exhibit little response to the endogenous ligand/CD1d *i*NKT cells expressed by DCs, the presence of excessive amount of IL-12 would remarkably augment the *i*NKT cell response to endogenous ligand, which leads to production of a large amount of IFNγ from *i*NKT cells. Thus, *i*NKT cells may act as crucial amplifiers of Th1 cells in the initial inflammatory response to the pathogens.

Of note, not only Th1 but Th2 cytokine response could also be amplified through a similar mechanism. We have recently revealed that in the presence of excessive IL-2, TCR recognition of putative endogenous ligand would trigger production of IL-5 and IL-13 from human $CD4^+$ *i*NKT cells (Sakuishi et al. 2007). These findings indicate that under physiological conditions, cytokine milieu would be decisive in directing *i*NKT cell responses towards Th1 or Th2, and are relevant for understanding the mechanism of how *i*NKT cells would regulate the adaptive immune response in vivo (Fig. 3).

Since α-anomeric glycolipids do not exist in mammalian tissues, a number of β-anomeric glycolipids have been evaluated for their possible role as an endogenous ligand for *i*NKT cells. The search has led to the identification of lysosomal glycolipid isoglobotrihexosylceramide (iGb3) as a putative endogenous ligand (Zhou et al. 2004; Mattner et al. 2005). However, it has recently been demonstrated that *i*NKT cells are normal in number and function in iGb3 synthetase deficient mice, despite of lacking endogenous iGb3 (Porubsky et al. 2007). Moreover, a highly sensitive HPLC assay has failed to detect the presence of iGb3 in various mouse tissues except for the dorsal root ganglion. Nor was iGb3 detected in any human tissue (Speak et al. 2007). Therefore, the search for endogenous ligand is still not over. Regarding the pathogenesis of MS, it is of key interest whether any myelin-derived lipid antigen may stimulate *i*NKT cells.

Another subject of growing interest is to use *i*NKT cell ligands as therapeutic agents for autoimmune diseases. The prototypical ligand α-GC showed some efficacy for autoimmune diseases (Hong et al. 2001). However, as it provokes production of a wide range of cytokines including proinflammatory ones, it may worsen some disease conditions. To overcome this problem, structurally altered analogs of α-GC were synthesized and their ability to inhibit the development of autoimmune disease has been examined. A work from our laboratory has demonstrated that an α-GC analog bearing a shorter sphingosine chain compared with α-GC (named as OCH) would selectively stimulate IL-4 production from *i*NKT cells, whereas α-GC stimulation induces both IL-4 and IFNγ (Miyamoto et al. 2001; Oki et al. 2004). Accordingly, OCH stimulation of *i*NKT cells favors a Th2 bias of immune response in vivo as compared with α-GC stimulation and showed better efficacy for treatment of various autoimmune disease models (Fig. 3) (see Sect. 3.3 as well).

3.2 Studies of iNKT Cells in MS

Using single-strand conformation polymorphism (SSCP), a method for examining the TCR repertoire, we have previously analyzed blood samples from subjects with MS as well as other neurological diseases (Illes et al. 2000). Expression of the

invariant Vα24-Jα18 rearrangement, the invariant TCR α-chain expressed by human *i*NKT cells, was greatly reduced in the blood lymphocytes of the patients with MS, compared with those from healthy subjects. The reduction was not observed in the patients with other autoimmune/inflammatory neurological diseases. Interestingly, the Vα24-Jα18 TCR was only rarely found in the CNS lesions of MS but was often detected in the biopsy samples from chronic inflammatory demyelinating polyneuropathy (CIDP).

More recently, we have reanalyzed the frequency of *i*NKT cells in the peripheral blood of MS by using flow cytometry. A striking reduction of the total number of *i*NKT cells was confirmed in the peripheral blood of the patients with MS in a drug-free remission state (Araki et al. 2003). Interestingly, when $CD4^+$ and DN *i*NKT cells were analyzed separately, a remarkable *i*NKT cell reduction was found to reflect a great reduction of DN *i*NKT cells, that are known to preferentially produce proinflammatory cytokines (Gumperz et al. 2002; Lee et al. 2002). Moreover, we found that the $CD4^+$ *i*NKT cell lines from MS patients were significantly biased for Th2: they produced much more IL-4 than those from healthy subjects, although the production of IFN-γ was not altered significantly (Araki et al. 2003). Collectively, the changes found in *i*NKT cells (a reduction of DN and Th2 bias of $CD4^+$ *i*NKT cells) are thought to be beneficial for maintaining the remission state of MS.

It is also worthwhile to mention that the currently available drugs may exert their actions through targeting *i*NKT cells. Although the drug-free remission state of MS was associated with a great reduction of *i*NKT cells in the peripheral blood (Araki et al. 2003), patients who were continuously given a low dose oral corticosteroid showed a normal frequency of *i*NKT cells in the blood, indicating that oral corticosteroid treatment may restore the frequency of *i*NKT cells (Araki et al. 2004). Interestingly, the cytokine profile of DN NKT cells from the corticosteroid-treated MS showed a trend for Th2 bias. This may represent one of the mechanisms of the corticosteroid effects in MS and other autoimmune diseases.

In a recent longitudinal study, IFN-β treatment significantly increased the number of *i*NKT cells in the peripheral blood mononuclear cell within same patients (Gigli et al. 2007). Furthermore, *i*NKT cells of IFN-β treated individuals showed a dramatically improved secretion of INF-γ, IL-4, and IL-5 in response to α-GC stimulation compared with those isolated from the same individuals before IFN-β treatment. The study also showed up-regulation of key costimulatory molecules expressed by DCs in the IFN-β treated patients. Thus, immune regulatory effect of IFN-β therapy in MS may possibly mediate *i*NKT cells.

3.3 iNKT Cells as a Therapeutic Target in MS/EAE

Results of EAE studies give us clues to understanding the role of *i*NKT cells in the pathogenesis of MS. It is well known that SJL/J mice are very susceptible to induction of EAE and other autoimmune diseases. In this strain of mice, *i*NKT cells are reduced in number and defective in IL-4 production (Yoshimoto et al. 1995),

allowing us to speculate that the *i*NKT cell defects may account for the autoimmune susceptible nature. On the contrary, transgenic overexpression of the invariant TCR of *i*NKT cells was found to protect NOD strain of mice from development of EAE. This EAE protection was associated with an inhibition of antigen-specific IFN-γ production but was independent of IL-4 (Mars et al. 2002). These results indicate an inverse correlation of *i*NKT cell numbers/functions with the susceptibility to EAE, raising a simple idea that expanding *i*NKT cells may be beneficial for treating patients with MS.

After α-GC was identified as a potent ligand for *i*NKT cells, several laboratories have examined whether in vivo injection of α-GC may modify the clinical course of EAE by stimulating *i*NKT cells. A study by Singh et al. showed that α-GC is capable of down-modulating EAE, by inducing Th2 bias of *i*NKT cells (Singh et al. 2001). Furlan et al. also showed an efficacy of α-GC in EAE, but they did not reveal a Th2 bias but rather showed an enhanced IFNγ production by the liver *i*NKT cells (Furlan et al. 2003). In an independent study by Jahng et al., injection of α-GC with aim to suppress EAE resulted in diverse outcome, which depends on the administration route, timing of injection, and dose of this glycolipid (Jahng et al. 2001). Although the reason for these discrepancies remain unclear, it is possible that source of the mice, quality of the animal facilities, or even gut flora might have influenced the results.

It was subsequently found that CD28-B7 costimulatory signals play a critical role in stimulating *i*NKT cells with α-GC. When *i*NKT cells were stimulated with α-GC in the presence of anti-B7 (CD80) antibody *in vitro*, they selectively produced Th2 cytokines (Pal et al. 2001). *In vivo* stimulation of *i*NKT cells along with blocking CD28-B7 interactions was found to suppress the onset of EAE (Pal et al. 2001). These results collectively indicated that proper stimulation of *i*NKT cells might lead to suppression of pathogenic Th1 responses. We have then explored whether a Th2 polarizing ligand could be identified among α-GC analogs. As discussed briefly in Sect. 3.1.2, we have found that an analog of α-GC, called OCH, bearing a shorter sphingosine chain could selectively induce production of IL-4 but not of IFN-γ and could modulate disease process of EAE when injected *in vivo* (Miyamoto et al. 2001). This protective effect against the development of EAE was abrogated by a simultaneous injection of anti-IL-4 antibody. Moreover, the protective effect of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from *i*NKT cells is involved in the disease suppression.

The molecular mechanism for the selective IL-4 production by OCH has been intensively studied in our laboratory. Owing to the truncation of sphingosine chain, OCH binds to CD1d molecule less stably compared to α-GC. We are proposing that the unstable OCH-CD1d interaction, which does not allow continuous TCR stimulation, is a key to understanding the Th2 polarizing character of OCH (Oki et al. 2004). When *i*NKT cells are stimulated by a-GC, IL-4 is produced within a few hours, which is then followed by production of a large quantity of IFN-γ (Pal et al. 2001). Of note is that de novo protein synthesis is required for the *i*NKT cell production of IFN-γ but not of IL-4 (Oki et al. 2004). Subsequent analysis has revealed that c-Rel protein is selectively induced, when *i*NKT cells are simulated by α-GC. Inhibiting c-Rel expression in *i*NKT cells has led to a selective IL-4 induction as a result of

suppressed production of IFN-γ, as seen with OCH stimulation. Taken together, it can be postulated that unstable binding of OCH with CD1d leads to disrupted TCR signaling, which does not induce expression of c-Rel and of its down-stream molecule IFN-γ. Compared with α-GC, which is capable of fully inducing c-Rel and IFN-γ, OCH would exhibit a unique Th2 polarizing effect on *i*NKT cells *in vitro* and *in vivo*. Intriguingly, in vivo injection of OCH induces defective IFN-γ production not only by NKT cells but also by NK cells (Oki et al. 2005). Mechanistic analysis has revealed that an injection of OCH induces an insufficient induction of CD40L in addition to lower primary IFN-γ production by the NKT cells, leading to a marginal IL-12 production by DCs. A combination of these differences between OCH and α-GC stimulation would account for the lower secondary IFN-γ production by NKT and NK cells by OCH. Of note, McCarthy et al. have recently confirmed that shortening of the phytosphingosine chain increased the rate of lipid dissociation from CD1d molecule and induced less sustained TCR signals (McCarthy et al. 2007). In this study, they have also demonstrated the decreased affinity of TCR to OCH bound-CD1d.

Other lipid chain truncated analogs of α-GC have been reported to display a similar skewing of cytokine profile towards Th2 but the mechanism seems to differ from that found in OCH (Goff et al. 2004; Yu et al. 2005). Taken together, altered glycolipid provides attractive means for *i*NKT cells mediated intervention of inflammatory autoimmune disease such as EAE and human MS.

4 MR1- Restricted Invariant T Cells in MS

Another novel invariant NK cell receptor-positive T cell population besides *i*NKT cells has been described in mice and humans. They are preferentially located in the gut lamina propria and are generally termed mucosal-associated invariant T (MAIT) cells (Treiner et al. 2003). Of interest, they are absent in germ-free mice, which indicates the role of gut flora for generation and maintenance of this lymphocyte. The discovery of this population is dated back to 1993, when DN T cell population expressing an invariant TCR α-chain was described along with the identification of Vα24 *i*NKT cells (Porcelli et al. 1993). It is now established that the new invariant T cells are distinct from *i*NKT cells in the expression of another conserved CDR3α sequence (Vα7.2-Jα33 in humans and Vα19-Jα33 in mice) and restricted use of Vβ2 and Vβ13 in mice and humans. Unlike *i*NKT cells selected by CD1d, they are selected by another MHC class Ib molecule, MR1, that is also highly conserved among species (Treiner et al. 2003). The mouse MAIT cells were isolated from $NK1.1^+$ T cells in the liver of CD1d deficient mice lacking "conventional" *i*NKT cells, allowing us to call the cells "Vα19-Jα33 NKT cells." As seen with "conventional" NKT cells, human MAIT cells constitutively express memory phenotype and some NK cell markers other than CD57 (Treiner et al. 2005) (Fig. 1). Several lines of evidence suggest that MR1 presents lipid ligands such as α-mannocylceramide (Shimamura et al. 2007). Although the function of MAIT cells is unclear at the moment, their cardinal features such as the semiinvariant repertoire, restriction by

monomorphic class I-like molecule and the natural memory phenotype suggest that *i*NKT cells and MAIT cells may exhibit similar and/or complementary functions.

When expression of Vα7.2 invariant TCR for human MAIT cells was investigated in MS patient samples, there was a striking difference between the MAIT and *i*NKT cell invariant TCR in their expression. Expression of the invariant TCR chain for NKT cells was clearly reduced in the peripheral blood of MS patients (Illes et al. 2000), whereas invariant TCR for MAIT cells was clearly detected in the great majority of the patients (Illes et al. 2004). Parallel analysis of CNS lesions from MS patients showed that MAIT cells would infiltrate the majority of the lesions, whereas *i*NKT cells do not (Illes et al. 2000, 2004). The differential expression of the two invariant chains in samples from MS suggests that MAIT cells and NKT cells may complement each other and MAIT cells may substitute deficiency of *i*NKT cells in MS.

The protective role of MAIT cells is further delineated by the study of mouse EAE. We found that overexpression of the invariant Vα19-Jα33 TCR in B6 mice is protective against EAE induction and progression (Croxford et al. 2006). Consistently, EAE was exacerbated in MR1 deficient mice, which lack Vα19-Jα33 invariant T cells. The protective effect was found to accompany a reduced production of inflammatory mediators as well as an increased secretion of IL-10. We have also demonstrated that IL-10 production occurred in part through interactions between B cells and Vα19 MAIT cells involving ICOS costimulatory molecule.

5 Concluding Remarks

NK cells and *i*NKT cells are groups of innate lymphocytes with multi potential qualities. Recent advances in cell biology of these cells have brought our attention to their ability in regulating autoimmune inflammatory responses. Selective induction of their regulatory properties could be an effective means for modification of autoimmune disease affecting the CNS. It is also notable that NK cells and *i*NKT cells change their phenotypes, number, and gene expression profile during disease course of MS. They could be good targets also for those who attempt to identify useful biomarkers for MS.

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Differential Enhancement of T Helper Type 1 (Th1)/Th2 Cytokine Production by Natural Killer T Cells Through Negative Feedback Regulation with Cytokine-conditioned Dendritic Cells

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Abstract: NKT cells can modulate the immune response through the production of type 1 T helper (Th1), Th2, or even Th17 cytokines and serve as a good target for immunotherapy. Selective enhancement of either Th1- or Th2-cytokine production upon stimulation may better control immune-mediated diseases according to the respective immunopathology. By employing a co-culture of NKT cells with differently treated dendritic cells (DC) and quantifying cytokines in the culture supernatants, we have developed novel methods to enhance either IFN-γ or IL-4 production by NKT cells. When α-galactosylceramide-loaded DCs were pre-treated with IL-4 or IFN-γ and then co-cultured with NKT cells, the enhanced production of IFN-γ or IL-4 by NKT cells was respectively induced, implying that NKT cells could produce a cytokine of the opposite response to the cytokine used for pre-treatment of the DCs. Dynamics of inhibitory ligand expression on DCs appear to be involved in this phenomenon. Utilization of negative feedback regulation may expand the utility of NKT cells for therapy for tumors, infectious diseases, and autoimmunity.

Keywords: Th1/Th2 balance, immune bias, negative feedback regulation, NKT cells.

INTRODUCTION

NKT cells are a unique subset of T cells that recognize lipid antigens in the context of CD1d [1]. Ligands, such as α-galactosylceramide (α-GC), can activate NKT cells to secrete copious amounts of a variety of cytokines (IL-2, 4, 5, 6, 10, 13, 17, 21, IFN-γ, TNF-α, GM-CSF, and TGF-β) and chemokines (MIP-1α, MIP-1β, LT, Eotaxin, RANTES) and to become cytocidal, *via* the expression of perforin, granzyme B, FasL, and TRAIL, as cytotoxic T lymphocytes and NK cells [2]. In a review, Matsuda *et al.* have described the NKT cell as a 'Swiss-Army knife' [2], since NKT cells indeed affect immune and inflammatory responses by recruiting, activating or inhibiting various immunocompetent cells *via* various molecular tools. If we could use an appropriate tool to selectively induce distinct cytokine production profiles in NKT cells at an appropriate time, the versatility of these cells could be better exploited for modulating immune responses and treating immune-mediated diseases with synthetic ligands [3]. One way to induce selective cytokines is dependent on the chemical species of the ligands. OCH is an α-GC analogue with a shorter sphingosine chain (-9 carbon atoms) that preferentially induces Th2 responses and ameliorated experimental autoimmune encephalomyelitis (EAE) in mice [4]. Likewise, C20:2, an *N*-acyl variant of α-GC preferentially induces IL-4 by NKT cells and is superior to α-GC in protecting NOD mice against diabetes [5]. On the other hand, α-C-GC (an α-GC analogue with a methylene, -CH_2-, between the sugar and ceramide, instead of -O- in the original α-GC) [6] preferentially stimulated Th1-type responses and gave prolonged production of IFN-γ. C-glycoside showed improved activity in anti-malarial and anti-tumor immunity [7].

Factors that control preferential cytokine production by NKT cells also include differences in NKT cell subsets, integrated signals from the TCR and other receptors, especially inhibitory receptors, and the environment where NKT cells are stimulated [3, 8]. As for the last factor, DCs play critical roles by sensing environments and producing cytokines, such as IL-12, followed by antigen (Ag) capture and presentation [9]. As potent Ag presenting cells, DCs can activate NKT cells and NKT cells can maturate DCs, suggesting that a close interaction between NKT cell and DC occurs through interactions *via* IL-12/IL-12R, CD40/CD154, and others [10, 11].

In this review, we introduce a unique method to potentiate a biased response by NKT cells to selectively enhance either Th1- (IFN-γ) or Th2-cytokine (IL-4) production with IL-4- or IFN-γ-pre-treated DCs, respectively [12]. The regulation of cytokine production from NKT cells by pre-treated DCs appears to operate through negative feedback mechanisms [12]. Pre-treatment of DCs by other cytokines, including IL-21 [13], and the Toll-like receptor (TLR) ligand CpG [14], were also performed, and the effects were analyzed. We then discuss possible mechanisms shared

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by these pre-treatments, likely through inhibitory receptors, and potential therapeutic applications to tumor immunity as well as infectious and autoimmune diseases.

1. EXPERIMENTAL SYSTEM

In Vitro Study

To study DC-NKT cell interactions, we employed a simple co-culture system of Nylon-wool non-adherent cells (mainly splenic T cells as the NKT cell source) and spleen-derived dendritic cells (SDDC) or a DC cell line (BC1; BALB/c mice origin) [15, 16]. SDDCs ($CD11b^{+}8^{-}45R^{-}$) or BC1 cells were loaded with α-GC for 24 hr and then incubated with either cytokine or TLR ligand for another 24 hr (referred to as DC / ligand / cytokine or TLR ligand). Unstimulated SDDCs or BC1 cells were used as immature DCs (iDC). The pre-treated DCs were washed and co-cultured with NKT cells for 48 hr, and the cytokines released into the supernatant were quantified with ELISA (Fig. **1A**). For further analyses, co-culture of sorted NKT cells with DCs and intra-cellular staining of cytokines in a gated population were performed.

In Vivo Study

To test whether the DC-NKT cell interaction observed *in vitro* also functions *in vivo*, DC/α-GC/cytokine or TLR-ligand were intrasplenically (*i.s.*) injected, and sera were collected for quantification of cytokines with ELISA (Fig. **1B**). Alternatively, the cytokine was intravenously (*i.v.*) or intraperitoneally (*i.p.*) administered beforehand, and the animal was later challenged with α-GC, followed by quantification of serum cytokines. For the use of IL-4, an IL-4/anti-IL-4 monoclonal antibody (mAb) immune complex was administered since this formulation had a long-lasting half-life [17]. The enhancement of cytokine effects involves several mechanisms such as a protection of cytokine molecules from breakdown or excretion [18] (FcγR-independent), or an FcγR-dependent focusing of cytokine-containing immune complex [19].

2. SELECTIVE ENHANCEMENT OF CYTOKINE PRODUCTION BY NKT CELLS- *IN VITRO* STUDIES

i) Enhancement of IFN-γ Production by NKT Cells

a) Pre-Treatment of DCs with IL-4 Enhances IFN-γ Production by NKT Cells

First, we simply co-cultured NKT cells with ligand-loaded iDCs (iDC/α-GC/-) and found that an increasing amount of IFN-γ was produced with the increasing ratio of added DC cells (Fig. **2A**) [12]. When NKT cells were co-cultured with DC/α-GC/IFN-γ, IFN-γ production by NKT

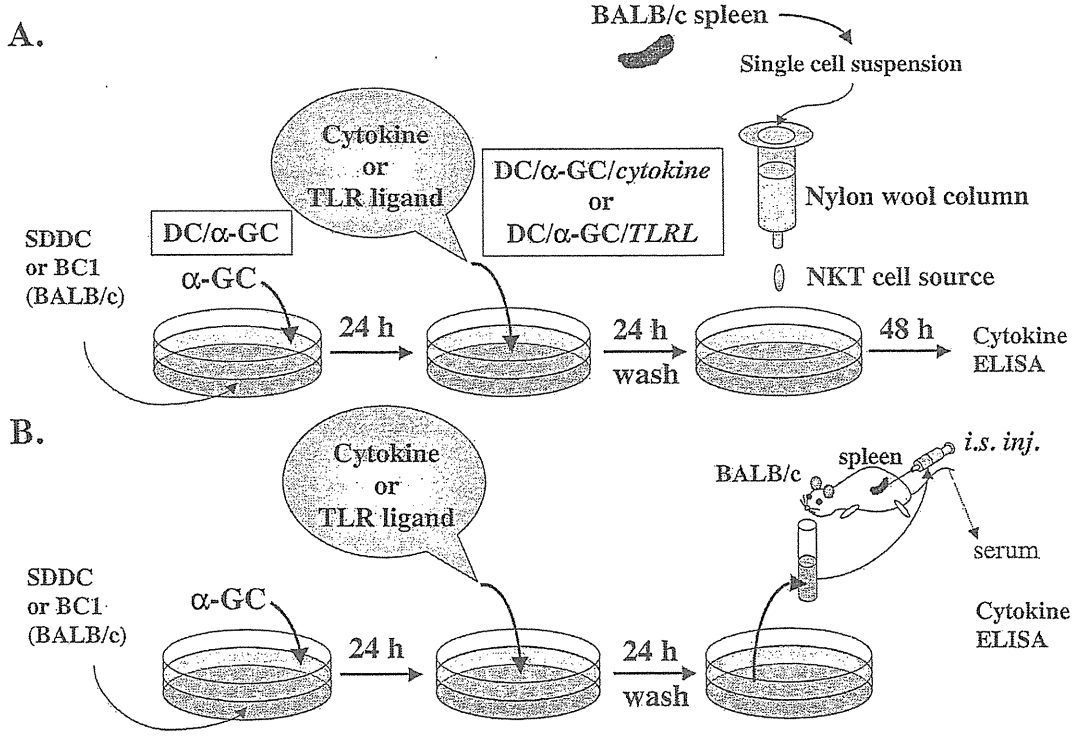

Fig. (1). Experimental system for studying DC-NKT cell interactions. (A) Co-culture of dendritic cells (DC) and natural killer T (NKT) cells. Spleen-derived dendritic cells (SDDC) and a dendritic cell line, BC1 (BALB/c), were incubated with α-galactosylceramide (α-GC) for 24 hr. Then, cytokine or Toll-like receptor (TLR) ligand were added to the culture and incubated for another 24 hr. The α-GC-loaded DCs that were pre-treated with IL-4 were referred to as DC/α-GC/IL-4 (DC (source) / vehicle (veh) or α-GC / cytokine or TLR ligand). After washing to remove α-GC, cytokine or TLR ligand, DC and T cell fractions (Nylon-wool non-adherent cells) from BALB/c mice were co-cultured for 48 hr. Cytokine concentrations in the culture supernatant were quantified with ELISA. (B) *In vivo* transfer of pre-treated DCs. Differently pre-treated DCs were prepared as depicted in (A) and collected after washing. A total of 5×10^5 cells in 50 μl were transferred into the spleen. Sera were serially collected and quantified with ELISA.

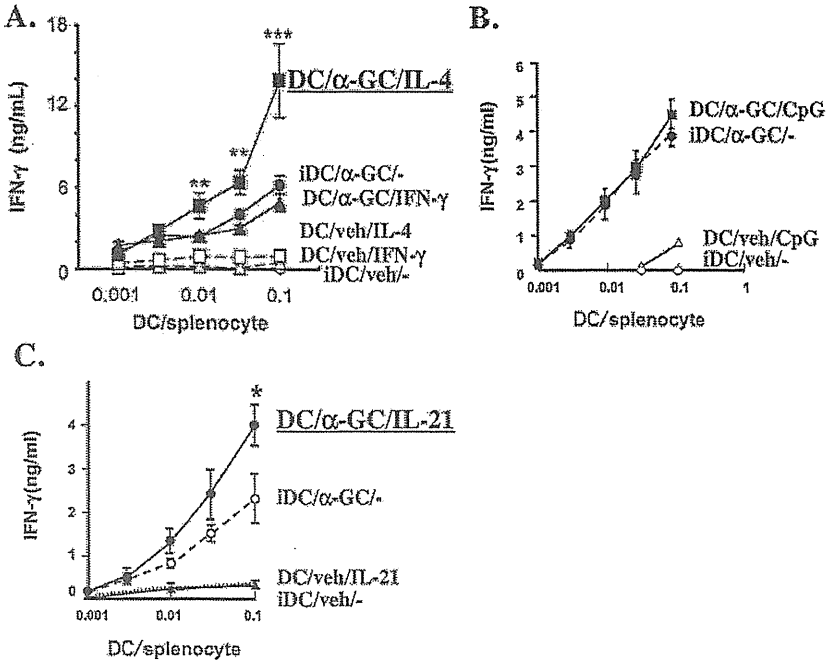

Fig. (2). IFN-γ production by splenocytes stimulated with α-GalCer-loaded, differently treated DCs *in vitro*. (A) IFN-γ production by splenocytes stimulated with various DC preparations with IL-4 or IFN-γ. DCs of each preparation are indicated as described in Fig. (1A). The T cell fraction and SDDCs, both from BALB/c mice, were cultured for 48 hr at the indicated DC/splenocyte ratios (0.001 to 0.1) in the x-axis. IFN-γ in the culture supernatant was quantified with ELISA. Each symbol represents the mean ± SE of 3 independent experiments. (B) IFN-γ production by splenocytes stimulated with various DC preparations with CpG oligodeoxynucleotide (CpG). The T cell fraction from BALB/c mice and a DC cell line, BC1, derived from BALB/c mice were co-cultured for 48 h. Each symbol represents the mean ± SE of 4 independent experiments. (C) IFN-γ production by splenocytes stimulated with various DC preparations with IL-21. The T cell fraction from BALB/c mice and BC1 were co-cultured for 48 h. Each symbol represents the mean ± SE of 4 independent experiments. BC1 cells were incubated with IL-21 for 4 d before co-culture with the splenocytes. Statistical significance was calculated by Student *t*-test (*p< .05; **p< .01; ***p< .001 *vs* iDC/α-GC/-).

cells was neither enhanced nor suppressed compared to control (iDC/α-GC/-). On the other hand, IFN-γ production by NKT cells was significantly enhanced when cultured with DC/α-GC/IL-4 compared to that of control (Fig. 2A), whereas IL-4 production was not affected by DC/α-GC/IL-4 (Fig. 3A). No enhancement of IFN-γ was observed with any DCs without α-GC (DC/veh/IL-4, DC/veh/IFN-γ, iDC/veh/-; veh – vehicle; Fig. 2A) or with DCs of any treatment from CD1d$^{-/-}$ mice (data not shown). To examine whether IL-12 was involved in the enhancement of IFN-γ production with DC/α-GC/IL-4, NKT cells were co-cultured with IL-12$^{-/-}$ DC/α-GC/IL-4, and the enhancement was re-produced, suggesting that the process was IL-12-independent (data not shown).

b) Pre-Conditioning of DCs with IL-21 Also Enhanced IFN-γ Production by NKT Cells

Next we treated DCs with IL-21 before co-culture with NKT cells. The IL-21/21R system resembles IL-2, -4, and -15, since this system utilizes the cytokine receptor common γ (γ_c) chain [20]. IL-21 has a pleiotropic effect depending on the cell type and demonstrates a negative effect on DC maturation [21]. For NKT cells, IL-21 has a proliferative effect in collaboration with IL-2 and IL-15 [22]. IL-21 also enhances both IL-4 and -13 productions from NKT cells, and NKT cells themselves produce IL-21 in response to cross-linking with anti-CD3 mAb or with α-GC [22]. Since IL-21 is a member of the IL-4 cytokine family, a similar effect as IL-4 in the pre-treatment of DCs was anticipated. Indeed, higher levels of IFN-γ were produced from NKT cells co-cultured with DC/α-GC/IL-21 than those with iDC/α-GC/- (Fig. 2C), as seen with DC/α-GC/IL-4. On the other hand, IL-4 production was not enhanced with DC/α-GC/IL-21 (Fig. 3C).

ii) Enhancement of IL-4 Production by NKT Cells

a) Pre-Treatment of DCs with IFN-γ Enhances IL-4 Production by NKT Cells

We co-cultured NKT cells with DC/α-GC/IFN-γ and quantified IL-4 with ELISA (Fig. 3A). Enhanced production of IL-4 was observed with DC/α-GC/IFN-γ compared to iDC/α-GC/- (Fig. 3A), whereas DC/α-GC/IFN-γ neither enhanced nor suppressed IFN-γ production (Fig. 2A). Together with the result that IL-4-treated DCs enhanced IFN-γ production (Fig. 2A), NKT cells produced a cytokine of the opposite response direction (Th1 or Th2) when stimulated with DCs that had been pre-treated with a cytokine of the other response direction (Th2 or Th1, respectively). This mode of cytokine production may function in counter-regulation of the immune response. In other words, cytokine production by NKT cells might counteract the biased immune response to which DCs are

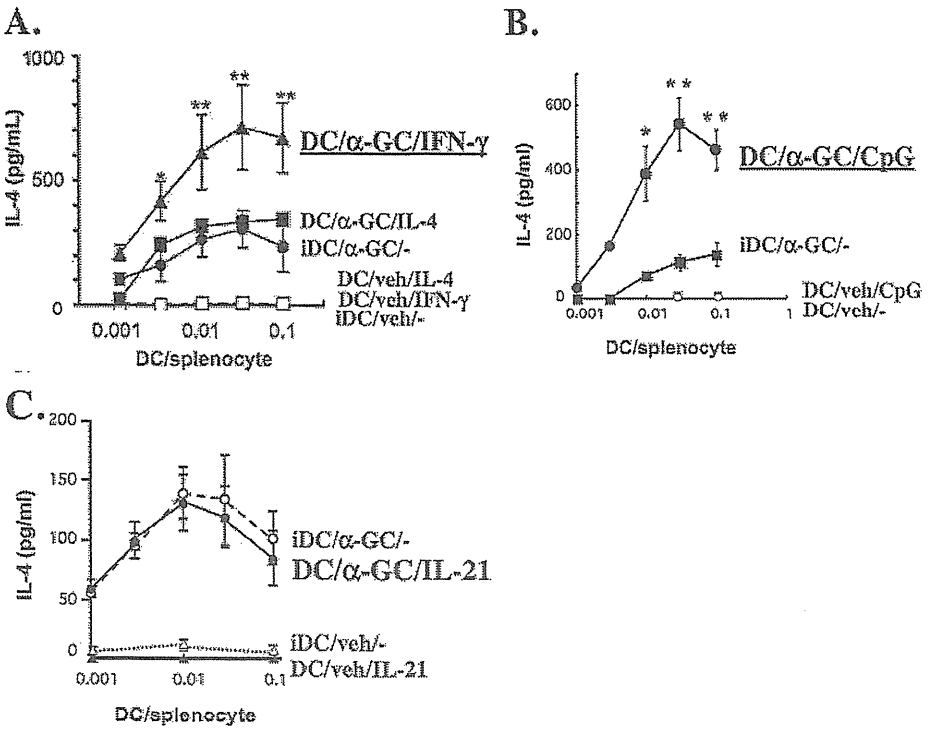

Fig. (3). IL-4 production by splenocytes stimulated with α-GalCer-loaded, differently treated DCs *in vitro*. (A) IL-4 production by splenocytes stimulated with various DC preparations with IFN-γ or IL-4. DCs of each preparation are indicated as described in (A). The T cell fraction and SDDCs, both from BALB/c mice, were cultured for 48 hr at the indicated DC/splenocyte ratios (0.001 to 0.1) in the x-axis. IL-4 in the culture supernatant was quantified with ELISA. Each symbol represents the mean ± SE of 3 independent experiments. (B). IL-4 production by splenocytes stimulated with various DC preparations with CpG. Nylon-wool non-adherent splenocytes obtained from BALB/c mice and a DC cell line, BC1, derived from BALB/c mice were co-cultured for 48 h. Each symbol represents the mean ± SE of 4 independent experiments. (C) IL-4 production by splenocytes stimulated with various DC preparations with IL-21. The T cell fraction from BALB/c mice and BC1 cells were co-cultured for 48 h. Each symbol represents the mean ± SE of 4 independent experiments. BC1 cells were incubated with IL-21 for 4 d before co-culture with the splenocytes. Statistical significance was calculated by Student *t*-test (*p< .05; **p< .01 *vs* iDC/α-GC/-).

normally exposed. This negative feedback regulation is specific to NKT cells, because positive feedback regulation appears to be operating in the cytokine responses of mainstream T cells [12].

b) Pre-Treatment of DCs with CpG Enhances IL-4 Production

CpG is a ligand of TLR9 and biases the immune response towards Th1 by inducing IL-12 from DCs [23]. If NKT cells were to be activated with DC/α-GC/CpG, an enhancement of the cytokine of the opposite response direction, IL-4, would be anticipated through the negative feedback mechanism. Indeed, IL-4 production was enhanced with DC/α-GC/CpG (Fig. **3B**). Again, IFN-γ production was not enhanced with DC/α-GC/CpG and was comparable to that with iDC/α-GC/- (Fig. **2B**).

3. MECHANISM FOR SELECTIVE ENHANCEMENT OF IFN-γ OR IL-4 CYTOKINE *VIA* DC-NKT INTERACTIONS

We have demonstrated that specific pre-treatments of DCs could selectively enhance Th1- or Th2-cytokine production by NKT cells. In each pre-treatment, the expression of surface molecules was analyzed with flow cytometry, and the results are listed in Table **1**.

The group that produces more IFN-γ did not show unified characteristics in the expression of surface molecules. IL-4-treated DCs showed decreased expression of CD1d but the same level of H-2K^d, I-A^d, CD40, and CD86 compared to control DCs. Of note, in IL-4 treated DCs, a down-modulation of Qa-1^b was observed. Since blockade of the Qa-1^b-CD94/NKG2 signal generated strong induction of IFN-γ, as reported by Ota *et al.* [24], down-modulation of Qa-1^b on IL-4 pre-treated DCs may be attributable to up-regulation of IFN-γ. Integration of a reduced TCR signal, implied by the down-modulation of CD1d, and a reduced inhibitory signal, implied by the down-modulation of Qa-1^b, might result in the enhancement of IFN-γ production (Fig. 4). Intriguingly, Ota *et al.* also demonstrated that OCH treatment followed by α-GC resulted in an exaggerated production of IFN-γ, and they explained this observation by showing that the differences in re-expression kinetics were distinct between TCR/CD28 (more rapid) and inhibitory receptors (delayed), which rendered the NKT cells hyper-reactive during certain periods of time [24]. Since OCH preferentially induces IL-4, OCH administration might

Table 1. Effect of DC Treatment on NKT Cell Response with α-GC Stimulation

Input	Original Bias	Output	Th1/2 Balance	Surface Ag on DC			Ref.
				CD1d	Class I MHC	Co-Stimulator	
DC/α-GC/IL-4	Th2	IFN-γ↑	Th1	↓	→	CD40 · 86→	[12]
DC/α-GC/IL-21	—	IFN-γ↑	Th1	↑	→	CD80 · 86↑	[13]
DC/α-GC/IFN-γ	Th1	IL-4↑	Th2	↑	↑	CD40 · 86↑	[12]
DC/α-GC/CpG	Th1	IL-4↑	Th2	↑	↑	CD80 · 86↑	[14]

Relationship between primary treatment of DC (input) and cytokine production (output) is summarized. Expression of surface Ag on DC with each treatment is compared with that of control and expressed with arrow. ↓: down-regulated; →: unchanged; ↑: up-regulated.

correspond to the pre-treatment of DCs with IL-4 in our *in vivo* system. In either case, the down-modulation of the inhibitory signal enhanced the net signal in NKT cells to generate a Th1-biased response.

In IL-21-treated DCs, increased expression of CD1d, CD40, and CD86 was noted, whereas class I MHC (K^d) and class II MHC ($I\text{-}A^d$) were not increased (Table **1**) [13]. CD40 cross-linking of DC/ - /IL-21 generated less IL-12p40 than that from iDC/ - /- with CD40 cross-linking, suggesting that the involvement of IL-12 in the enhanced production of IFN-γ was unlikely. On the other hand, anti-CD86 blockade in a co-culture of NKT cells with DC / α-GC / IL-21 partially decreased the production of IFN-γ, suggesting that the CD86/CD28 pathway may in part play a role in the enhancement by IL-21-treated DCs.

However, in groups demonstrating Th2-immune bias (enhanced IL-4 production; DC with IFN-γ or CpG pre-treated), CD1d, class I MHC, and co-stimulators were up-regulated. Intriguingly, the expression of $H\text{-}2D^d$ molecules was up-regulated in CpG-treated DCs. To examine the effect of the up-regulation of $H\text{-}2D^d$ on the enhancement of IL-4 production, anti-$H\text{-}2D^d$ mAb was added to the co-culture of CpG-treated DCs and NKT cells. The enhanced production of IL-4 was down-modulated with the addition of anti-D^d mAb compared to control (control IgG added; Fig. **5**). On the other hand, IFN-γ production was up-regulated with the addition of anti-$H\text{-}2D^d$ mAb (Fig. **5**). The above results are consistent with the previous finding that IFN-γ production was enhanced in H-2D-deficient mice [25]. The TCR signal and the D^d/Ly49 inhibitory signal are integrated in IL-4 production, which is concordant with the notion that IL-4 is an immunomodulatory cytokine. Again, in NKT cell responses, the inhibitory signal appears to be essential for tuning the Th1/Th2 immune bias so as to restore a neutral cytokine production profile. As for IFN-γ-treated DCs, we have no information on the inhibitory ligand/receptor on

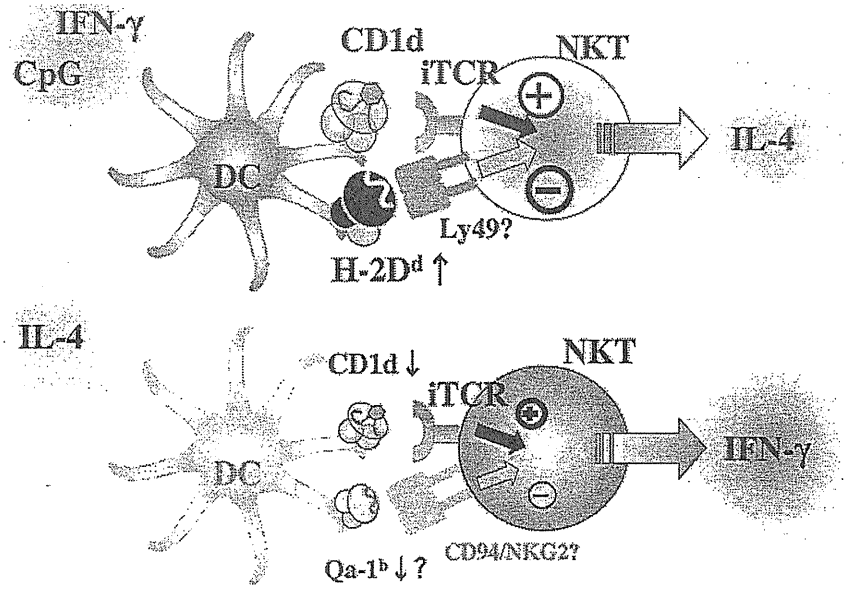

Fig. (4). Regulation of the Th1/Th2 cytokine balance is mediated by an interaction of DC and NKT cells in a negative feedback fashion IFN-γ- or CpG-treated DCs express elevated levels of CD1d and $H\text{-}2D^d$. Signals through the TCR and an inhibitory receptor, probably an Ly49 subtype, are integrated, and the resultant NKT cells produce IL-4. Inputs (IFN-γ or CpG) that usually induce a Th1 response give rise to the opposite response, Th2 cytokine production. IL-4-treated DCs express reduced levels of CD1d and $Qa\text{-}1^b$. Signals through the TCR and an inhibitory receptor, probably CD94/NKG2, are integrated, and the resultant NKT cells produce IFN-γ in this setting. Again, the input (IL-4) that usually induces a Th2 response gives rise to the opposite response, Th1 cytokine production. The mode of regulation appears to be a negative feedback regulation.

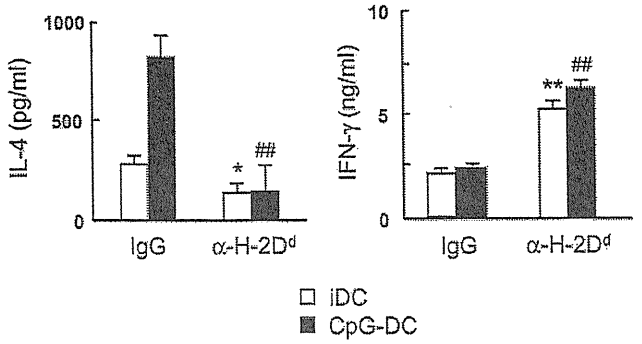

Fig. (5). Anti-H-2D^d mAb blocks the enhancement of IL-4 production by NKT cells co-cultured with CpG-treated DCs. The T cell fraction from BALB/c mice was co-cultured with either iDC/α-GC/- (iDC) or DC/α-GC/CpG (CpG-DC) that had been pre-treated with control Ig (IgG) or anti-H-2D^d mAb (α-H-2D^d) for 48 hr. BC1 cells were used as the DCs. The supernatant was collected, and IL-4 (left panel) or IFN-γ (right panel) were quantified with ELISA. Each column represents the mean ± SE of 4 independent experiments. Statistical significance was calculated by Student's *t*-test (*p< .05; **p< .01 *vs* control IgG-treated iDC; #p< .05; ##p< .01 *vs* control IgG-treated CpG-DC).

both DCs and NKT cells that could be linked to the enhanced production of IL-4.

The corresponding molecule on NKT cells to D^d on DCs has not yet been elucidated. Although Ly49 is likely, there are at least 10 subtypes of Ly49 (A, C, D, F, G, O, P, R V and W) that can bind to the D^d molecule [26]. Of note, DC/α-GC/CpG could not enhance IL-4 production in C57BL/6 mice [14]. Since H-2D^b binds to A, C, O, and V subtypes, these may not be involved in the response. At any rate, we should pursue the corresponding molecule to D^d in BALB/c mice in further studies.

4. SELECTIVE ENHANCEMENT OF CYTOKINE PRODUCTION BY NKT CELLS- *IN VIVO* STUDIES

i) Enhancement of IFN-γ Production by NKT Cells

a) Intrasplenic Injection of Pre-Treated DCs or Intravenous Administration of Cytokines

As the next step from the development of *in vitro* studies, we applied intrasplenic injection of DCs treated as in Fig. (**1B**). When α-GC loaded DCs that had been treated with either IL-4 or IL-21 (5 x 10^5) were injected (50 μl) *i.s.*, enhanced production of IFN-γ was detected 12 hr later in sera compared to mice injected with control DCs [12, 13]. Thus, when DCs modulated with IL-4 or IL-21 *in vitro* were transferred, those DCs could prime NKT cells toward a Th1-immune bias. This result suggests that systemic administration of cytokines may also modulate DCs *in situ* if the cytokine level could be sustained high enough as to modify the nature of DCs. When we injected IL-21 (100 ng/head) *i.p.* followed by α-GC (2 μg/head *i.p.*) 2 d later, enhanced IFN-γ production was again reproduced, whereas no enhancement of IL-4 was observed. However, the enhancement of IFN-γ was not as marked as observed *in vitro*, suggesting that an effective concentration of IL-21 could not be sustained *in vivo*.

b) IL-4/Anti-IL-4 mAb Formulation Enhances IFN-γ Production by NKT Cells In Vivo

When we administered IL-4, we employed a long-lasting formulation of IL-4, which consisted of IL-4 and anti-IL-4 mAb immune complexes (IL-4C) [17]. This formulation protects IL-4 from degradation and enables the slow release of biologically active IL-4. The half-life of IL-4 is prolonged from a few minutes to approximately 24 hr with this formulation [17]. First we examined the effect on cytokine production by splenocytes from mice administered with IL-4C. Splenocytes from IL-4C-treated mice indeed showed enhanced production of IFN-γ (Fig. **6**) with an unaltered composition of lymphocytes, especially invariant NKT cells [12]. IL-4 production, on the other hand, was suppressed in mice receiving IL-4C [12]. As shown in the *in vitro* study, IL-4 pre-conditioning was also vital *in vivo* for the enhanced production of IFN-γ.

ii) Enhancement of IL-4 Production by NKT Cells

a) Intrasplenic Injection of Pre-Treated DCs

When IFN-γ-treated, α-GC-loaded DCs were injected *i.s.*, enhanced production of IL-4 was detected 6 hr after transfer. Thus, IFN-γ-treated DCs could function *in vivo* as observed *in vitro*. We have not examined the simple transfusion of IFN-γ or the administration of an IFN-γ/anti-IFN-γ mAb complex (IFN-γC), which has been developed for IL-4 but not for IFN-γ. If IFN-γC were as efficacious as IL-4C *in vivo*, we would examine whether IFN-γC administration enhances IL-4 production. A few cytokine/anti-cytokine mAb immune complexes have actually been shown to exhibit stable activity of IL-2 [19, 27] or IL-7 [28].

iii) Therapeutic Application

a) Tumor Immunity

Stronger induction of IFN-γ is very critical for anti-tumor immunity and immunity against many infections. We demonstrated that the administration of IL-4C and subsequent stimulation with α-GC induced a higher level of IFN-γ *in vivo*. This treatment enhanced the cytotoxicity of splenocytes against the renal cell carcinoma cell line, RenCa (Fig. 7A), and against YAC-1 and CT 26 tumor cells (data not shown) *ex vivo* [12]. To test whether the effect on the enhancement of cellular cytotoxicity with IL-4C administration was vital *in vivo*, the inhibitory effect on lung metastasis was assessed in mice receiving murine renal cell carcinoma cells (RenCa) as depicted in Fig. (**7B**). Mice

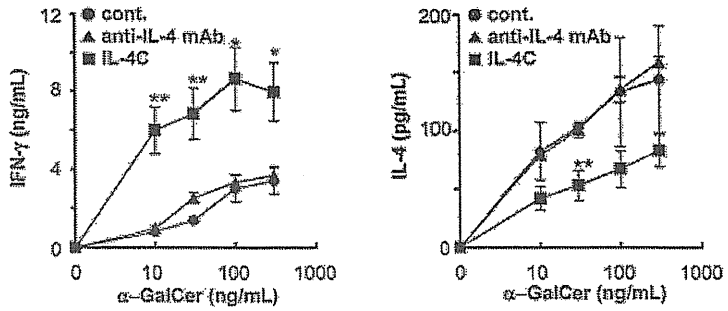

Fig. (6). Cytokine production by splenocytes from mice pre-treated with IL-4/anti-IL-4mAb immune complex (IL-4C). BALB/c mice were injected *i.v.* with PBS, anti-IL-4 Ab alone, and IL-4C. Three days later, a single cell suspension was prepared from the spleens of each group of mice. The splenocytes were incubated with various concentrations of α-GC for 48 hr, and cytokines in the supernatant were quantified with ELISA. Each symbol represents mean ± SE of 3 independent experiments. Statistical significance was calculated by Student's *t*-test (*p< .05; **p< .01 *vs* control).

received PBS or IL-4C 3 days before the intravenous administration of 5 x 10^5 RenCa cells followed by α-GC or vehicle injection. Three weeks later, the number of lung metastases was enumerated. Although the results are just schematically recapitulated in Fig. (**7B**), the IL-4C/α-GC group showed the least lung metastases *in vivo*. Thus, the most potent protection was achieved in a group of mice in which the highest IFN-γ induction was produced with IL-4C + α-GC.

The ultimate goal is to apply the negative feedback regulation for rejection of solid tumor that has already been grown clinically visible in size. To attain the effective modulation of NKT cells *in vivo*, we first need to know the local cytokine environment induced around or in the tumor and the systemic influence, and then to control them in the particular host. Apparently there are many obstacles to be removed, which should be studied in further investigation.

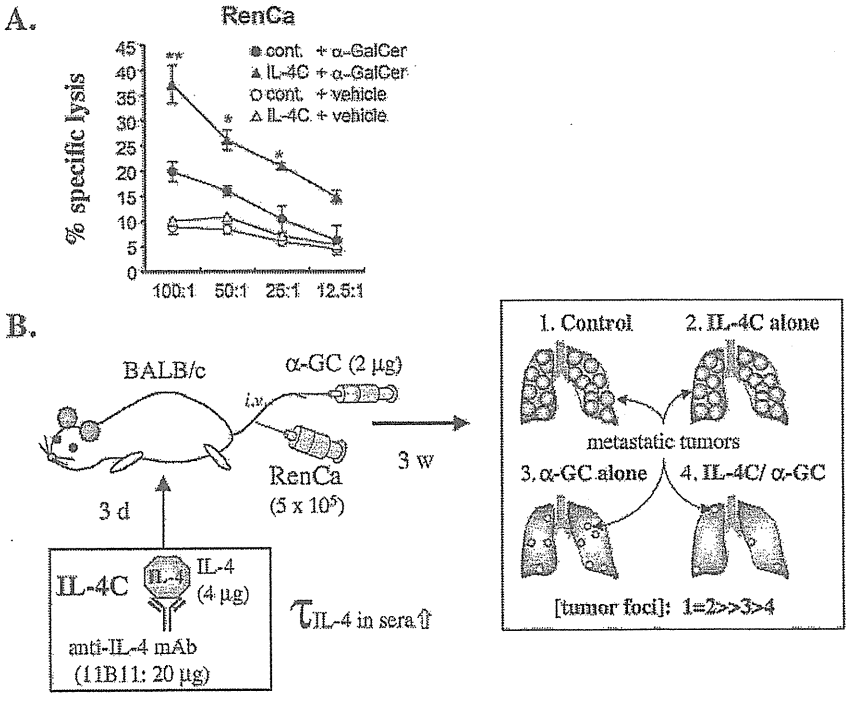

Fig. (7). Anti-tumor effect of IL-4C pre-treatment in combination with α-GC administration. (A) Effect of IL-4C on α-GC-induced cytotoxicity. BALB/c mice were pre-treated with IL-4C followed by the *i.v.* administration of α-GC. After 24 hr, splenocytes were prepared and co-cultured with a ^{51}Cr-labeled renal cell carcinoma cell line (RenCa) for 4 hr. Percent specific lysis (cytotoxicity) was calculated as [(experimental release-spontaneous release)/(maximal release-spontaneous release)] x 100. (B) Anti-tumor effect of IL-4C with α-GC *in vivo*. BALB/c mice were administered with PBS or IL-4C and intravenously injected with 5 x 10^5 RenCa cells with or without α-GC (2 μg/head) 3 d later. After 3 weeks, the number of lung metastases was enumerated in 4 groups. The number of metastatic foci in the lung was in the order: [IL-4C + α-GC] < α-GC alone << IL-4C alone ≈ control.

Table 2. Infectious and Autoimmune Diseases in which NKT Cells have Beneficial Effects

Diseases of Better Prognosis with NKT + α-GC or C-Glycoside (IFN-γ)	Ref.
Infectious Diseases (Pathogens)	
Mycobacterium tuberculosis	[38, 39]
Steptococcus pneumonia	[40]
Pseudomonas aerginosa	[41]
Plasmodium yoeli, Plasmodium berghei	[7, 42]
Trypanosoma cruzi	[43]
Cryptococcus neoformans	[44]
Hepatitis B virus	[45]
Respiratory syncytial virus	[46]
Diseases of Better Prognosis with NKT + α-GC or OCH (IL-4)	
Autoimmune Disease	
Type 1 diabetes (NOD mice)	[47]
Experimental allergic encephalomyelitis	[4]
Type II collagen arthritis (B6, SJL)	[48]
Antibody-induced arthritis	[49]
Dextran sulfate sodium-induced colitis	[50]

Pathogens of each infectious diseases (upper) and the murine model of autoimmune diseases (lower) are listed with representative references (more detailed informations up to 2005 are described in ref. 25). Pathogens and disease models having ambivalent outcomes in the presence or absence of NKT cells (ameliorating vs aggravating) are not listed to avoid confusion.

b) Infectious Diseases

As demonstrated in tumor immunotherapy, higher IFN-γ in the immune response is usually thought to better control infectious diseases, unless tissue damage surpasses the beneficial effects. As indicated in Table 2, a considerable number of examples for good prognosis have been reported in the presence of NKT cells or α-GC, and these effects were even more dramatic with stronger agonists (such as C-glycoside) [7, 29, 30]. For those diseases, the enhancement of IFN-γ production with IL-4- or IL-21-treated DCs could be examined for treatment. However, it is not known whether IL-4C administration is beneficial for the course of these diseases by enhancing IFN-γ production *in vivo* because the level of the cytokine of the opposite response direction, IL-4, is elevated, albeit temporarily. The combination of OCH followed by α-GC administration could induce elevated levels of IFN-γ with an initial increase of IL-4 [24]. To avoid this, Qa-1^b-CD94/NKG2 blockade could be employed instead, and is readily applicable to B6 background [24].

In some diseases such as influenza infection, in which NKT cell-deficiency is neutral to the onset or outcome [31], NKT cell activation is still sometimes effective [32-34]. Thus, more applications of α-GC with novel ways of enhancing IFN-γ production should be undertaken for the treatment of infectious diseases caused by various pathogenic organisms.

iv) Autoimmune Diseases

Although we have not focused on inducing Th2-immune bias *in vivo* in this review, Th2-immune bias has important roles in ameliorating autoimmune diseases. Autoimmune diseases ameliorated with NKT cell manipulation are listed in Table 2 [29]. To skew toward the Th2 response, OCH is a good inducer and has been applied for experimental therapeutics [4]. For the DC-based modulation, the administration of DCs pre-treated with CpG or IFN-γ may be preferable, since the systemic administration of CpG or IFN-γ may worsen Th1-mediated tissue damage. However, IFN-γC, if available and efficacious, may also be tested for the prolonged biological activity of IFN-γ in order to enhance IL-4 production by NKT cells.

In some murine models of autoimmune and allergic diseases, the induction of IFN-γ from NKT cells ameliorates disease [35, 36]. Similarly, in some infectious disease models, the induction of IL-4 production ameliorates disease [29]. For both of these cases, appropriate immunomodulation regimens should be developed according to the underlying immunopathogenesis.

a) NKT Cells as Cellular Medicine

The above manipulations have been considered in a situation in which a normal population of NKT cells is present. However, NKT cells are absent or severely reduced in some pathogenic situations [1]. In such cases, the generation of NKT cells from progenitor cells has to be considered. To this end, embryonic stem (ES) cell-based generation of NKT cells may serve as a promising therapeutic measure when we employ NKT cells as a magic bullet for many immune-based diseases [37, Wakao *et al.* this issue].

CONCLUSION

We have developed DC immunotherapy based on the negative feedback regulation of NKT cell responses, likely through inhibitory ligand/receptor interactions. Elucidation of the mechanism involved will be very important, not only for the application to immune-mediated diseases but also for understanding a novel regulatory mechanism of NKT cell responses.

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