermoScript Reverse Transcriptase system (Invitrogen). Real-time PCR was performed using SYBR Green Master Mix (Bio-Rad) and 20 ng cDNA per well in duplicates for each sample on a CFX96 real-time PCR machine (Bio-Rad). The Ct values were collected for the housekeeping gene β-actin and the genes of interest during the log phase of the cycle. Gene of interest levels were normalized to β-actin and compared with the values obtained for the test sample. Each gene was compared with every normalizer in succession, and the ΔCt was calculated (ΔCt = Ct Gene of interest − Ct Normalizer). The normalized expression (ΔΔCt) of the gene of interest was calculated using the CFX manager software (Bio-Rad). The following PCR primers were used: IL-12 P35: forward, 5′-AAATGAGGTCTGACCTTCG-3′ and reverse, 5′-TCACCCTGTGATGTCACCG-3′; IL-23 P19, forward, 5′-TAT- CAGGTGGAGAATGGTTG-3′ and reverse, 5′-CATTAAAGGCTCAG- TCAGATTG-3′; iNOS: forward, 5′-CTGCTCGTCTGCTGCGAGA-3′ and reverse, 5′-TGGCAACAGAGGAAGCAGC-3′; IL-6: forward, 5′-GA- GGATTACCTCCTCAACAAGACC-3′ and reverse, 5′-AAAGTCATCATG- TTGTTCAAC-3′; TGFB: forward, 5′-TGACGCTACCTGAGTTTGAGAC- G-3′ and reverse, 5′-GGCTGTCATTGCTGCTG-3′; IL-10, forward, 5′-ATGCTGCTGCTTCTTACTGACTG-3′ and reverse, 5′-CCAAATAGC- CCTAATACTCGC-3′; Arg1: forward, 5′-ACAGTCTGGGATTTGGAAGGATC-3′ and reverse, 5′-GGAGTTCCCTGGAAATCC-3′; β-actin: forward- 5′-TACAGCTTCACACAAGGAC-3′ and reverse, 5′-AAGGAGAAGCCTGAAAGACG-3′.

Statistical analyses

Statistical significance between the groups was determined by application of an unpaired two-tailed Student t test using GraphPad Prism software; p < 0.05 was considered significant.

Results

Pathogenic IL-17A–producing T cells in MOGp-challenged and α-GalCer–treated mice are actively suppressed

It is now generally accepted that Th17 cells specific for the immunogenic Ag are required for the progression of EAE disease in B6 mice (33–37). Consequently, mice deficient in IL-23, a cytokine that is required for expansion of Th17 cells, are resistant to the development of EAE (38). In sharp contrast, IL-12 and IFN-γ, which are critical for the development and effector functions of Th17 cells, might in some situations exhibit a protective role during the development of EAE, as mice deficient in either of these cytokines exhibit exacerbated and fulminant EAE (34). We therefore measured production of the cytokines IL-17A and IFN-γ by MOGp-stimulated splenocytes or LN cells from vehicle- or α-GalCer–treated mice at day 11 after EAE induction. Compared with vehicle-treated mice, splenocytes and to a lesser extent LN cells from α-GalCer–treated mice were induced to produce profoundly reduced levels of IL-17A (Fig. 1A). IFN-γ production in these cultures was less affected by α-GalCer treatment (Fig. 1A), which is consistent with the nonpathogenic role of IFN-γ–producing T cells in this EAE model. However, we considered that the pathogenic activities of IL-17A–producing MOGp-specific T cells might be ac- tively suppressed. Therefore, we measured the frequency of IL- 17A–producing CD4+ T cells among purified CD4+ T cells stimulated with MOGp-pulsed DCs by ELISPOT assay. We found that such cells were induced in the spleen and LN of vehicle- and α-GalCer–treated mice at comparable levels (Fig. 1B).

To further evaluate the pathogenic potential of MOGp-specific T cells in α-GalCer–treated mice, we tested the capacity of CD4+ T cells from these animals to induce disease in a pEAE model. For this purpose, splenic cells from MOGp-challenged mice treated with vehicle or α-GalCer were depleted of CD11b+ cells and restimulated with MOGp in vitro for 3 d. The T cells obtained from these cultures were then used to induce pEAE in sublethally irradiated B6 mice. Results showed that T cells obtained from vehicle- and α-GalCer–treated mice were equally competent in causing EAE disease in recipient mice, suggesting that Th17 cells with pathogenic potential are induced in the spleen of α-GalCer–treated mice but that these cells are rendered inca- pable of causing EAE, possibly by accessory cells that keep the pathogenic T cells in check.

α-GalCer promotes expansion of MDSCs early after EAE induction

We previously observed splenic hyperplasia in α-GalCer– but not vehicle-treated animals between days 6 and 12 after EAE induc-
tion (Fig. 2A). Because these effects of α-GalCer might provide a clue to the underlying mechanism of protection, we analyzed the cells that contributed to this transient splenomegaly. We found that cells coexpressing the CD11b and Gr1 markers, a surface phenotype characteristic of MDSCs (22, 39), contributed significantly to the splenic cellularity (Fig. 2B, 2C). At day 6 after EAE induction, the percentages of MDSCs in vehicle- and α-GalCer–treated mice were comparable, but absolute numbers of these cells were slightly increased in α-GalCer–treated mice as compared with vehicle-treated mice (Fig. 2B, 2C). At day 11 after EAE induction, at the peak of the pathogenic T cell response, both the CD11b + cells and were stimulated with MOGp for 72 h in vitro. Live cells (5 × 10⁶) were adoptively transferred into irradiated B6 mice to induce pEAE. EAE clinical scores were determined as described in Materials and Methods.

**Infiltration of CD4+ T cells and MDSCs into the CNS**

Next, we assessed the infiltration of cells into the CNS at day 21 when vehicle-treated, MOGp-challenged mice had fulminant EAE disease with hind limb paralysis (clinical score ~4), but α-GalCer–treated mice had few signs of EAE (clinical score ~1). We found that the percentage of CD4+ T cells was 10-fold lower in α-GalCer–treated mice compared with vehicle-treated mice (Fig. 3A, 3B). CD4+ T cells that infiltrated the spinal cord of α-GalCer–treated mice produced lower levels of IL-17A or IFN-γ than did those in vehicle-treated mice (Fig. 3C). Interestingly, CD11b+Gr1+ MDSCs were found to infiltrate the spinal cords of α-GalCer–treated mice, which is consistent with the higher scattered profile of cells from α-GalCer–treated mice (Fig. 3A). We also found that mature CD11b+Ly6C+Ly6G−/− granulocytes were reduced in the spinal cord of α-GalCer–treated mice compared with vehicle-treated mice (Fig. 3B). These results suggest that MDSCs induced after iNKT cell activation infiltrate the CNS.

**Effects of MDSC depletion or adoptive transfer**

Next, we performed experiments to test whether selective depletion of MDSCs modulates protection against EAE conferred by iNKT cell activation. Selective depletion or functional inactivation of MDSCs can be achieved with the chemotherapeutic agent gemcitabine (30, 31, 40). To ensure that such treatment does not affect the pathogenic T cells, we treated animals at days 6 and 9 after EAE induction, at a dose (20 mg/kg body weight) that is much lower than that used for treating cancer (~100–120 mg/kg) (30, 31). We found that such treatment consistently depleted >80%...
FIGURE 2. α-GalCer–mediated expansion of MDSCs in EAE-induced mice. B6 mice were induced with EAE and treated with vehicle or α-GalCer. (A) Splenic and lymph node cellularity in vehicle- or α-GalCer–treated mice induced for development of EAE. B6 mice were induced with EAE and treated with vehicle or α-GalCer. At the indicated times the cellularity of spleen and lymph nodes was determined. The data presented are the mean ± SEM of six mice per group and representative of at least three individual experiments. (B) At the indicated times after EAE induction, splenocytes were stained with anti-CD11b and anti-Gr1 Abs, or with anti-CD11b, -Ly6G, and -Ly6C Abs, as indicated. CD11b+Ly6GhiLy6C-i cells represent G-MDSCs and CD11b+Ly6G−Ly6C+i cells represent M-MDSCs. (C) The absolute numbers of CD11b+Gr1+ MDSCs, CD11b+Ly6Ghi G-MDSCs, and CD11b+Ly6C+i M-MDSCs were determined based on splenic cellularity. (D) Absolute numbers of MDSCs in wild-type and CD1d−/− mice at day 11 after EAE induction. (E) The ratio of absolute numbers of CD11b+Ly6C+i M-MDSCs/CD11b+Ly6Ghi G-MDSCs was calculated at various time points after EAE induction. (F) BrdU was injected in mice starting from day 7 after EAE induction, twice a day for two days. The cells were stained with anti-CD11b, -Ly6G, and -Ly6C Abs followed by staining with anti-BrdU Ab or its isotype control Ab. Representative plots of three independent experiments are shown. (G) Percent of CD4+ T cells and the ratio of MDSCs/CD4+ T cells are depicted. The results are plotted as the mean ± SEM for six mice and are representative of five independent experiments. *p < 0.05.
of MDSCs in both vehicle- and α-GalCer–treated mice (Fig. 4A). Treatment with gemcitabine for such a short time period specifically depleted MDSCs without noticeable effects on other cell populations in the spleen and LN when measured 12 d after EAE induction (Table I). The results showed that depletion of MDSCs by gemcitabine in vehicle-treated mice modestly exacerbated EAE disease (Fig. 4B), which is consistent with the documented regulatory role of these cells in EAE (21, 32, 41, 42). In α-GalCer–treated mice, MDSC depletion completely abrogated disease protection conferred by iNKT cell activation (Fig. 4B).

To complement the depletion studies, we next tested the capacity of MDSCs expanded during EAE to protect against induction of pEAE in an adoptive transfer model. The results showed that adoptive transfer of MDSCs derived from α-GalCer–treated mice significantly prevented induction of pEAE, whereas no protection was observed with MDSCs derived from vehicle-treated mice (Fig. 4C).

Collectively, these results provide strong evidence that MDSCs induced in α-GalCer–treated mice exhibit immune-suppressive functions and have a critical role in protection against EAE.

MDSCs suppress pathogenic T cells

Next, we tested the capacity of MDSCs derived from vehicle- or α-GalCer–treated mice to suppress MOGp-specific, IL-17A–producing CD4+ T cells ex vivo. Purified CD4+ T cells from MOGp-immunized mice were stimulated with DCs and MOGp in the

FIGURE 3. Infiltration of MDSCs and CD4 T cells into the CNS. B6 mice were induced with EAE, treated with vehicle or α-GalCer, and sacrificed 21 d later. Cells infiltrating the spinal cord were isolated. (A and B) Cells were stained with anti-CD4 Abs, anti-CD11b, and -Gr1 Abs, or anti-CD11b anti-Ly6G and -Ly6C Abs. Representative data are shown in (A). Note the change in the scatter profile of cells isolated from α-GalCer–treated mice (top panels), which is predominantly due to infiltration of MDSCs. A summary of the percentage of cells from six individual mice from three independent experiments is shown. *p < 0.05. (C) Cells were stimulated with ionomycin (ION) plus PMA in the presence of Golgi Plug for 5 h. The cells were then surface-stained with anti-CD4 Ab followed by intracellular staining with anti–IL-17A and anti–IFN-γ Abs. One representative of three experiments is shown.
The presence of varying numbers of MDSCs isolated from MOGp-immunized mice treated with vehicle or α-GalCer. Mice were then treated at days 6 and 9 after EAE induction with PBS or gemcitabine at 20 mg/kg body weight by i.p. injection. (A) At day 11 after EAE induction, spleen cells were analyzed for the prevalence of MDSCs. Note that >80% depletion was observed. (B) EAE clinical scores were determined as described in Materials and Methods. (C) Mice induced with αEAE were treated with vehicle or α-GalCer and sacrificed 11 d after EAE induction. MDSCs were enriched from the spleen using magnetic sorting and pulsed with MOGp at 100 μg/ml for 1 h; 5 × 10⁶ cells was adoptively transferred into B6 mice on days 1, 4, and 9 following induction of pEAE with MOGp-specific T cells. EAE clinical scores were determined as described for active EAE. Combined data for two experiments with four mice in each group are shown.

Table I. Effect of gemcitabine on MDSCs and other cells of the immune system

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Vehicle + PBS</th>
<th>Vehicle + Gemcitabine</th>
<th>α-GalCer + PBS</th>
<th>α-GalCer + Gemcitabine</th>
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</thead>
<tbody>
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<td>LN CD11b⁺Gr1⁺ cells</td>
<td>1.175 ± 0.125</td>
<td>0.11 ± 0.013*</td>
<td>2.02 ± 0.076</td>
<td>0.13 ± 0.027*</td>
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<tr>
<td>Spleen CD11b⁺Gr1⁺ cells</td>
<td>22.13 ± 0.436</td>
<td>2.61 ± 0.28*</td>
<td>27.73 ± 1.325</td>
<td>1.63 ± 0.275*</td>
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<tr>
<td>LN NK cells</td>
<td>1.32 ± 0.254</td>
<td>1.19 ± 0.245</td>
<td>0.83 ± 0.083</td>
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<tr>
<td>Spleen NK cells</td>
<td>2.96 ± 0.226</td>
<td>2.88 ± 0.361</td>
<td>2.49 ± 0.226</td>
<td>2.3 ± 0.086</td>
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<td>LN B cells</td>
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<td>54.32 ± 3.29</td>
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</tr>
<tr>
<td>Spleen B cells</td>
<td>39.12 ± 0.961</td>
<td>40.27 ± 5.577</td>
<td>38.6 ± 4.12</td>
<td>33.85 ± 3.09</td>
</tr>
<tr>
<td>LN CD4⁺ T cells</td>
<td>18.95 ± 2.26</td>
<td>20.65 ± 2.364</td>
<td>19.82 ± 1.024</td>
<td>17.67 ± 0.757</td>
</tr>
<tr>
<td>Spleen CD4⁺ T cells</td>
<td>15.32 ± 2.01</td>
<td>15.8 ± 1.277</td>
<td>15.92 ± 0.370</td>
<td>13.52 ± 1.084</td>
</tr>
<tr>
<td>LN CD11b⁺Gr1⁺ cells</td>
<td>7.7 ± 0.779</td>
<td>5.66 ± 0.581</td>
<td>6.42 ± 0.742</td>
<td>6.54 ± 0.374</td>
</tr>
<tr>
<td>Spleen CD11b⁺Gr1⁺ cells</td>
<td>3.602 ± 0.103</td>
<td>3.167 ± 0.231</td>
<td>3.057 ± 0.381</td>
<td>3.45 ± 0.122</td>
</tr>
</tbody>
</table>

EAE was induced in B6 mice, and these animals were treated with vehicle or α-GalCer. Mice were also treated at days 6 and 9 after EAE induction with PBS or gemcitabine at 20 mg/kg body weight by i.p. injection. At day 12, mice were sacrificed, and the splenic and LN cells were stained with Abs against MDSCs, NK cells, B cells, CD4⁺ T cells, and neutrophils. The data presented are the mean ± SEM percentage of cells for six mice and are representative of three independent experiments.

* p < 0.05 compared with PBS treatment.
Results showed that repeated α-GalCer treatment was able to expand MDSCs with superior suppressive functions in an iNKT cell-dependent manner (Fig. 5D). These results suggest that expansion of MDSCs and acquisition of immune-suppressive functions in these cells by activated iNKT cells can occur in the absence of an inflammatory context.

MDSCs in α-GalCer–treated mice are enriched for expression of immune-suppressive markers

It has been shown that the immunosuppressive functions of MDSCs are associated with high expression of enzymes involved in arginine metabolism, including iNOS and Arg1 (23, 26). To understand the mechanism by which MDSCs from α-GalCer–treated mice exert superior immune-suppressive activities, we measured mRNA expression levels of Arg1 and iNOS in M- and G-MDSCs derived from MOGp-challenged mice treated with vehicle or α-GalCer. IL-17A production in the culture supernatants was measured by ELISA. (D) Naive B6 mice were injected i.p. with 2 μg α-GalCer at days 0, 4, and 7. At day 11, the percentage (top) and absolute numbers (bottom left) of MDSCs and the ability of these cells to inhibit MOGp-specific Th17 cells (bottom right) was determined. The results are the mean ± SEM for three mice and are representative of at least three experiments. *p < 0.05.
It has been proposed that N1-type cells have a hypersegmented and lobular nuclear morphology, whereas N2-type cells have an immature nuclear morphology. We therefore enriched G-MDSCs from vehicle- or α-GalCer–treated mice at day 11 after EAE induction and determined their nuclear morphology after cytospin followed by Giemsa staining. The results showed that the majority of G-MDSCs derived from vehicle-treated mice exhibited a hypersegmented nuclear morphology that is reminiscent of the N1-like phenotype. In contrast, the majority of G-MDSCs isolated from α-GalCer–treated mice exhibited a ring-shaped nucleus, a phenotype that is typically seen in CD11b+Gr1+ immature cells of the bone marrow during steady state conditions and is suggestive of an N2-like phenotype. These results suggested that both populations of MDSCs from α-GalCer–treated mice are enriched in cells with a suppressive M2/N2-like phenotype.

We also measured mRNA expression levels of cytokines relevant to EAE, including IL-12, IL-23, and IL-6, and the inhibitory cytokine TGF-β in both populations of MDSCs. We found that IL-12p40 expression was greatly induced in G-MDSCs from α-GalCer–treated mice, and the opposite was observed for M-MDSCs. TGF-β expression was mainly restricted to M-MDSCs, and its expression in these cells was only modestly upregulated in M-MDSCs from α-GalCer–treated mice. IL-23p19 and IL-6 mRNA were not detected in either MDSC population (data not shown).

Role of factors expressed by MDSCs and iNKT cells

High expression of iNOS and Arg1 in MDSCs is associated with the immunosuppressive functions of these cells. We therefore tested the role of these factors in the capacity of α-GalCer to protect mice against EAE. First, we tested the course of EAE in iNOS−/− mice treated with vehicle or α-GalCer. Vehicle-treated iNOS−/− mice developed fulminant EAE. α-GalCer treatment resulted in a delay in the onset of EAE in iNOS−/− mice, but severity of disease was unaffected. Because we observed high expression of Arg1 in MDSCs derived from α-GalCer–treated mice, we used the chemical inhibitor of this enzyme, BEC, in an attempt to restore the function of pathogenic T cells in an in vitro culture assay. We enriched CD4+ T cells from MOGp-immunized mice and stimulated these cells in vitro with DCs and MOGp in the presence of MDSCs derived from MOGp-challenged mice treated with vehicle or α-GalCer. The results showed significant restoration of IL-17A production by CD4+ T cells cultured with MDSCs from α-GalCer–treated mice in the presence of BEC, whereas such treatment had no effect on cultures with MDSCs from vehicle-treated mice (Fig. 7B). These results suggested that both enzymes involved in arginine metabolism, iNOS and Arg1, have an important role in the capacity of α-GalCer to protect mice against EAE.

Because we found high expression of IL-10 mRNA in M-MDSCs of α-GalCer–treated mice (Fig. 6A), we tested whether...
anti–IL-10 Abs restored the function of Th17 cells in vitro. Results showed that anti–IL-10 Abs significantly restored IL-17A production by MOGp-specific T cells in cultures with M-MDSCs derived from α-GalCer-treated mice. Anti–IL-10 Abs had no effect on IL-17A production in cultures containing M-MDSCs from vehicle-treated mice (Fig. 7C, top panel) or G-MDSCs derived from either treatment group (data not shown). Furthermore, M-MDSCs derived from IL-10−/− mice were significantly impaired in their capacity to suppress pathogenic Th17 cells in vitro (Fig. 7C, bottom panel). These results are consistent with a role of IL-10 in the immunosuppressive functions of MDSCs in α-GalCer–treated mice.

To understand further the mechanism of MDSC activation in α-GalCer–treated mice, we next assessed the role of cytokines,

![Image](http://www.jimmunol.org/Downloaded/from)
including IL-4, IFN-γ, and GM-CSF, produced by iNKT cells, in
inducing immunosuppressive functions in MDSCs. For this pur-
pose, we treated naïve B6 mice with CFA and stimulated spleno-
cytes from these animals 3 d later in vitro with α-GalCer for 24 h
in the presence of neutralizing Abs against these cytokines. MDSCs
were then enriched and tested for their capacity to suppress pathogenic Th17 cells. Results showed that anti-GM-CSF Abs
significantly reduced numbers of MDSCs in this culture system,
but had no effect on the suppressive functions of these cells (Fig.
7D). In contrast, anti-IFN-γ and anti-IL-4 Abs did not affect MDSC
numbers, but significantly impaired the suppressive activities of
these cells (Fig. 7D).

Finally, we assessed the contribution of direct contact between
iNKT cells and MDSCs for the generation of functionally sup-
pressive MDSCs in α-GalCer–treated mice. For this purpose, we
mixed bone marrow chimeras, using bone marrow from
wild-type and CD1d−/− donor mice and wild type B6 recipient
mice. CD1d−/− iNKT cells in such animals can develop by
interacting with CD1d expressed by wild type thymocytes (47)
(Fig. 7E). We then induced EAE in these animals, sacrificed them
11 d later, and compared the capacity of wild-type and CD1d−/−
MDSCs to suppress pathogenic Th17 cells in vitro. The results
showed that α-GalCer induced similar expansion of wild-type and
CD1d−/− MDSCs in these animals (Fig. 7E). Furthermore, wild-
type and CD1d−/− MDSCs enriched from α-GalCer– or vehicle-
treated chimeric mice using congenic markers were equally
effective in suppressing pathogenic Th17 cells (Fig. 7F). Collect-
ively, these results suggest that cytokines secreted by iNKT cells
have a critical role, whereas direct contact between iNKT cells
and MDSCs might not be required for the induction of suppressive
MDSCs by α-GalCer.

**Discussion**

Glycolipid-activated iNKT cells produce a wide variety of cyto-
kines and interact with a number of other cell types of the immune
system, permitting these cells to regulate immune responses in
different disease conditions (10, 12). Prior studies have suggested
a role for several subsets of the myeloid lineage, including tol-
erogenic DCs (48) and suppressive macrophages (49), in iNKT
cell-mediated protection against EAE. In this study, we provide
strong evidence for cooperation between iNKT cells with MDSCs,
a heterogeneous population of immature myeloid cells with po-
tent immunosuppressive properties, in protection against au-
toimmunity in the CNS conferred by the iNKT cell agonist α-
GalCer.

Activation of iNKT cells in mice induced for EAE resulted in
the expansion of both subpopulations of MDSCs that possess
superior capacity to suppress pathogenic T cells. Because 70–80% of
MDSCs in these animals were G-MDSCs, it is likely that this
MDSC subset has a dominant role in preventing autoimmunity in
our model. The pathogenic T cell suppressive function of MDSCs
in α-GalCer–treated mice is consistent with the expression of
MHC class II molecules by MDSCs, which permits these cells
to actively interact with Ag-specific CD4+ T cells (50). We also
found that MDSCs express CD1d (data not shown), but we found
that CD1d expression by these cells was not required for their
expansion or induction of suppressive functions in response to
α-GalCer stimulation. Nevertheless, we cannot exclude that
interactions between iNKT cells and MDSCs via other surface
molecules are required for the capacity of α-GalCer to induce the
generation of functional MDSCs.

Our findings are consistent with prior studies reporting a pro-
tective role of MDSCs in CNS autoimmunity (21, 32, 41, 42, 50,
51). Studies by Zhu et al. (32, 42) showed a predominant role for
M-MDSCs and iNOS in the capacity of MDSCs to regulate au-
toimmunity, and these investigators showed that activated M-
MDSCs can prevent EAE upon adoptive transfer. In contrast,
Ioanou et al. (41) described a role of G-MDSCs expressing
program death ligand-1 in suppressing pathogenic T cells in vitro
and in vivo (41). Our findings similarly demonstrated that both
subpopulations of MDSCs possess immunosuppressive functions
and that iNKT cell activation induces increased numbers of both
subtypes of MDSCs with superior capacity to suppress pathogenic
T cells.

M-MDSCs and G-MDSCs express different sets of anti-in-
flammatory proteins, and therefore use different mechanisms to
suppress T cell functions (21). We found high expression of Arg1
in both M- and G-MDSCs from α-GalCer–treated mice, whereas
iNOS and IL-12 were predominantly expressed by G-MDSCs,
and IL-10 and TGF-β were expressed by M-MDSCs. These results
suggested that the suppressive effects of G-MDSCs predominantly
involve iNOS, Arg1, and IL-12, whereas those of M-MDSCs mostly
involve Arg1, IL-10, and TGF-β. We further demonstrated a direct
role of iNOS expression in the capacity of α-GalCer to protect mice
against EAE, and a role for Arg1 and IL-10 in the capacity of
MDSCs from α-GalCer–treated mice to suppress pathogenic
T cells.

MDSCs are derived from hematopoietic bone marrow progenitor
cells in response to inflammatory stimuli (20–24). Consistently,
evidence suggests that iNKT cells influence and regulate hema-
topoiesis in vivo and in vitro (52, 53). It has been shown that
a single injection of α-GalCer in B6 mice results in rapid secretion
of GM-CSF and IL-3 by iNKT cells, and increased CFU fre-
quency in peripheral blood and spleen (54). These studies there-
fore suggest that GM-CSF and IL-3 secreted by iNKT cells may
directly influence hematopoiesis and promote the recruitment
of MDSCs to the spleen during EAE. Consistent with this notion, we
found that GM-CSF neutralization in vitro inhibited the capacity
of activated iNKT cells to promote MDSC survival. In addition
to GM-CSF and IL-3, additional cytokines produced by glycolipid-
activated iNKT cells could contribute to the expansion of MDSCs
and induction of immunosuppressive functions in these cells.
MDSCs that are recruited to tumor cells were shown to require
expression of IL-4Rα for their immunosuppressive function and
expansion (55). We similarly observed IL-4Rα expression on
MDSCs from vehicle- and α-GalCer–treated mice induced for
EAE (data not shown). Therefore, it is tempting to speculate that
IL-4 secreted by iNKT cells could directly be involved in acti-
vating MDSCs to expand these cells in the spleen and promote
their immunosuppressive activities. This possibility is also con-
sistent with the critical role of IL-4 in the capacity of α-GalCer to
protect mice against MOG-ρ–induced EAE (13). Similarly, IFN-γ
released by iNKT cells and by NK cells activated in response to
α-GalCer treatment was shown to be required for the protective
effects of α-GalCer against EAE (18, 19). Furthermore, the pro-
tective effects of IFN-γ in EAE have been shown to relate directly
to iNOS expression and NO production (32), a finding that was
also confirmed in our studies. Consistent with this proposed sce-
nario, in vitro Ab blockade of IL-4 or IFN-γ in our studies sug-
gested a direct role of these cytokines in the induction of
suppressive functions in MDSCs. Thus, a variety of cytokines
produced by activated iNKT cells might contribute to the recruit-
ment of MDSCs to the spleen and in inducing the immunosup-
pressive activities of these cells.

A few previous studies have reported interactions between
iNKT cells and MDSCs (56–58). De Santo et al. (56) showed that
the presence of iNKT cells suppressed the development of MDSCs
during influenza virus infection, and studies by Ko et al. (57) and


