Kinjo Y, Wu D, Kim G, Xing G, Poles M, Ho D, et al (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. Nature 434:520–525

Kirwan S, Burshtyn D (2007) Regulation of natural killer cell activity. Curr Opin Immunol 19:46–54

Koehler N, Genain C, Giesser B, Hauser S (2002) The human T cell response to myelin oligodendrocyte glycoprotein: a multiple scleorsis family-based study. J Immunol 168:5920–5927

Kondo T, Yamamura T, Inobe J, Ohashi T, Takahashi K, Tabira T (1996) TCR repertoire to proteolipid protein (PLP) in multiple sclerosis (MS): homologies between PLP-specific T cells and MS-associated T cells in TCR junctional sequences. Int Immunol 8:123–130

Koseki H, Asano H, Inaba T, Miyashita N, Moriwaki K, Lindahl K, et al (1991) Dominant expression of a distinctive V14+ T-cell antigen receptor a chain in mice. Proc Natl Acad Sci U S A 88:7518–7522

Kronenberg M (2005) Toward understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol 2005: 877–900

Kyewski B, Derbinski J (2004) Self-representation is the thymus: an extended view. Nat Rev Immunol 4:688–698

Lee P, Benlagha K, Teyton L, Bendelac A (2002) Distinct functional lineages of human Vα24 natural killer T cells. J Exp Med 195:637–641

Lopez-Botet M, Perez-Villar J, Carretero M, Rodriguez A, Melero I, Bellon T (1997) Structure and function of the CD94 C-type lectin receptor complex involved in the recognition of HLA class I molecules. Immunol Rev 155:165–174

Mars L, Laloux V, Goude K, Desbois S, Saoudi A, Van Kaer L, et al (2002) Cutting edge: Vα14-Jα 281 NKT cells naturally regulate experimental autoimmune encephalomyelitis in nonobese diabetic mice. J Immunol 168:6007–6011

Martin R, Howell M, Jaraquemada D, Flerlage M, Richert J, Brostoff S (1991) A myelin basic protein peptide is recognized by cytotoxic T cells in the context of four HLA-DR types associated with multiple sclerosis. J Exp Med 173:19–24

Matsumoto Y, Kohyama K, Aikawa Y, Shin T, Kawazoe Y, Suzuki Y, et al (1998) Role of natural killer cells and TCR γδ T cells in acute autoimmune encephalomyelitis. Eur J Immunol 28:1681–1688

Mattner J, Debord K, Ismail N, Goff R, Cantu 3rd C, Zhou D, et al (2005) Exogenous, and endogenous glycolipid antigens activate NKT cells during microbial infection. Nature 434:525–529

McCarthy C, Shepherd D, Floire S, Stronge V, Koch M, Illarionov P, et al (2007) The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. J Exp Med 204:1131–1144

Medzhitov R, Janeway JC (1997) Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 9:4–9

Mempel M, Ronet C, Suarez F, Gilleron M, Puzo G, Van Kaer L, et al (2002) Natural killer T cells restricted by the monomorphic MHC class 1b CD1d1 molecules behave like inflammatory cells. J Immunol 168:365–371

Mendel I, Kerlero de Rosbo N, Bennun AA (1995) Myeline oligodendrocyte glycoportein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2β mice: fine specificity and T cell receptor Vb expression of encephalitogenic T ells. Eur J Immunol 25:1951–1959

Miyamoto K, Miyake S, Yamamura T (2001) A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 413:531–534

Miyara M, Sakaguchi S (2007) Natural regulatory T cells: mechanisms of suppression. Trends Mol Med 13:108–116

Morelli A, Larregina A, Shufesky W, Zahorchak A, Logar A, Papworth G, et al (2003) Internalization of circulating apoptopic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. Blood 101:611–620

Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari M (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19:197–223

Morse RH, Seguin R, McCrea EL, Antel JP (2001) NK cell-mediated lysis of autologous human oligodendrocytes. J Neuroimmunol 116:107–115

Munschauer F, Hartrich L, Stewart C, Jacobs L (1995) Circulating natural killer cells but not cytotoxic T lymphocytes are reduced in patients with active relapsing multiple slcerosis and little clinical disability as compared to controls. J Neuroimmunol 62:177–181

Ohashi T, Yamamura T, J-i Inobe, Kondo T, Kunishita T, Tabira T (1990) Analysis of proteolipid protein (PLP)-specific T cells in multiple sclerosis: identification of PLP 95–116 as an HLA-DR2,w15-associated determinant. Int Immunol 7:1771–1778

Oki S, Chiba A, Yamamura T, Miyake S (2004) The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. J Clin Invest 113:1631–1640

Oki S, Tomi C, Yamamura T, Miyake S (2005) Preferential T_h2 polarization by OCH is supported by incompetent NKT cell induction of CD40L and following production of inflammatory cytokine by bystander cells in vivo. Int Immunol 17:1619–1629

Ota K, Matsui M, Milford E, Mackin G, Weiner H, Hafler D (1990) T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature 346:183–187

Pal E, Tabira T, Kawano T, Taniguchi M, Miyake S, Yamamura T (2001) Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of Va14 NK T cells. J Immunol 166:662–668

Pelfrey C, Trotter J, Tranquill L, McFarland H (1993) Identification of a novel T cell epitope of human proteolipid protein (residues 40–60) recognized by proliferative and cytolytic $CD4^+$ T cells from multiple sclerosis patients. J Neuroimmunol 46:33–42

Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G (1998) Cutting edge. Differentiation of human NK cells into NK1 and NK2 subsets. J Immunol 161:5821–5824

Pette M, Fujita K, Wilkinson D, Altmann D, Trowsdale J, Giegerich G, Wekerle H (1990) Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple slcerosis patients and healthy donors. Proc Natl Acad Sci U S A 87:7968–7972

Pillarisetty V, Katz S, Bleier J, Shah A, Dematteo R (2005) Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-γ via autocrine IL-12. J Immunol 174:2612–2618

Porcelli S, Yockey C, Brenner M, Balk S (1993) Analysis of T cell antigen receptor (TCR) expression by human peripheral blood $CD4^-CD8^-$alph/β T cells demonstrates preferential use of several Vβ genes and an invariant TCRa chain. J Exp Med 178:1–16

Porubsky S, Speak A, Luckow B, Cerundolo V, Platt F, Grone H (2007) Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. Proc Natl Acad Sci U S A 104:5977–5982

Rauch H, Montgomery I, Kaplan J (1985) Natural killer cell activity in multiple sclerosis and myasthenia gravis. Immunol Invest 14:427–434

Rice G, Casali P, Merigan T, Oldstone M (1983) Natural killer cell activity in patients with multiple sclerosis given a interferon. Ann Neurol 1983: 333–338

Richert J, Robinson E, Deibler G, Martenson R, Dragovic L, Kies M (1989) Human cytotoxic T-cell recognition of a synthetic peptide of myelin basic protein. Ann Neurol 26:342–346

Roncarolo M, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings M (2006) Interleukin-10-secreting type I regulatory T cells in rodents and humans. Immunol Rev 212:28–50

Sakuishi K, Oki S, Araki M, Porcelli S, Miyake S, Yamamura T (2007) Invariant NKT cell biased for IL-5 production act as crucial regulators of inflammation. J Immunol 179:3452–3462

Santoli D, Hall W, Kastrukoff L, Lisak R, Perussia B, Trinchieri G, et al (1981) Cytotoxic activity and interferon production by lymphocytes from patients with multiple sclerosis. J Immunol 126:1274–1278

Saraste M, Irjala H, Airas L (2007) Expansion of $CD56^{bright}$ natural killer cells in the peripheral blood of multiple sclerosis patients treated with interferon-b. Neurol Sci 28:121–126

Screpanti V, Wallin R, Grandien A, Ljunggren H (2005) Impact of FASL-induced apoptosis in the elimination of tumor cells. Mol Immunol 42:495–499

Shi F, Van Kaer L (2006) Reciptocal regulation between natural killer cells and autoreactive T cells. Nat Rev Immunol 6:751–760

Shi F, Ljunggren H, Sarventnick N (2001) Innate immunity and autoimmunity: from self-protection to self-destruction. Trends Immunol 22:97–101

Shimamura M, Huang YY, Okamoto N, Suzuki N, Yasuoka J, Morita K, et al (2007) Modulation of Vα19 NKT cell immune responses by α-mannosyl ceramide derivatives consisting of a series of modified sphingosines. Eur J Immunol 37:1836–1844

Singh A, Wilson M, Hong S, Olivares-Villagomez D, Du C, Stanic A, et al (2001) Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. J Exp Med 194:1801–1811

Skulina C, Schmidt S, Dornmair K, Babbe H, Roers A, Rajewsky K, Wekerle H, Hohlfeld R, Goebels N (2004) Multiple sclerosis: brain-infiltrating $CD8^+$ T cells persist as clonal expansions in the cerebrospinal fluid and blood. Proc Natl Acad Sci U S A 101:2428–2433

Smeltz R, Wolf N, Swanborg R (1999) Inhibition of autoimmune T cell response in the DA rat by bone marrow-derived NK cells in vitro: implication for autoimmunity. J Immunol 163:1390–1397

Smyth M, Swann J, Cretney E, Zerafa N, Yokoyama W, Hayakawa Y (2005) NKG2D function protects the host from tumor initiation. J Exp Med 202:583–588

Sospedra M, Martin R (2005) Immunology of multiple sclerosis. Annu Rev Immunol 23:683–747

Speak A, Salio M, Nerville D, Fontaine J, Priestman DP, Platt N, Heare T, et al (2007) Implications for invariant natural killer T cell ligands due to the restricted presence of isoglobotrihexosylceramide in mammals. Proc Natl Acad Sci U S A 104:5971–5976

Stacker S, Springer T (1991) Leukocyte intergrin P150, 95 (CD11c/CD18) function as an adhesion molecule binding to a counter-receptor on stimulated endothelium. J Immunol 146:648–655

Steinman L (2001) Multiple sclerosis: a two-stage disease. Nat Immunol 2:762–764

Takahashi K, Miyake S, Kondo T, Terao K, Hatakenaka M, Hashimoto S, et al (2001) Natural killer type 2 bias in remission of multiple sclerosis. J Clin Invest 107:R23–R29

Takahashi K, Aranami T, Endoh M, Miyake S, Yamamura T (2004) The regulatory role of natural killer cells in multiple sclerosis. Brain 127:1917–1927

Takeda K, Hayakawa Y, Smyth M, Kayagaki N, Yamaguchi N, Kakuta S, et al (2001) Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 7:94–100

Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H (2003) The regulatory role of Va14 NKT cells in innate and acquired immune response. Annu Rev Immunol 21:483–513

Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al (2003) Selection of evolutionary conserved mucosal-associated invariant T cell by MR1. Nature 422:164–169

Treiner E, Duban L, Moura I, Hansen T, Gilfillan S, Lantz O (2005) Mucosal-associated invariant T (MAIT) cells: an evolutionarily conserved T cell subset. Microbes Infect 7:552–559

Trinchieri G (1989) Biology of natural killer cells. Adv Immunol 47:187–193

Vranes Z, Poljakovic Z, Marusic M (1989) Natural killer cell number and activity in multiple sclerosis. J Neurol Sci 94:115–123

Walker L, Abbas A (2002) Keeping self-reactive T cells at bay in the periphery. Nat Rev Immunol 2:11–19

Warren H, Smyth M (1999) NK cells and apoptosis. Immunol Cell Biol 77:64–75

Yamamura T, Sakuishi K, Illes Z, Miyake S (2007) Understanding the behavior of invariant NKT cells in autoimmune disease. J Neuroimmunol 191:8–15

Yoshimoto T, Bendelac A, Hu-Li J, Paul W (1995) Defective IgE production by SJL mice is linked to the absence of $CD4^+$ $NK1.1^+$ T cells that promptly produce interleukin 4. Proc Natl Acad Sci U S A 92:11931–11934

Yu K, Im J, Molano A, Dutronc Y, Illarionov P, Forestier C, et al Modulation of CD1d-restrotced NKT cell responses by using N-acyl variants of α-galactosylceramides. Proc Natl Acad Sci U S A (2005) 102:3383–3388

Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T (1997) Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. J Exp Med 186:1677–1687

Zhou D, Mattner J, Cantu 3rd C, Schrantz N, Yin N, Gao Y, et al (2004) Lysosomal glycosphingolipid recognition by NKT cells. Science 306:1786–1789

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

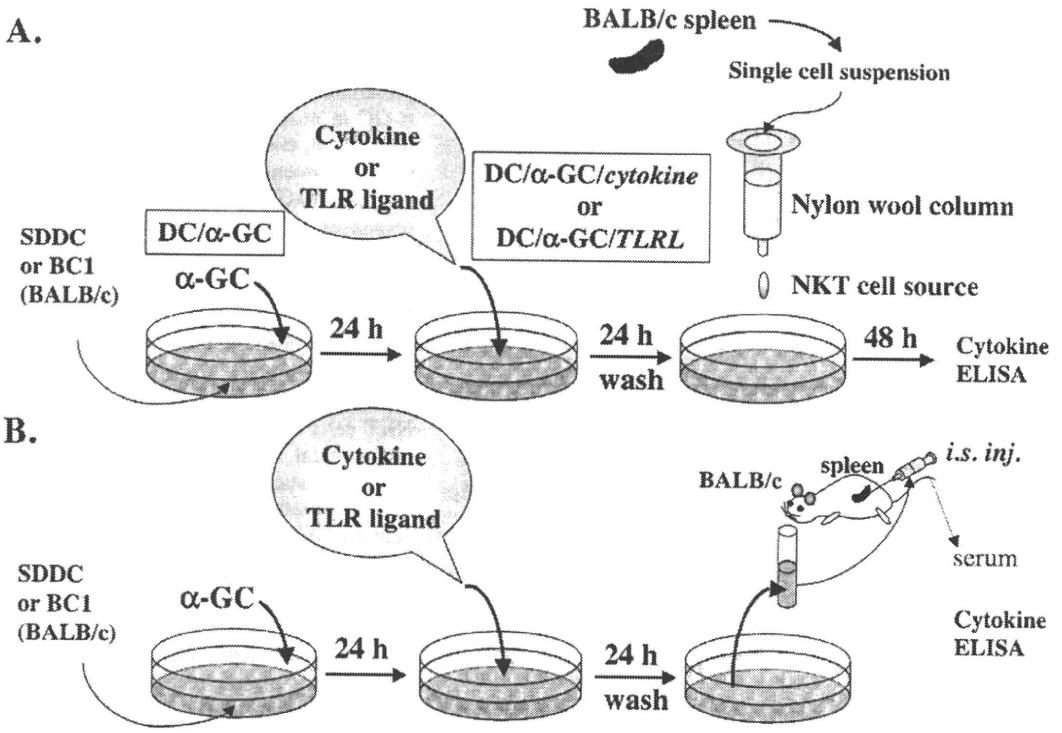

Fig. (1). Experimental system for studying DC-NKT cell interactions. (**A**) Co-culture of dendritic cells (DC) and natural killer T (NKT) cells. Spleen-derived dendritic cells (SDDC) and a dendritic cell line, BC1 (BALB/c), were incubated with α-galactosylceramide (α-GC) for 24 hr. Then, cytokine or 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.

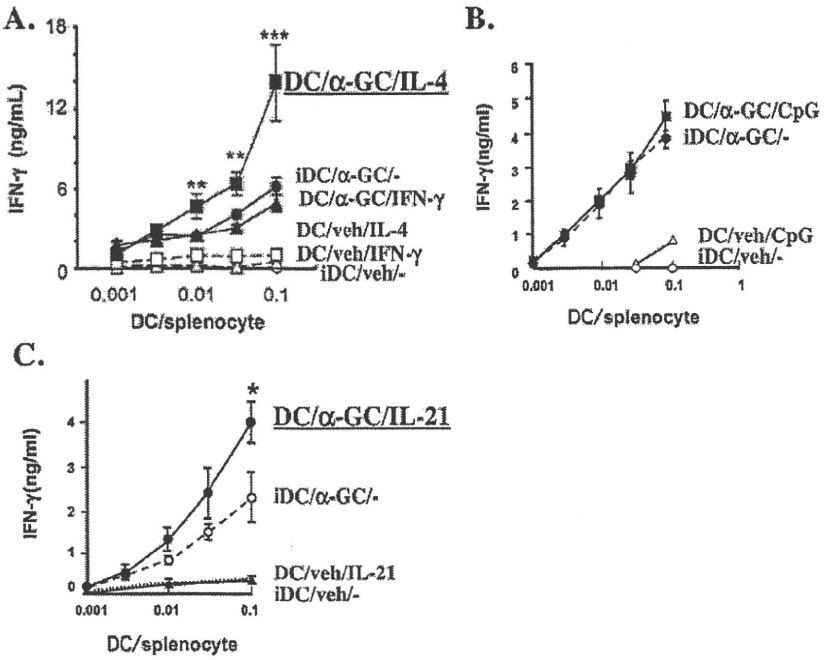

Fig. (2). IFN-γ production by splenocytes stimulated with α-GalCer-loaded, differently treated DCs *in vitro*. (**A**) IFN-γ production by splenocytes stimulated with various DC preparations with IL-4 or IFN-γ. DCs of each preparation are indicated as described in Fig. (**1A**). The T cell fraction and 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 $IL\text{-}12^{-/-}$ 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

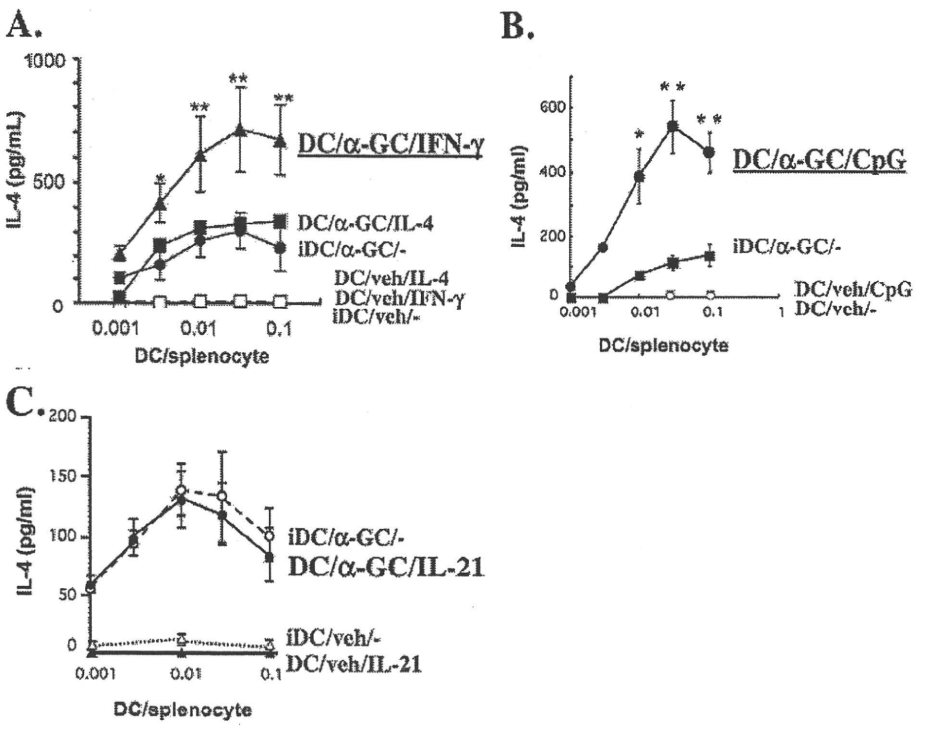

Fig. (3). IL-4 production by splenocytes stimulated with α-GalCer-loaded, differently treated DCs *in vitro*. (**A**) IL-4 production by splenocytes stimulated with various DC preparations with IFN-γ or IL-4. DCs of each preparation are indicated as described in (**A**). The T cell fraction and SDDCs, 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

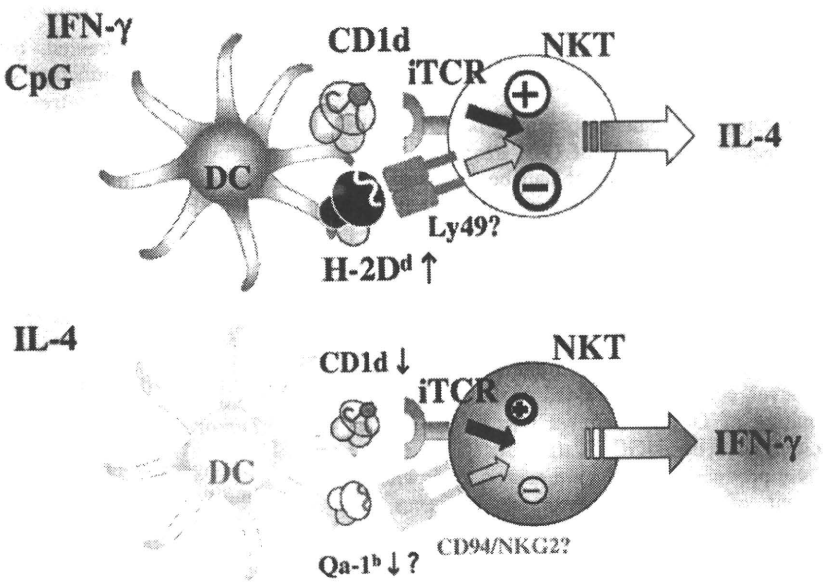

Fig. (4). Regulation of the Th1/Th2 cytokine balance is mediated by an interaction of DC and NKT cells in a negative feedback fashion IFN-γ- or CpG-treated DCs express elevated levels of CD1d and $H\text{-}2D^d$. Signals through the TCR and an inhibitory receptor, probably an Ly49 subtype, are integrated, 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.

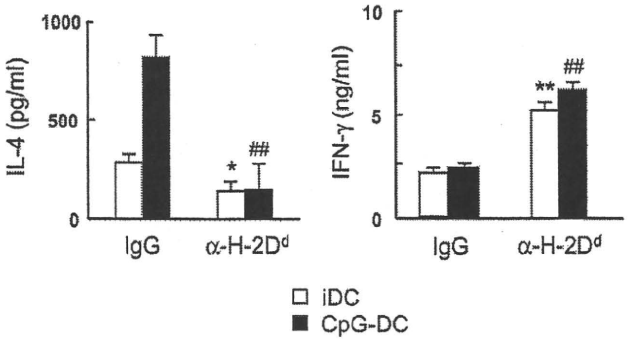

Fig. (5). Anti-H-2D^d mAb blocks the enhancement of IL-4 production by NKT cells co-cultured with CpG-treated DCs. The T cell fraction from BALB/c mice was co-cultured with either iDC/α-GC/- (iDC) or DC/α-GC/CpG (CpG-DC) that had been pre-treated with control Ig (IgG) or anti-H-2D^d mAb (α-H-2D^d) for 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

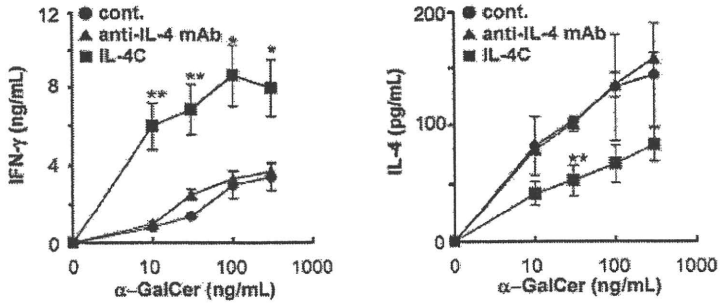

Fig. (6). Cytokine production by splenocytes from mice pre-treated with IL-4/anti-IL-4mAb immune complex (IL-4C). BALB/c mice were injected *i.v.* with PBS, anti-IL-4 Ab alone, and IL-4C. Three days later, a single cell suspension was prepared from the spleens of each group of mice. The splenocytes were 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.

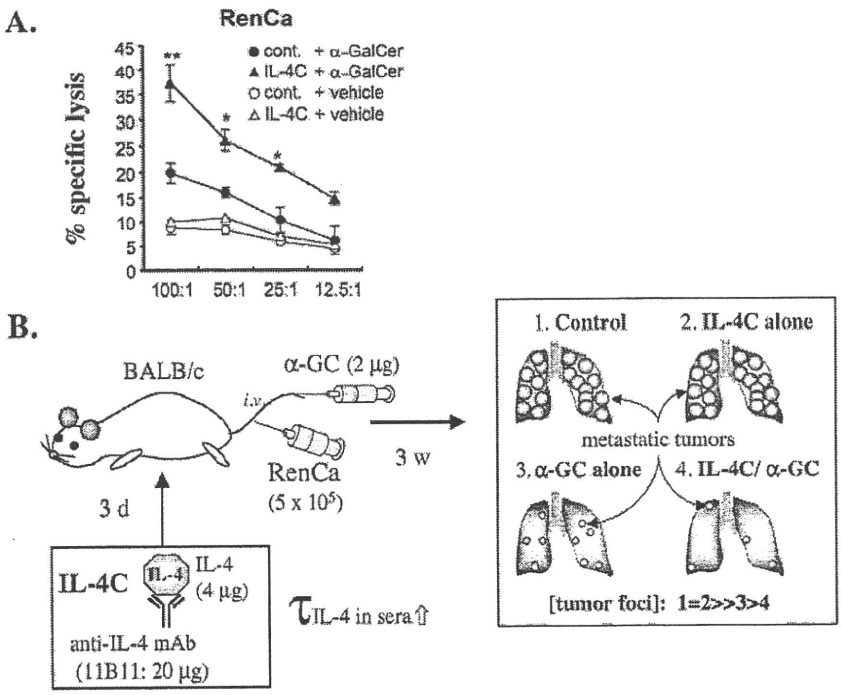

Fig. (7). Anti-tumor effect of IL-4C pre-treatment in combination with α-GC administration. (**A**) Effect of IL-4C on α-GC-induced cytotoxicity. BALB/c mice were pre-treated with IL-4C followed by the *i.v.* administration of α-GC. After 24 hr, splenocytes were prepared and co-cultured with a ^{51}Cr-labeled 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.

ACKNOWLEDGEMENTS

K. I. is supported by a Grant-in-Aid for Scientific Research (B) (#20390106) from the Japan Society for the Promotion of Science (JSPS), the Global COE Program 'Establishment of International Collaboration Center for Zoonosis Control' from the Ministry of Education, Culture, Science, Sports and Technology (MEXT), a Grant for Researchers on Behçet's Disease, Ministry of Health, Labour and Welfare, Japan, and grants from The Suhara Memorial Foundation and Heisei Ijuku Tomakomai East Hospital.

REFERENCES

[1] Bendelac A, Savage PB, Teyton L. The biology of the NKT cells. Annu Rev Immunol 2007; 25: 297-336.

[2] Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. Curr Opin Immunol 2008; 20: 358-68.

[3] Van Kaer L. NKT cells: T lymphocytes with innate effector functions. Curr Opin Immunol 2007; 25: 354-64.

[4] Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 2001; 413: 531-4.

[5] Forestier C, Takaki T, Molano A, *et al.* Improved outcomes in NOD mice treated with a novel Th2 cytokine-biasing NKT cell activator. J Immunol 2007; 178: 1415-25.

[6] Yang G, Schmieg J, Tsuji M, Franck RW. The C-glycoside analogue of the immunostimulant a-galactosylceramide (KRN7000): synthesis and striking enhancement of activity. Angew Chem Int Ed Engl 2004; 43: 3818-22.

[7] Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand a-galactosylceramide. J Exp Med 2003; 198: 1631-41.

[8] Kronenberg M. Toward an understanding of NKT cell biology: Progress and paradoxes. Annu Rev Immunol 2005; 26: 877-900.

[9] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392: 245-51.

[10] Kitamura H, Iwakabe K, Yahata T, *et al.* The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J Exp Med. 1999; 189: 1121-8.

[11] Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-g producing NKT response induced with a-galactosylceramide-loaded DCs. Nat Immunol 2002; 3: 867-74.

[12] Minami K, Yanagawa Y, Iwabuchi K, *et al.* Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance *via* dendritic cell and natural killer T cell interactions. Blood 2005; 106: 1685-93.

[13] Maeda M, Yanagawa Y, Iwabuchi K, *et al.* IL-21 enhances dendritic cell ability to induce interferon-g production by natural killer T cells. Immunobiol 2007; 212: 537-47.

[14] Mizuuchi K. Yanagawa Y, Iwabuchi K, *et al.* H-2Dd-mediated upregulation of interleukin-4 production by natural killer T cell and dendritic cell interaction. Immunol 2008; 124: 102-111.

[15] Yanagawa Y, Onoé K. CCL19 induces rapid dendritic extension of murine dendritic cells. Blood 2002; 100: 1948-56.

[16] Yanagawa Y, Onoé K. CCR7 ligands induce rapid endocytosis in mature dendritic cells with concomitant up-regulation of Cdc42 and Rac activities. Blood 2003; 101: 4923-29.

[17] Finkelman FD, Madden KB, Morris SC, *et al.* Anti-cytokine antibodies as carrier proteins. Prolongation of *in vivo* effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. J Immunol 1993; 151: 1235-44.

[18] Phelan JD, Orekov T, Finkelman FD. Mechanism of enhancement of *in vivo* cytokine effects by anti-cytokine monoclonal antibodies. J Immunol 2008; 180: 44-8.

[19] Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science 2006; 311: 1924-7.

[20] Leonard WJ, Spolski R. Interleukin-21: Basic Biology and implications for cancer and autoimmunity. Annu Rev Immunol 2008; 26: 57-79.

[21] Brandt K, Bulfone-Paus S, Foster DC, Ruckert R. Interleukin-21 inhibits dendritic cell activation and maturation. Blood 2003; 102: 4090-8.

[22] Coquet JM, Kyparissoudis K, Pellicci DG, *et al.* IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J Immunol 2007; 178: 2827-34.

[23] Wagner H. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Curr Opin Microbiol 2001; 5: 62-9.

[24] Ota T, Takeda K, Akiba H, *et al.* IFN-g mediated negative feedback regulation of NKT-cell function by CD94/NKG2. Blood 2005; 106; 184-92.

[25] Ikarashi Y, Mikami R, Bendelac A, *et al.* Dendritic cell maturation over-rules H-2D-mediated natural killer T (NKT) cell inhibition: critical role for B7 in CD1d-dependent NKT cell interferon g production. J Exp Med 2001; 194: 1179-86.

[26] Dimasi N, Biassoni R. Structural and functional aspects of the Ly49 natural killer cell receptors. Immunol Cell Biol 2005; 83: 1-8.

[27] Webster KE, Walters S, Kohler RE, *et al. In vivo* expansion of Treg cells with IL-2-mAb complexes:induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. J Exp Med 2009; 206: 751-60.

[28] Boyman O, Ramsey C, Kim DM, Sprent J, Surh CD. IL-7-anti-IL-7 mAb complex restore T cell development and induce homeostatic T cell expansion without lymphopenia. J Immunol 2008; 180: 7265-75.

[29] Yu KOA, Porcelli SA. The diverse functions of CD1d-restricted NKT cells and their potential for immunotherapy. Immunol Lett 2005; 100: 42-55.

[30] Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. Nat Rev Microbiol. 2007; 5: 405-17.

[31] Benton KA, Misplon JA, Lo C-Y, Brutkiewicz RB, Prasad SA, Epstein SL. Heterotypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or gd T cells. J Immunol 2001; 166: 7437-45.

[32] Youn H-J, Ko S-Y, Lee K-A, *et al.* A single nasal immunization with inactivated influenza virus and a-galactosylceramide induces long-term protective immunity without redirecting antigen to the central nervous system. Vaccine 2007; 15: 5189-98.

[33] Ho L-P, Denney L, Luhn K, Teoh D, Clelland C, McMichael AJ. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. Eur J Immunol 2008; 38: 1-10.

[34] Kamijuku H, Nagata Y, Jian X, *et al.* Mechanism of NKT cell activation by intranasal coadministration of a-galactosylceramide, which can induce cross-protection against influenza viruses. Mucosal Immunol 2008; 3: 208-18.

[35] Hachem P, Lisbonne M, Michel ML, *et al.* a-galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN-g. Eur J Immunol 2005; 35: 2793-802.

[36] Matsuda H, Suda T, Sato J, *et al.* a-galactosylceramide, a ligand of natural killer T cells, inhibits allergic airway inflammation. Am J Respir Cell Mol Biol 2005; 33: 22-31.

[37] Wakao H, Wakao R, Sakata S, Iwabuchi K, Oda A, Fujita H. *In vitro* induction of natural killer T cells from embryonic stem cells prepared by somatic cell nuclear transfer. FASEB J 2008; 22: 2223-31.

[38] Chackerian A, Alt J, Perera V, Behar SM. Activation of NKT cells protect mice from Tuberculosis. Infect Immun 2002; 70: 6302-9.

[39] Sada-Ovalle I, Chiba A, Gonzales A, Brenner MB, Behar SM. Innate Invariant NKT Cells Recognize *Mycobacterium tuberculosis*-Infected Macrophages, Produce Interferon-g, and Kill Intracellular Bacteria. PloS Pathog 2008; 4: e1000239.

[40] Kawakami K, Yamamoto N, Kinjo Y, *et al.* Critical role of Va14^{+} natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. Eur J Immunol 2003; 33: 3322-30.

[41] Hazlett LD, Li Q, Liu J, McClellan S, Du W, Barrett RP. NKT cells are critical to initiate an inflammatory response after *Pseudomonas aeruginosa* ocular infection in susceptible mice. J Immunol 2007; 179: 1138-46.

[42] Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M, *et al.* a-galactosylceramide-activated Va14 natural killer T cells mediate protection against murine malaria. Proc Natl Acad Sci USA 2002; 97: 8461-6.

[43] Duthie MS, Kahn SJ. During acute *Trypanosoma cruzi* infection highly susceptible mice deficient in natural killer cells are protected by a single alpha-galactosylceramide treatment. Immunology 2006; 119: 355-61.

[44] Kawakami K, Kinjo Y, Yara S, *et al.* Activation of Va14(+) natural killer T cells by a-galactosylceramide results in development of Th1 response and local host resistance in mice infected with *Cryptococcus neoformans*. Infect Immun 2001; 69:213-20.

[45] Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural killer T cell activation inhibits hepatitis B virus replication *in vivo*. J Exp Med 2000; 192: 921-30.

[46] Johnson TR, Hong S, Van Kaer L, Koezuka Y, Graham BS. NKT cells contribute to expansion of CD8^{+} T cells and amplification of antiviral immune responses to respiratory syncytial virus. J Virol 2002; 76: 4294-303.

[47] Mizuno M, Masumura M, Tomi C, *et al.* Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice. J Autoimmun 2004; 23: 293-300.

[48] Chiba A, Oki S, Miyamoto K, Hashimoto H, Yamamura T, Miyake S. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of a-galactosylceramide. Arthritis Rheum 2004; 50: 305-13.

[49] Takagi D, Iwabuchi K, Maeda M, *et al.* Natural killer T cells ameliorate antibody-induced arthritis in macrophage migration inhibitory factor transgenic mice. Int J Mol Med 2006; 18: 829-36.

[50] Ueno Y, Tanaka S, Sumii M, *et al.* Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of Va14 natural killer T cells in mice. Inflamm Bowel Dis 2005; 11: 35-41.

Received: May 10, 2009 Revised: June 21, 2009 Accepted: June 25, 2009

向精神薬の治験の進め方

——抗うつ薬の臨床試験を中心に——

●

中林哲夫

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はじめに

世界保健機構（WHO: World Health Organization）による世界疾病負荷調査（GBD: The Global Burden of Disease Study）では、精神疾患は、主な機能障害の原因の1つであることが指摘（WHO (2009)；WHO (2004 update2008)）され、疾病全体の中でも最も重要な疾患として位置づけられている。また海外における中枢領域の臨床試験の登録件数は悪性疾患領域についで2位であり、なかでもうつ病と統合失調症を対象とした試験の登録件数が多く（Karlberg (2008)）、当該領域の新薬開発は活発である。

臨床試験においては、科学的に有効性と安全性を示す必要があることは言うまでもない。本邦における向精神薬の治験は、これまでは実薬対照非劣性試験または同等性治験が中心であった。しかし、ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use、日米EU医薬品規制調和国際会議）のE10ガイドライン（厚生省医薬局審査課長（2001）でも指摘されているとおり、非劣性試験または同等性試験は、内部妥当性を示す指標が存在せず無効同等の可能性が排除できないことより、有効性に関する結果解釈が困難となる問題がある。近年は、塩酸セルトラリンやミルタザピンのようにプラセボ対照試験により承認された向精神薬が登場しており、明確なエビデンスが蓄積されるようになったと言える。

しかし、既承認の統合失調症治療薬の約4分の1が、そして抗うつ薬の約半数がプラセボ対照試験に失敗しており（Laughren (2001)）、プラセボに対する優越性を示すことも容易なことではない。また、初回の臨床試験から医薬品として承認に至るまで割合は10%未満と高

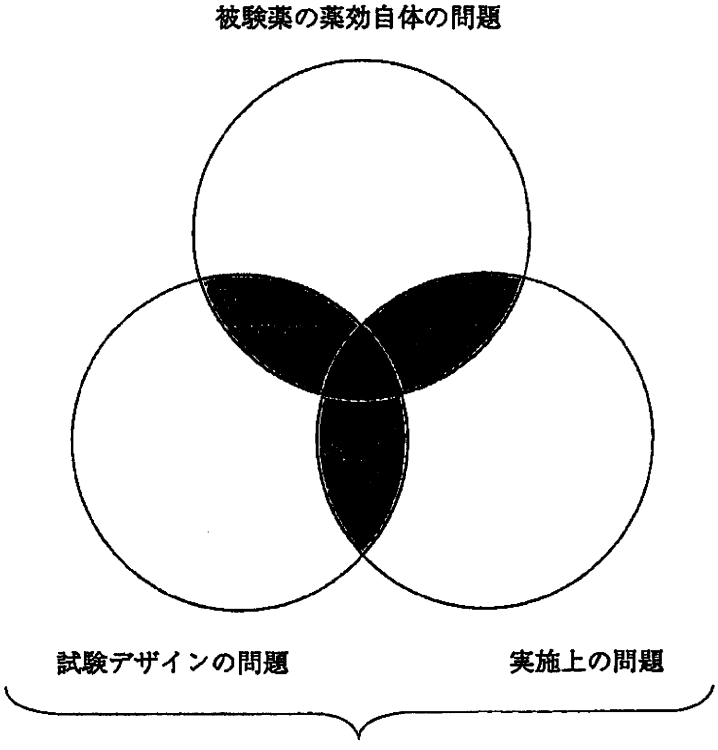
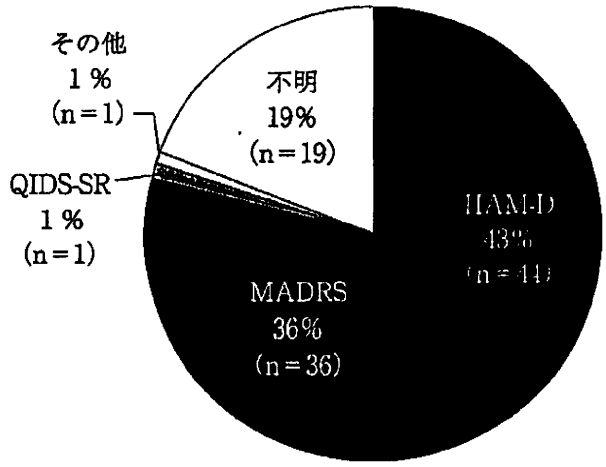


図１　臨床試験の成否に影響を及ぼす要因

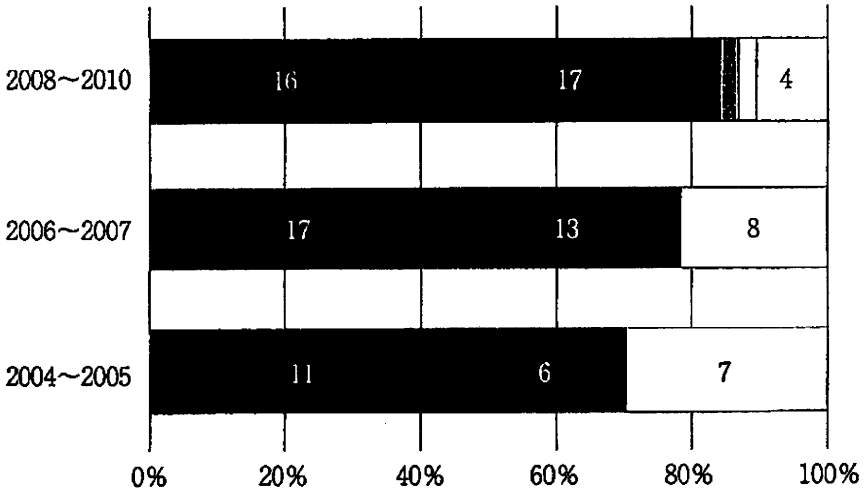
くはなく（Kola, et al (2004)）、多くの場合は被験薬の有効性を示すことができない（Frank, et al (2003)）ことが原因する。臨床試験の成否には、薬効自体の問題もあるが、試験デザインや試験実施上の問題等の方法論的問題も影響（図１）する。適切に臨床試験を実施していくためには、よく計画された試験計画が必要なだけではなく、治験を行うわれわれ臨床医が実施上の問題についても十分理解する必要がある。このため、本稿では、抗うつ薬の治験を例にその有効性評価に影響を及ぼす実施上の問題を取り上げ説明する。

抗うつ薬の臨床試験の特徴

米国国立衛生研究所（NIH: National Institutes of Health）の臨床試験登録データベース（http://www.clinicaltrials.gov/）には、企業主導によるうつ病を対象としたプラセボ対照の無作為化二重盲検比較試験（2004年から2010年1月までに開始された第Ⅱ相もしくは第Ⅲ相試験）のうち、安全性評価を主目的とした試験およびランダム化治療中止試験を除外すると、101試験が登録されている。これらの臨床試験の有効性の主要評価項目は、HAM-D（Hamilton Depression Rating Scale）またはMADRS（Montgomery-Åsberg Depression Rating Scale）が多く使用されている（図2a)。また最近では、HAM-Dと比較してMADRSの主要評価項目としての使用が増加する傾向もある（図2b)。対象となる被験者の重症度は、選択基準において症状評価尺度のカットオフポイントとして設定されるが、各カットオフポイントはHAM-D（17項目）では20点以上、MADRSでは22点以上と設定されることが多い（図3)。登録されている101試験のうち、選択基準および主要評価項目で設定されている症状評価尺度がいずれも公開されているのは37試験のみであるが、このうち12試験は選択基準および主要評価項目では同一の症状評価尺度が使用され、残り25試験は選択基準と主要評価項目とで異なる評価尺度（例えば、選択基準はHAM-D、主要評価項目はMADRS）が用いられている。以上の試験の投薬期間は、多くが6週週間もしくは8週間と設定されている（表1)。



(a) 2004年～2010年1月



(b) 開始年別（数値は試験数を表示）

図2　主要評価項目に使用された症状評価尺度

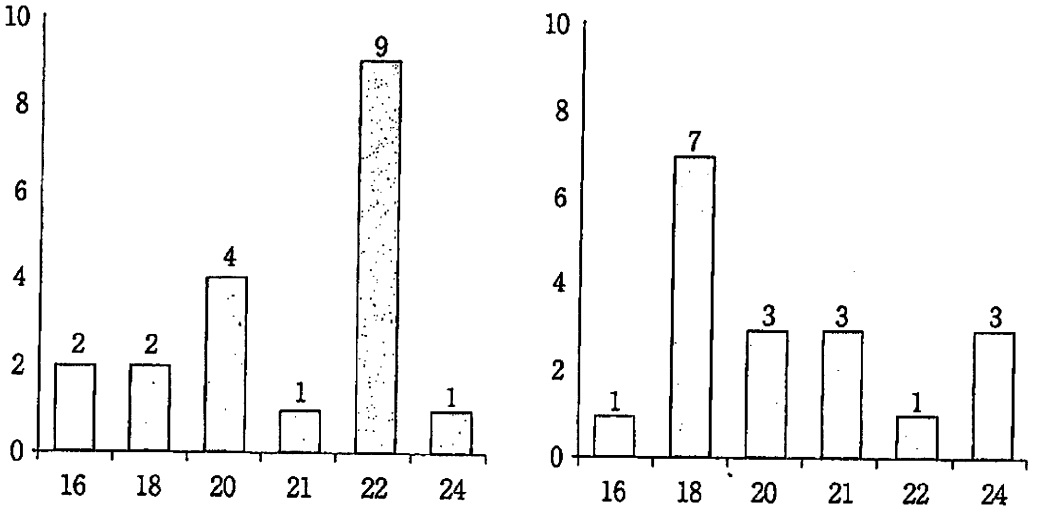


図3　選択基準における症状評価尺度のカットオフ・ポイント
(2004年-2010年1月)

投与期間	試験数
4週間	2
6週間	18
8週間	53
9～10週間	6
11～12試験	3
不明	19
合計	101

表1　各試験ごとの投与期間の分布
(2004年～2010年1月)

有効性評価に関わる問題

●プラセボに対する反応性

向精神薬の臨床試験においては、主要な有効性評価は、症状評価尺

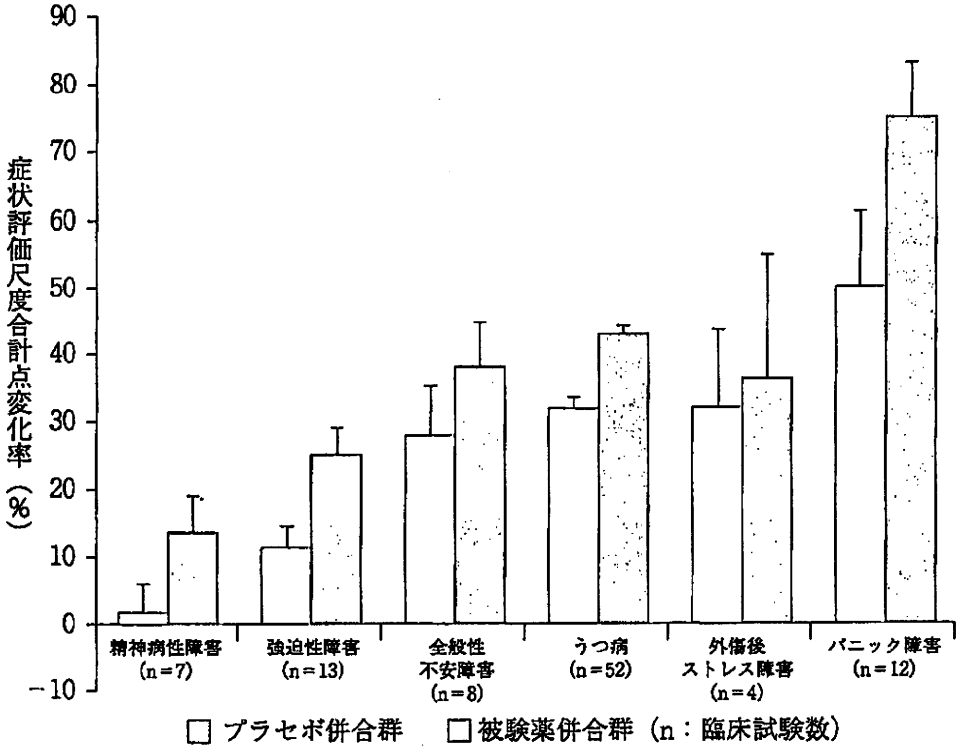


図４　疾患領域ごとのエフェクトサイズ

Knan, et al（2005）改編。

度の合計点のベースラインから最終観察時までの変化量が検討される。薬効はエフェクトサイズ（被験薬群とプラセボ群の差）が検討されるため、当然のことながらプラセボに対する反応性が影響する。精神疾患領域のなかでは、うつ病や全般性不安障害においてプラセボ反応性が高いことが知られている（図4）。プラセボ反応性に影響する要因についてもさまざまな検討（Walsh, et al（2002）；Fava, et al (2003)）が行われているが、明確にはなっていない。治験薬に対しても、被験者のみならず担当医師も新たな治療としての効果を期待したバイアスが発生（Marks（2009））するため、このような事実を把握したうえで、より客観的に症状評価を行うことが重要である。症状評価は臨床試験の質に大きく影響するが、詳細は別章（樋口「日本の治験の現状と課題」）で解説する。