Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: Programmed Death-1/Programmed Death Ligand 1 Interaction Regulates the Induction and Maintenance of Invariant NKT Cell Anergy[1]

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Invariant NKT (*i*NKT) cells are a distinct subset of T lymphocytes that recognize glycolipid Ags. Upon TCR stimulation, *i*NKT cells promptly secrete a wide range of cytokines and therefore have been investigated as a target for immunotherapy. However, after primary activation, *i*NKT cells become hyporesponsive toward their ligand (anergy). The further mechanism behind *i*NKT cell anergy is poorly understood. We found that a low level of programmed death-1 (PD-1) was constitutively expressed on *i*NKT cells and that PD-1 expression was increased after stimulation and lasted at least 2 mo. Moreover, not only did blocking of the PD-1/PD ligand 1 (PD-L1) pathway prevent the induction of anergy in *i*NKT cells, but anergic *i*NKT cells also recovered responsiveness and these "rescued" cells efficiently mediated antitumor immunity. Our findings suggest that the PD-1/PD-L1 interaction is essential for the induction and maintenance of *i*NKT cell anergy. ***The Journal of Immunology,*** **2008, 181: 6707–6710.**

Natural killer T (NKT)[3] cells are a distinctive population of T lymphocytes that can recognize glycolipids presented by CD1d, an MHC class I like-molecule (1). A major subset of NKT cells, called type I NKT cells or invariant NKT (*i*NKT) cells, express an invariant TCR composed of Vα14-Jα18 chains in mice (Vα24-Jα18 in humans). Upon TCR stimulation with a ligand such as α-galactosylceramide (αGC), *i*NKT cells rapidly produce a wide range of cytokines including IL-4, IFN-γ, and IL-12 (1, 2). This response enables *i*NKT cells to enhance or regulate the activity of various immune cells in innate and acquired immunity (3). These immunomodulatory roles of *i*NKT cells are found in diverse diseases, promoting tumor rejection or regulating autoimmune disorders (4–6).

Another unique feature of *i*NKT cells is that they become unresponsive after stimulation with their ligands. For instance, *i*NKT cells that have been stimulated with αGC have reduced proliferation and cytokine production upon secondary stimulation with the same ligand (7, 8). This *i*NKT cell anergy is a major obstacle in immunotherapeutic trials targeting *i*NKT cells; however, the mechanism behind the anergy is not clear. A classic concept of anergy in conventional T cells is that the cells become anergic when they receive a TCR signal with insufficient costimulatory signals. In contrast, it has recently been suggested that coinhibitory molecules may actively anergize or tolerize T cells by delivering inhibitory signals into TCR-stimulated T cells (9). Moreover, in cases of chronic viral infection, blockade of the programmed death-1 (PD-1) signal can reverse the anergic phenotype of CD8 T cells (10, 11).

PD-1 is well known as a coinhibitory molecule on T cells. In conventional T cells, it is not expressed on naive T cells but is inducibly expressed after T cell activation. The interactions of PD-1 with the PD ligands (PD-L1 and PD-L2) can transduce inhibitory or costimulatory signals into the T cells (12). It is well established that PD-1 plays a critical role in the regulation of immune tolerance and autoimmunity (10, 13, 14).

Several costimulatory molecules have been well established in the *i*NKT cells (15, 16), but their role in *i*NKT cell anergy has been elusive. The goal of our study was to delineate the mechanism of *i*NKT cell anergy. Our results show that PD-1 expressed on *i*NKT cells is up-regulated after stimulation and that blocking of the PD-1/PD-L1 pathway allows the anergic *i*NKT cells to recover their responsiveness. Moreover, anergic *i*NKT cells recovered by PD-1 blockade have potent antitumor activity. Therefore, we suggest that PD-1 plays an important role in the induction and maintenance of *i*NKT cell anergy.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from Orient Bio. All mice were bred and maintained in specific pathogen-free conditions. All studies

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Received for publication July 21, 2008. Accepted for publication September 25, 2008.

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[1] This study was supported by Korea Science and Engineering Foundation (KOSEF) National Research Laboratory Program Grant No. R0A-2008-000-20113-0 funded by the Korean government (Ministry of Education, Science, and Technology).

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[3] Abbreviations used in this paper: NKT, natural killer T; *i*NKT, invariant NKT; αGC, α-galactosylceramide; PD-1, programmed death-1; PD-L1, PD ligand 1; PD-L2, PD ligand 2.

conformed to the principles for laboratory animal research outlined by Seoul National University (Seoul, Korea).

Reagents and antibodies

αGC provided by Dr. S. Kim (Seoul National University) was dissolved in 0.5% Tween 20 in PBS as a vehicle. Hybridoma clones producing blocking mAbs against mouse PD-1 (clone RMP1-14; rat IgG2a), PD-L1 (clone MIH-5; rat IgG2a), and PD-L2 (Ty25; rat IgG2a) were generated as described previously (17–19) and prepared from the ascites of nude mice by using caprylic acid purification.

i*NKT cell anergy*

Mice were injected i.p. with 2 μg of αGC. Seven days or 1 mo later, splenocytes were isolated and cultured with 100 ng/ml αGC plus 50 μg/ml control rat IgG or each blocking mAb in vitro. The supernatants were assayed by ELISA to detect levels of IL-2 after 12 h of culture and levels of IL-4 and IFN-γ after 96 h of culture. For proliferation assays, [^{3}H]thymidine was added to the wells after 48 h of culture and cells were cultured for an additional 16 h before cell harvest and the measurement of radioactivity uptake. For in vivo models, 200 μg of the control IgG or each blocking mAb was administered into mice 1 day before αGC treatment. Two weeks later, αGC was reinjected and sera were prepared 2 and 12 h later for assay of IL-4 and IFN-γ levels, respectively. Splenocytes were prepared at 2 h for assay of intracellular cytokine staining, which was performed by BD Cytofix/Cytoperm Plus with Golgi-Plug kit (BD Biosciences).

B16F10 melanoma metastasis model

Mice given 200 μg of each blocking mAb or control rat IgG at day −1 were i.v. inoculated with 2×10^5 B16F10 tumor cells at day 0. At days 0, 4, and 8 the mice were treated with 500 ng of αGC plus 200 μg of each blocking mAb or control rat IgG. Fourteen days later, lungs were weighed. To induce the *i*NKT cell anergy in the tumor model, 1 day after mice were given 200 μg of each blocking mAb or control rat IgG, they were treated i.p. with 2 μg of αGC. Seven days after αGC treatment, 5×10^5 B16F10 tumor cells were inoculated. Then, mice were treated with 500 ng of αGC at 0, 4, and 8 days after the inoculation. Fourteen days later, lungs were isolated and metastatic nodules were counted.

Flow cytometric analysis

To analyze the *i*NKT cell population, we used αGC-loaded CD1d dimer complex as described previously (15). For analysis of PD-1, PD-L1, and PD-L2 expression, cells were stained with anti-PD-1-PE mAb (Biolegend), anti-PD-L1-PE mAb (BD Biosciences), or anti-PD-L2-PE mAb (BD Biosciences), respectively. For intracellular cytokine staining, we used anti-IFN-γ-allophycocyanin mAb and anti-IL-4-allophycocyanin mAb (Biolegend). All cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

Results are expressed as mean ± SEM. When appropriate, we used the Student's *t* test. For results that did not show normal distribution, a Wilcoxon two-sample rank-sum test (Mann-Whitney *U* test) was used; $p < 0.05$ was considered significant.

Results and Discussion

We first analyzed the expression of PD-1 and its ligands on *i*NKT cells upon αGC stimulation. After mice were injected with αGC or vehicle, splenocytes were analyzed at different time points. As depicted in Fig. 1, PD-1 was constitutively expressed on *i*NKT cells at a low level. Its expression was up-regulated and persisted for at least 2 mo after αGC stimulation. In contrast, PD-L1 expression was temporarily increased on αGC-stimulated *i*NKT cells but declined toward naive state levels within 72 h; however, PD-L2 was not expressed (Fig. 1). Although, PD-1 has been expressed in Vβ8 transgenic mice (20), our data convincingly showed constitutive expression of PD-1 on *i*NKT cells and its up-regulation upon activation by staining with αGC/CD1d:Ig dimer.

We next investigated whether PD-1 signal has any role in the activation of *i*NKT cells by using anti-PD-1, anti-PD-L1, or anti-PD-L2 blocking mAbs. After activating *i*NKT cells by αGC with each blocking mAb or control IgG in vitro and in vivo, anti-PD-1 and anti-PD-L1 mAbs significantly increased IFN-γ production

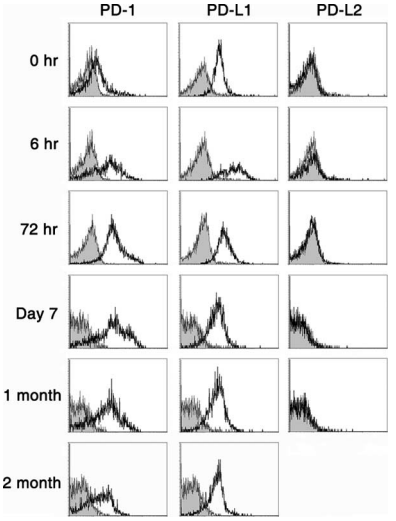

FIGURE 1. Expression of PD-1 and PD-L1 on iNKT cells. Total splenocytes were isolated from 2 μg of αGC-treated mice at the indicated time points after treatment. Cells were stained with anti-PD-1-PE, anti-PD-L1-PE, anti-PD-L2-PE, or isotype control-PE mAbs, respectively. *i*NKT cells were gated on B220$^-$TCR-β^{int} αGC/CD1d:Ig$^+$ population (where "int" is "intermediate"). PD-1, PD-L1, and PD-L2 expression (open histograms) was analyzed by flow cytometry. Shaded histograms indicate staining with isotype control mAbs.

from *i*NKT cells (supplemental figure 1).[4] Therefore, the PD-1/PD-L1 interaction delivered a coinhibitory signal during *i*NKT activation, particularly in IFN-γ-secreting features.

*i*NKT cells stimulated by αGC readily become unresponsive to αGC restimulation, and this anergic phenotype lasts at least 1 mo (8). Our observation described above prompted us to investigate the role of the PD-1 signal in induction or maintenance of the *i*NKT cell anergy. Therefore, we investigated the role of PD-1/PD-L1 interaction in *i*NKT cell anergy. To this end, mice were injected with αGC to induce *i*NKT cell anergy. Seven days later, splenocytes were restimulated with αGC plus each blocking mAb. Consistent with previous findings (8), cytokine levels of αGC-pretreated mice were significantly less than those of vehicle-pretreated mice, indicative of the anergic status of *i*NKT cells (Fig. 2*A*). Surprisingly, the levels of IFN-γ in the anti-PD-1 or anti-PD-L1 mAb-treated group, but not those of the group treated with anti-PD-L2, were remarkably higher than that of the control IgG group and reached the levels of the vehicle-pretreated group (Fig. 2*A*). The IL-2 and IL-4 levels were also higher in the anti-PD-1 mAb-treated group than in the control group and were in an intermediate range in the anti-PD-L1-treated group. In addition, cell proliferation, another characteristic of activated *i*NKT cells, was also recovered from the anergic state by treatment of the mAbs (Fig. 2*A*). The recovery of IFN-γ and IL-4 production by treatment of the mAbs was showed until 1 and 2 mo after αGC treatment (Fig. 2*B*; data not shown). These data demonstrate that blocking the PD-1/PD-L1 signal during restimulation reverses the established anergic phenotype of *i*NKT cells.

We next addressed whether the PD-1/PD-L1 interaction is required for induction of *i*NKT cell anergy in vivo. We injected mice with each blocking mAb or control IgG before injecting the mice with αGC. Two weeks later, all mice were injected with secondary

[4] The online version of this article contains supplemental material.

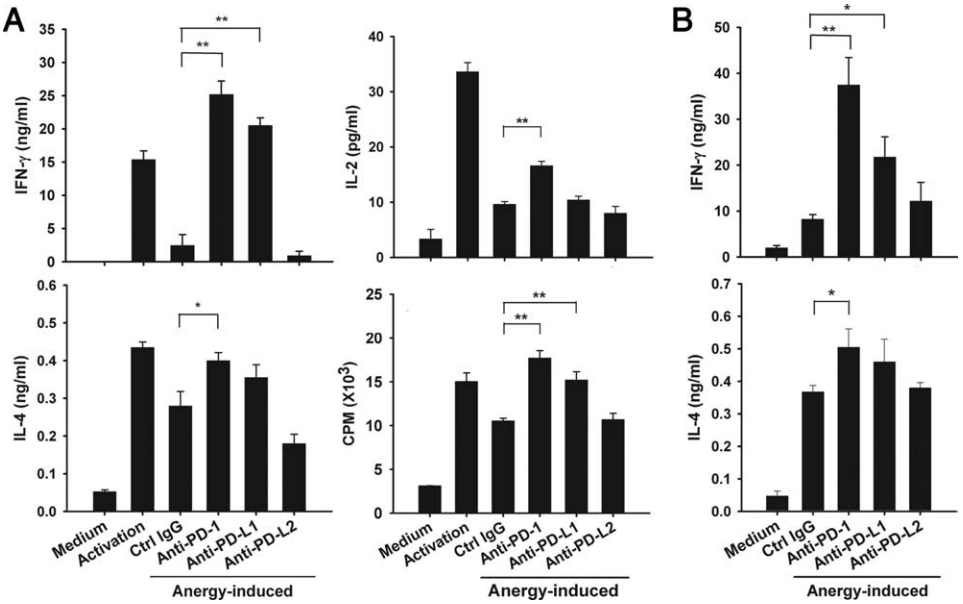

FIGURE 2. Blockade of PD-1/PD-L1 interaction restores the function of iNKT cells from anergic state. *A*, Total splenocytes were isolated 7 days after mice were treated with 2 μg of αGC followed by cultured with 100 ng/ml αGC plus 50 μg of each indicated mAb. Culture supernatants were evaluated for IL-2, IFN-γ, and IL-4 levels by ELISA 12 h and 3 days after culture. Alternatively, cell proliferation was assessed by [^{3}H]thymidine incorporation. *B*, Total splenocytes were isolated 1 mo after mice were treated with 2 μg of αGC and cultured as described in *A*. Supernatants were evaluated for IFN-γ and IL-4 levels by ELISA. To compare with results in the activated state, splenocytes from naive mice were cultured under identical αGC conditions, shown as "activation." *, $p < 0.05$; and **, $p < 0.01$ vs control IgG group.

αGC and the levels of cytokines in sera were analyzed. Mice pretreated with αGC produced significant less IFN-γ and IL-4 upon secondary αGC injection, indicative of anergic *i*NKT cells. However, mice treated with anti-PD-1 mAb during primary αGC treatment produced remarkably higher levels of IFN-γ and IL-4 than the control IgG-treated group upon secondary αGC injection (Fig. 3*A*). Intracellular cytokine staining showed that the increased cytokine production was from *i*NKT cells (Fig. 3*B*). Of note, we did not observe any improved cytokine production in the anti-PD-L1 mAb-treated group in this experimental setting. We speculated that it might be difficult for blockade of the PD-L1 molecule with mAb to abolish the induction of *i*NKT cell anergy due to broad expression and distribution of PD-L1 in vivo. Alternatively, there might be other reasons that remain unknown. We then tested for differences of CD69 expression, a typical activation marker, on *i*NKT cells and NK cells. We found that the CD69 expression on *i*NKT and NK cells upon secondary αGC injection was increased by treatment with anti-PD-1 mAbs (supplemental figure 2).

Others have reported that the blockade of PD-1 allows tolerized CD8 T cells to restore their responses (10) and that inhibition of the PD-1/PD-L1 pathway restores the function of exhausted CD8 T cells in a chronic infection model (21). In this study, we found that blocking the PD-1/PD-L1 pathway prevented the induction of *i*NKT cell anergy in vivo and that blockade of the PD-1/PD-L1 interaction resulted in the recovery of cytokine production in the anergized *i*NKT cells induced by αGC treatment. These observations indicate that PD-1/PD-L1 interaction plays an essential role in the induction as well as the maintenance of *i*NKT cell anergy.

It is well established that administration of *i*NKT ligand triggers antitumor activity against lung metastasis of B16F10 melanoma, depending on IFN-γ production by activated *i*NKT and NK cells (22, 23). To identify whether the inhibition of PD-1/PD-L1 interaction would enhance the antitumor effects of *i*NKT cells, we injected each blocking mAb 24 h before B16F10 inoculation, followed by an αGC injection with control IgG or each blocking mAb. As a result, the weight of B16F10 metastatic lungs was significantly reduced in the anti-PD-1 mAb-treated group compared with lungs from the group given control IgG (Fig. 4*A*). These results indicate that the antitumor effects of *i*NKT cells can be enhanced by blockade of the PD-1 coinhibitory pathway.

Finally, we asked whether *i*NKT cells rescued from anergy induction by blocking of the PD-1 pathway retain antitumor activity. To this aim, we injected 2 μg of αGC into mice pretreated with the respective blocking mAbs. One week later, all mice were inoculated with B16F10 tumor cells and treated with a suboptimal dose of αGC (500 ng/injection). In mice treated with control IgG, the number of tumor nodules was comparable to those seen in the vehicle-treated group, indicating that

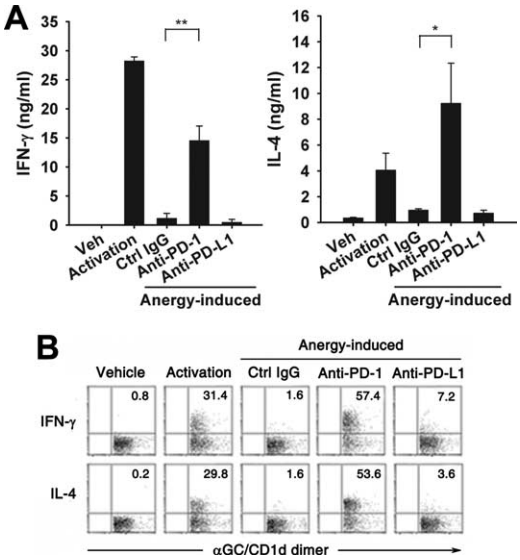

FIGURE 3. Blockade of PD-1/PD-L1 interaction prevents the induction of iNKT cell anergy. Two hundred micrograms of indicated mAb was injected i.p. into mice 1 day before the first αGC treatment followed by injection of 2 μg of αGC on days 0 and 14. *A*, Sera obtained at 12 h for IFN-γ assay and at 2 h for IL-4 assay after a second αGC treatment. Ctrl, Control; Veh, vehicle. *B*, Splenocytes were prepared 2 h after secondary αGC treatment and cultured for 2 h with Golgi-Plug. *i*NKT cells were gated on B220$^-$TCR-β^{int} αGC/CD1d: Ig$^+$ population (where "int" is "intermediate") and IFN-γ^+ or IL-4$^+$ *i*NKT cells were analyzed by flow cytometry.

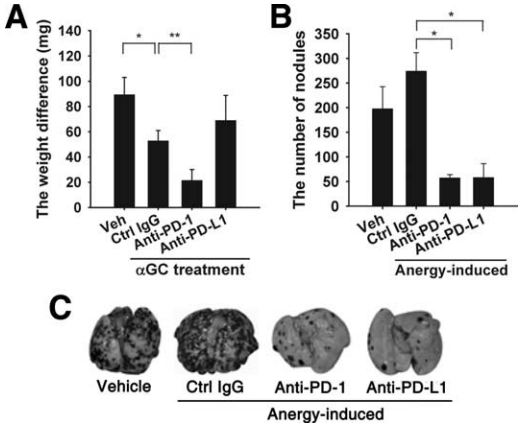

FIGURE 4. Antitumor effects of iNKT cells are enhanced or restored by blockade of PD-1/PD-L1 interaction. *A*, Mice given 200 μg of each indicated mAb at day -1 were i.v. inoculated with 2×10^5 B16F10 tumor cells at day 0. At days 0, 4, and 8 the mice were treated with 500 ng of αGC plus 200 μg of each indicated mAb. Fourteen days later, lungs were weighed and differences between metastatic and normal lungs were determined. *B* and *C*, One day after mice were given 200 μg of each indicated mAb, they were treated with 2 μg of αGC. Seven days later, they were inoculated with 5×10^5 B16F10 tumor cells. At days 0, 4 and 8 after inoculation, mice were treated with 500 ng of αGC. Fourteen days later, lungs were isolated and metastatic nodules were counted.

αGC treatment during tumor inoculation did not suppress tumor growth. However, the numbers of tumor nodules in the anti-PD-1- and PD-L1-treated groups were significantly reduced compared with those in the control IgG-treated group and in the vehicle-treated group (Fig. 4, *B* and *C*). These results indicate that blocking the PD-1/PD-L1 interaction during the induction phase of *i*NKT cell anergy can restore the antitumor activity of this T cell subset.

Several studies have shown improved antitumor activity of *i*NKT cells with the use of various tools or materials (24, 25). In our study, αGC-mediated antitumor effect was significantly increased by administration of anti-PD-1 mAb at the time of αGC treatment. Furthermore, administration of anti-PD-1 or anti-PD-L1 mAb prevented anergy induction of *i*NKT cells, which resulted in maintenance of the intrinsic antitumor effect of *i*NKT cells. Anti-PD-L1 mAb treatment triggered a strong antitumor response in this model, although we did not observe a restored cytokine production in a similar experimental setting. The reason for this discrepancy is not clear, but could be due to the expression of PD-L1 on tumor cells or to different restimulation strategies in the two in vivo models. Particularly, it is well established that various tumor cells can express PD-L1, enabling them to evade the antitumor immune responses (26, 27). Therefore, we speculate that the blockade of anti-PD-L1 mAb may enhance the antitumor immunity of various effector cells.

In conclusion, our study provides a direct basis for the activation of *i*NKT cells in response to repeated ligand stimulation without loss of their immunostimulatory activity and may be of use for improving current immunotherapeutic trials of *i*NKT cells.

Disclosures

The authors have no financial conflict of interest.

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