Generation of mCD1d Mutants

Mouse CD1d mutants were generated using Quick Change II Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies) according to the manufacturer's instructions with the primers indicated below. Mutated constructs were purified with the Qiagen Miniprep Kit (Qiagen) and the presence of the mutation confirmed by sequencing. The mutated birA-tag mCD1d/β2m were expressed and purified using the same method described above for mCD1d/β2m.

Primer sequences: L84V 5′-ttaccagggacatacaggaagtagtcaaaatgatgtcacc-3′; L84V_antisense 3′-aatggtccctgtatgtccttcatcagttttactacagtgg-5′; L84F 5′-accagggacatacaggaattcgtcaaaatgatgtcacc-3′; L84F_antisense 3′-tggtccctgtatgtccttaagcagttttactacagtgg-5′; V149L 5′-cttggttagacttgcccatcaaattgctcaacgctg-3′; V149L_antisense 3′-gaaccaatctgaacgggtagtttaacgagttgcgac-5′; L150V 5′-cttgcccatcaaagtggtcaacgctgatcaagg-3′; L150V_antisense 3′-gaacgggtagtttcaccagttgcgactagttcc-5′; M69A 5′-gtgggagaagttgcagcatgcgtttcaagtctatcgagtc-3′; M69A_antisense 3′-gtgggagaagttgcagcatgcgtttcaagtctatcgagtc-5′; M162A 5′-caagtgcaaccgtgcaggcgctcctgaatgacacct-3′; M162A_antisense 3′-caagtgcaaccgtgcaggcgctcctgaatgacacct-5′.

Cell Lines and Culture Conditions

A20/CD1d cells are derived from murine B cell lymphoma A20 (American Type Culture Collection, Rockville, MD), with stable expression of wild type mouse CD1d [7,32]. A20/CD1d and iNKT hybridomas cell lines Hy2C12, Hy1.2, and Hy1.4 were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 mg/ml each of penicillin and streptomycin, 50 mM 2-mercaptoethanol, and 10% FBS.

A20 Lipid Antigen Presentation Assay

Mouse iNKT cell hybridoma 1.2 (Vα14/Vβ8.2) has been described previously [3,6]. 1×10^6 A20/CD1d cells expressing wild-type mCD1d were cultured in complete medium containing indicated amounts of lipid antigens or vehicle (56 mg/ml sucrose, 7.5 mg/ml histidine, and 5 mg/ml Tween-20 [pH 7.2]) overnight. On the second day of culture, A20/CD1d were collected, washed thoroughly, and 1×10^5 APCs were seeded in the presence of 5×10^4 iNKT cell hybridomas per well in a 96-well plate for 24 h, and IL-2 in the supernatant was measured by ELISA according to the manufacturer's instructions (BD Biosciences).

APC Free Antigen Presentation Assay

Stimulation of mouse iNKT cell hybridomas on microwell plates coated with soluble mCD1d was carried out according to published protocols [3,6,33], with a few modifications. Briefly, the indicated amounts of compounds were incubated for 24 h in microwells that had been coated with 1.0 μg of mCD1d. After washing, 5×10^4 iNKT cell hybridoma cells were cultured on the plate for 16 h, and IL-2 in the supernatant was measured by ELISA according to the manufacturer's instructions (R&D systems).

Surface Plasmon Resonance

Surface Plasmon Resonance studies using a refolded and biotinylated Vα14Vβ8.2 TCR were carried out as previously reported [11] with 300–500 response units (RU) of biotinylated mCD1d-vehicle or mCD1d-ligand immobilized on the chip. Serial dilutions of Vα14Vβ8.2 were injected with increasing concentrations (0.002–1.25 μM) over a streptavidin CAPture chip (GE Healthcare). The experiment was performed twice. Loading efficiency was measured by immobilizing the biotinylated mCD1d-ligand (after incubation for 16 h in the presence of 1 μg/ml of α-GalCer) complex on a CAPture chip (400–500 RU) followed by the injection of a saturating concentration (1 μM) of the Fab portion of the mCD1d-α-GalCer specific antibody L363 [20]. 100% glycolipid loading efficiency is achieved when the increase in RU upon Fab binding is equal to the RU of CD1d-glycolipid coated on the chip, as mCD1d-glycolipid and Fab have a comparable molecular weight.

Supporting Information

Figure S1 Shake omit map of the Glc-DAG-s2 ligand. Side (A) and top (B) view of the mCD1d binding groove with the ligand in yellow. A shake-omit Fo-Fc map contoured at 2σ is shown as a green mesh around the ligand.
(TIF)

Figure S2 Modeling of Gal-DAG-s2 in the ternary complex. Detailed view of the Gal-DAG-s2 ligand at the mCD1d-TCR interface with the ligand in green, mCD1d in grey, and the iNKT TCR α chain in cyan. The different position of the 4′-OH group for Glc-DAG-s2 is shown in yellow for comparison. Distances between the 4′-OH group of the antigen and Asn30α on the TCR and Gly155 on mCD1d are shown as dashed lines with the corresponding length expressed in Å.
(TIF)

Table S1 Molecular contacts in the TCR complex. The program CONTACT [26] was used to analyze the molecular interactions within the complexes. Cutoffs of 4 Å (van der Waals interactions), 3.5 Å (hydrogen bonds), and 4.5 Å (salt bridges) were applied.
(DOC)

Acknowledgments

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: EG EDY YL YK DMZ. Performed the experiments: EG EDY NT BP JW. Analyzed the data: EG EDY YL YK MK DMZ. Contributed reagents/materials/analysis tools: PI. Wrote the paper: EG MK DMZ.

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REVIEW

*i*NKT cells in microbial immunity: recognition of microbial glycolipids

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ABSTRACT

Natural killer T cells expressing an invariant T cell antigen receptor (*i*NKT cells) are cells of the innate immune system. After recognizing glycolipid antigens presented by CD1d molecules on antigen presenting cells (APCs), *i*NKT cells rapidly produce large quantities of cytokines, thereby stimulating many types of cells. Recent studies have described several mechanisms of *i*NKT cell activation and the contribution of these cells to antimicrobial responses. *i*NKT cells can be activated by endogenous antigens and/or inflammatory cytokines from APCs. However, *i*NKT cells also recognize certain microbial glycolipids by their invariant T cell antigen receptor (TCR), and they contribute to pathogen clearance in certain microbial infections. These findings indicate that the *i*NKT TCR is useful for detecting certain microbial pathogens. Moreover, recent studies suggest that *i*NKT cell glycolipid antigens may be useful in antimicrobial therapy and vaccines.

Key words antigen, CD1d, glycolipid, *i*NKT cell.

Natural killer T cells are lymphocytes that express both $\alpha\beta$ TCRs and NK receptors (1–4). These T cells have several unique features. First, the majority of NKT cells express an invariant (*i*) TCRα chain, which is encoded by a Vα14-Jα18 rearrangement in mice and a Vα24-Jα18 rearrangement in humans (1–4). These cells are referred to as Vα14*i*NKT cells and Vα24*i*NKT cells, respectively; collectively they are referred to as *i*NKT cells (4). Second, in contrast to conventional T cells, which recognize peptide antigens presented by MHC class I or class II, *i*NKT cells recognize glycolipids presented by the CD1d molecule. Third, *i*NKT cells rapidly (within 1–2 hr) produce large quantities of cytokines (including IFNγ and IL-4) following glycolipid antigen recognition by their invariant TCRs. Consequently, *i*NKT cells stimulate many types of cells including APCs, NK cells, conventional T cells and B cells. Because of these unique features, *i*NKT cells are able to participate in various immune responses including

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List of Abbreviations: αGalCer, α-galactosylceramide; APCs, antigen presenting cells; β2m, β2 microglobulin; BbGL, *Borrelia burgdorferi* glycolipid; *B. burgdorferi*, *Borrelia burgdorferi*; CD, cluster of differentiation; *C. neoformans*; *Cryptococcus neoformans*; COPD, chronic obstructive pulmonary disease; *C. pneumoniae*, *Chlamydophila pneumoniae*; DAG, diacylglycerol; DCs, dendritic cells; *E. coli*, *Escherichia coli*; GalA, galacturonic acid; GalAGSL, galacturonic acid containing glycosphingolipid; GlcA, glucuronic acid; GlcAGSL, glucuronic acid containing glycosphingolipid; GSL, glycosphingolipid; *i*, invariant; IAV, influenza A virus; IFN, interferon; IL, interleukin; *i*NKT cells, natural killer T cells expressing an invariant T cell antigen receptor; *L. donovani*, *Leishmania donovani*; *L. monocytogenes*, *Listeria monocytogenes*; LPG, lipophosphoglycan; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein; MDSCs, myeloid derived suppressor cells; MHC, major histocompatibility complex; MIP-2, macrophage inflammatory protein-2; *M. tuberculosis*, *Mycobacterium tuberculosis*; *N. aromaticivorans*, *Novosphingobium aromaticivorans*; NKT, natural killer T cell; *P. aeruginosa*, *Pseudomonas aeruginosa*; PBC, primary biliary cirrhosis; PDC-E2, pyruvate dehydrogenase complex E2; PIM_4, phosphatidylinositol tetramannosides; *S. capsulata*, *Sphingomonas capsulata*; *S. pneumoniae*, *Streptococcus pneumoniae*; Spp., subspecies; *S. typhimurium*, *Salmonella typhimurium*; *S. yanoikuyae*, *Sphingobium yanoikuyae*; TCR, T cell antigen receptor; TLR, toll like receptor; TNF tumor necrosis factor.

tumor immunity, microbial immunity, and initiation and/or regulation of autoimmune diseases and asthma.

CD1 is an MHC class I-like antigen presenting molecule (5–8). Humans express five CD1 proteins (CD1a-e), but mice and rats have CD1d only (6–8). Similar to MHC class I, CD1 molecules have three extracellular domains (α1, α2 and α3), which bind to β2 microglobulin. CD1 molecules have deep, narrow and hydrophobic antigen-binding grooves that are suitable for lipid antigen presentation (5–8). CD1a, CD1b and CD1c proteins present lipid antigens from mycobacteria or endogenous lipids to CD1 restricted T cells, and CD1e functions in antigen processing (6–8). The CD1d protein is necessary for thymic development of *i*NKT cells and glycolipid antigen presentation to these cells (1–4).

Many studies have shown that *i*NKT cells participate in the response to various microbial pathogens (2, 4, 9, 10). *i*NKT cell deficient mice are susceptible to certain microbial pathogens including bacteria, fungi, parasites and viruses (2, 4, 9, 10). However, in some cases, *i*NKT cells do not play a role in the clearance of microbes, and they may have a detrimental impact on the host (2, 4, 9, 10). In this article, we review recent findings on the role of *i*NKT cells in the response to microbial pathogens and the mechanisms by which *i*NKT cells contribute to antimicrobial responses. We also describe how *i*NKT cell TCR contributes to the response to certain microbial pathogens by recognizing microbial glycolipid antigens. Furthermore, we summarize data indicating that *i*NKT cell glycolipid antigens may be useful as stimulatory agents that augment immune responses to certain microbial pathogens.

Natural killer T cells stimulate the innate immune response to microbial pathogens

Natural killer T cells expressing an invariant T cell antigen receptor are considered innate type lymphocytes because of their rapid cytokine production and NK receptor expression. *i*NKT cells participate in the response to certain microbial pathogens in the early phase of infection. For example, a role for NKT cells was shown in mice infected with *S. pneumoniae*, a leading causative agent of community acquired pneumonia and secondary bacterial pneumonia following influenza virus infection (11). Jα18 deficient mice, which specifically lack *i*NKT cells due to their inability to form the invariant TCRα chain (12), are highly susceptible to *S. pneumoniae* infection, showing high bacterial counts in the lungs and a high mortality rate (11). Neutrophil numbers and the amount of chemokines/cytokines in the lungs are markedly lower in Jα18 deficient mice compared to wild type mice after intratracheal infection with *S. pneumoniae* (11). Furthermore, data suggest that IFNγ derived from *i*NKT cells plays an important role in recruiting neutrophils to the lungs through increased production of MIP-2 and TNF by CD11b^{bright} cells after *S. pneumoniae* infection (13) (Fig. 1). These results indicate that *i*NKT cells contribute to the clearance of *S. pneumoniae* by enhancing neutrophil recruitment to the lungs.

Mouse *i*NKT cells are capable of inhibiting *M. tuberculosis* growth in macrophages *in vitro* (14). IFNγ derived from *i*NKT cells stimulates *M. tuberculosis* infected macrophages to synthesize nitric oxide, which inhibits bacterial replication (14). IL-12 and IL-18 are both involved in this response. These data suggest that *i*NKT cells inhibit the growth of intracellular microbes by stimulating infected APCs (Fig. 2). It has previously been reported that mice deficient in CD1d, which lack both *i*NKT cells and NKT cells with diverse TCRs due to an inability of these cells to differentiate in the thymus in the absence of CD1d (15–17), are not more susceptible to *M. tuberculosis* infection (18, 19). Similarly, Jα18 deficient mice are not more susceptible to *M. tuberculosis* infection (20, 21). However, in lethally irradiated mice, adoptive transfer of *i*NKT cells decreases bacterial numbers in the lungs following aerosol infection by *M. tuberculosis* (14), suggesting that *i*NKT cells inhibit the growth of this bacterium. Because CD1d expressing cells are found in granulomas of tuberculosis patients (22), *i*NKT cells may play a role in the response to *M. tuberculosis* in humans.

Natural killer T cells stimulate the acquired immune response to microbial pathogens

Cryptococcus neoformans is a fungal pathogen that primarily infects the lungs, but it can disseminate to the central nervous system and cause meningitis in immunocompromised patients. *i*NKT cells have been shown to accumulate in the lungs in the early phase (day 3 post-infection) of *C. neoformans* infection in a CCL-2 (MCP-1) dependent manner (23). Jα18 deficient mice show a significantly attenuated Th1 response (23), and Th1 is a critical component of the response to *C. neoformans*. Consistent with this, Jα18 deficient mice take longer to clear *C. neoformans* from their lungs than do wild type mice (23). These data suggest that *i*NKT cells contribute to the development of an effective Th1 response to *C. neoformans*. An influence of *i*NKT cells on acquired immunity was also reported in a mouse pneumonia model using *Chlamydophila* (formerly called *Chlamydia*) *pneumoniae*, an intracellular bacterium that causes acute and chronic respiratory diseases. Following intranasal infection with *C. pneumoniae*, *i*NKT cells accumulate in the lungs during the early phase (day 3 post infection) and express intracellular IFNγ (24, 25). CD8α^+ DCs from Jα18 deficient mice show lower

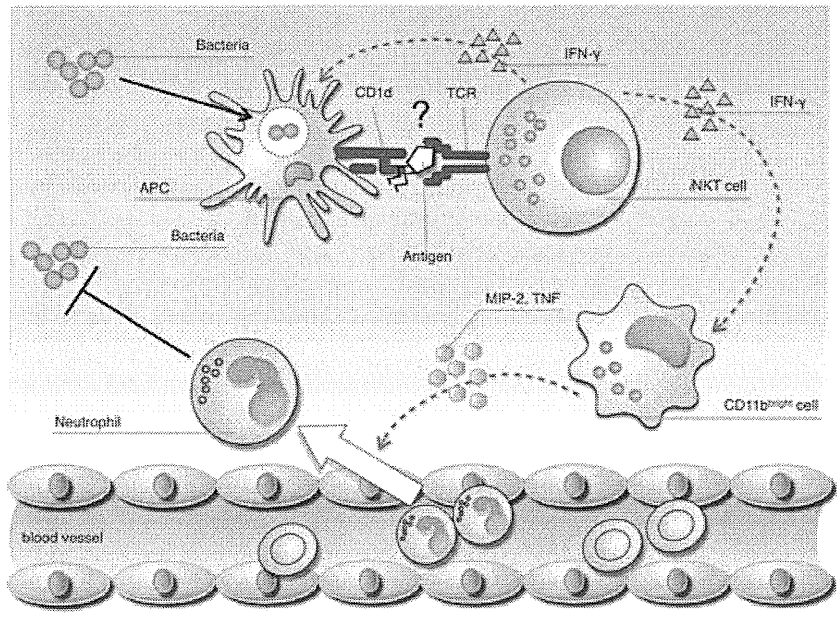

Fig. 1. *i*NKT cells contribute to neutrophil recruitment during bacterial infection. In the early phase of *S. pneumoniae* infection of the lungs, *i*NKT cells contribute to neutrophil recruitment by stimulating MIP-2 and TNF release from $CD11b^{bright}$ cells. IFNγ is important in this response, and data indicate that *i*NKT cells are a source of IFNγ. It is unknown if recognition of antigens presented by CD1d is involved in this response.

CD40 expression and intracellular IL-12 compared to wild type mice, which results in decreased IFNγ production by $CD4^+$ and $CD8^+$ T cells (26). IL-12 production by $CD8\alpha^+$ DCs is dependent on IFNγ and CD40-CD40L interaction (26). These findings suggest that *i*NKT cells enhance the Th1 response by stimulating DCs via IFNγ and co-stimulatory molecules during certain microbial infections (Fig. 3).

Natural killer T cells expressing an invariant T cell antigen receptor also participate in the response to viruses. Jα18 deficient mice and CD1d deficient mice are highly susceptible to influenza A virus, showing high virus titers and high mortality (27). In *i*NKT cell deficient mice, MDSCs expand and IAV specific CD8 T cells are suppressed (27). Adoptively transferring *i*NKT cells into Jα18 deficient mice, but not into CD1d deficient mice, restores IAV specific CD8 T cells and increases the survival rate by diminishing the suppressive function of MDSCs (27). In addition, *in vitro* experiments have shown that CD1d and CD40-CD40L interaction inhibit MDSC function (27). These data show that *i*NKT cells play an important role in the development of an effective IAV specific immune response by directly inhibiting the suppressive function of MDSCs (Fig. 4). MDSCs are present in the peripheral blood of IAV infected patients. However, suppression of the human T cell response by MDSCs from IAV infected patients is reduced by *i*NKT cell activation (27). These results indicate that *i*NKT cells may play a role in the response to certain microbial pathogens in humans.

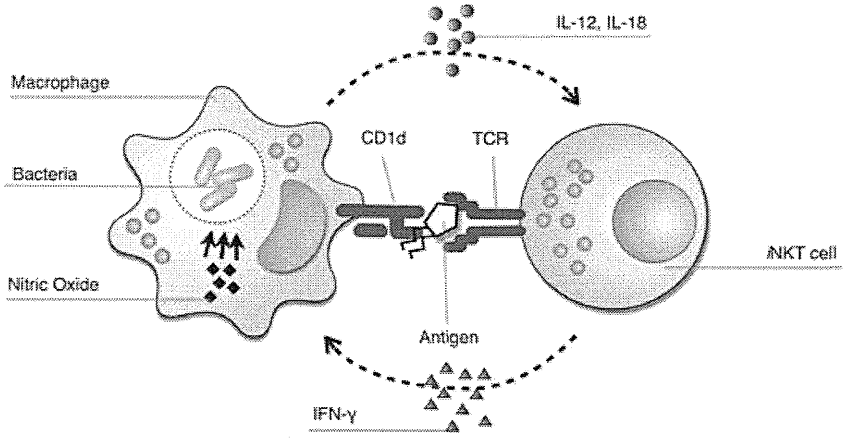

Fig. 2. *i*NKT cells enhance the killing of bacteria in macrophages. *i*NKT cells stimulate nitric oxide synthesis in *M. tuberculosis* infected macrophages by IFNγ secretion, enhancing the killing of bacteria. CD1d mediated antigen recognition and cytokines (IL-12 and IL-18) are involved in this response.

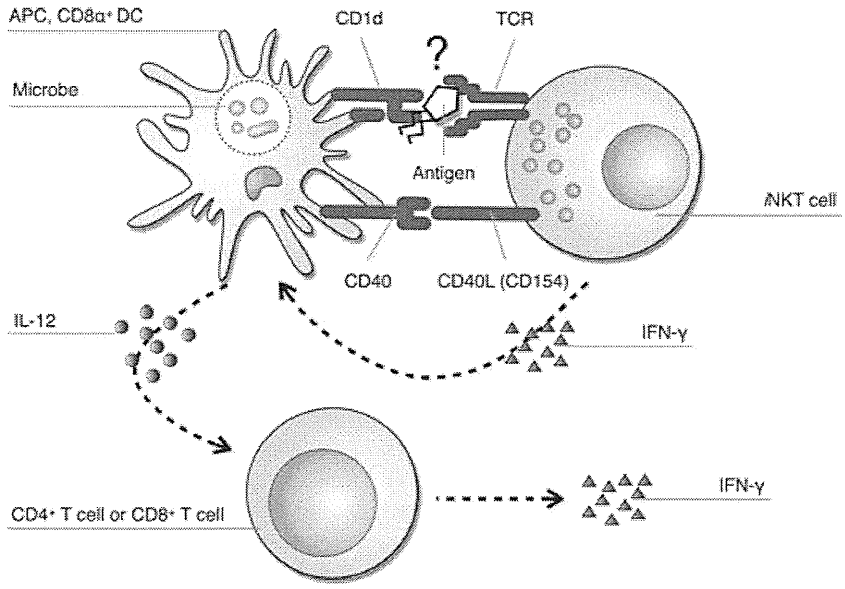

Fig. 3. *i*NKT cells augment IFNγ production by CD4 and CD8 T cells. *i*NKT cells enhance the Th1-type response during *C. pneumoniae* and *C. neoformans* infections. During *C. pneumoniae* infection, CD8α^+ DCs, which are activated by *i*NKT cells, produce IL-12 and stimulate IFNγ production by CD4 and CD8 T cells. CD40/CD40L interactions and IFNγ are involved in the activation of CD8α^+ DCs by *i*NKT cells. It is unknown if recognition of antigens presented by CD1d is involved in this response.

Natural killer T cells participate in the pathogenesis of lung inflammation during viral infections

Natural killer T cells expressing an invariant T cell antigen receptor have been shown to participate in the pathogenesis of infection induced inflammation in a mouse model of chronic inflammatory lung disease that resembles asthma and COPD. Mice infected with Sendai virus exhibit chronic airway disease that manifests as mucous cell metaplasia and airway hyper-reactivity (28). IL-13 production by macrophages is necessary in this response. The interaction of *i*NKT cell TCRs with CD1d on macrophages and IL-13 derived from *i*NKT cells is necessary to activate macrophages to produce IL-13 (28). Importantly, lung tissue from patients with severe COPD exhibits mucous cell metaplasia and an increased number of IL-13$^+$ CD68$^+$ macrophages compared to non-COPD controls (28). Moreover, Vα24*i*NKT cells are increased in COPD subjects (28). This study suggests that *i*NKT cells are involved in chronic inflammation in certain viral infections.

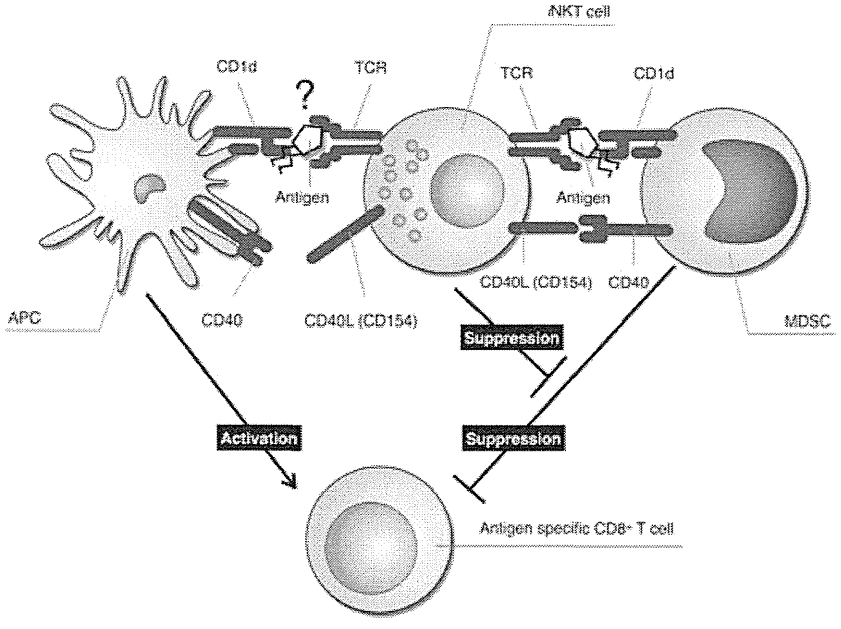

Fig. 4. *i*NKT cells augment antigen specific CD8 T cell responses by inhibiting the suppressive function of MDSCs. Following influenza A virus infection, *i*NKT cells inhibit the suppressive function of MDSCs and enhance antigen specific CD8 T cell responses. CD1d recognition and the CD40/CD40L interaction are involved in this response. It is unknown if the recognition of antigens presented by CD1d is involved in this response.

Natural killer T cells stimulation by inflammatory cytokines and/or endogenous antigens

Natural killer T cells expressing an invariant T cell antigen receptor participate in the response to various microbial pathogens. How do these cells respond to microbes? Recent studies have revealed several mechanisms of *i*NKT cell activation during microbial infection. *i*NKT cells in the liver produce IFNγ 2–3 days after intravenous infection with *S. typhimurium*, although this production is greatly inhibited by anti-IL-12 or anti-CD1d antibodies (29). LPS containing *S. typhimurium* extract and purified LPS, but not the lipid fraction of *S. typhimurium*, stimulates IFNγ release from *i*NKT cells in an IL-12 dependent manner (29). These results show that *i*NKT cells can be activated by a combination of IL-12 produced by APCs and weak TCR stimulation by endogenous antigens in the presence of LPS. However, in some cases, inflammatory cytokines are sufficient to stimulate *i*NKT cells to release IFNγ. *i*NKT cells produce IFNγ in response to *E. coli* LPS when cultured with DCs from wild type mice, but not with DCs from IL-12 or IL-18 deficient mice (30). Interestingly, DCs from CD1d deficient mice also induce IFNγ production by *i*NKT cells (30). Furthermore, *i*NKT cells produce IFNγ in response to both IL-12 and IL-18 *in vitro*, even in the absence of DCs (30). Similarly, it has been reported that CD1d mediated stimulation is dispensable for *i*NKT cell activation in response to CpG oligodeoxynucleotides and mouse cytomegalovirus (31–33). Thus, in some cases, inflammatory cytokines are sufficient for *i*NKT cell activation. These studies show that *i*NKT cells produce cytokines during microbial infection by activating APCs even in the absence of microbial glycolipid antigens. This feature allows *i*NKT cells to respond to various microbial pathogens, including viruses that do not have glycolipid antigens. We speculate that this feature is very important for the *i*NKT cell response to certain microbial pathogens. However, in some cases, *i*NKT cells do not contribute to the clearance of microbes despite their cytokine production (29, 34, 35). These findings indicate that there is another mechanism of *i*NKT cell activation in response to microbial pathogens.

Microbial glycolipid antigens for natural killer T cells

The synthetic antigen αGalCer was the first glycolipid shown to be presented by CD1d and thereby stimulate *i*NKT cell TCR (36) (Fig. 5). αGalCer is a very close structural analog of a glycolipid isolated from a marine sponge (37, 38). A unique feature of this glycolipid is its unusual α linkage of the sugar to the lipid (36). Using αGalCer and its analogues, the features and functions of *i*NKT cells have been elucidated (1–4). However, it remained unknown if the *i*NKT cell TCR can recognize microbial lipids. A subset of mouse and human *i*NKT cells respond to a purified glycolipid extracted from *Mycobacterium* cell wall containing PIM_4 (39). Amprey *et al.* showed that a LPG from *L. donovani* simulates a subset of *i*NKT cells in the liver (40). Compared to wild type mice, CD1d deficient mice are more susceptible to *L. donovani* infection, showing increased parasite burden and decreased granuloma formation (40). The *L. donovani* glycolipid LPG binds to CD1d and stimulates a subset of *i*NKT cells in the liver *in vivo* (40). However, only a small portion of *i*NKT cells respond to PIM_4 or LPG (39, 40). Therefore, it remains to be determined if the majority of *i*NKT cells detect microbial glycolipids.

The invariant T cell antigen receptor of natural killer T cells recognizes bacterial glycosphingolipids

We and other groups found that the *i*NKT cell TCR recognizes GSL from *Sphingomonas* spp (41–43). *Sphingomonas* are Gram-negative bacteria that are abundant in the environment (both soil and ocean) (44) and also present in human intestines (45). *Sphingomonas* spp. lack LPS, but instead have a GSL with a monosaccharide, GalA or GlcA (41, 46–48) (Fig. 5). The *Sphingomonas* GSL with GalA and the GSL with GlcA are called GalAGSL and GlcAGSL, respectively (Fig. 5). The structures of the *Sphingomonas* GSLs are very similar to that of αGalCer, including an unusual α-linkage of the sugar to the lipid (41, 46–48). GalAGSL and GlcAGSL bind to mouse CD1d and stimulate Vα14*i*NKT cells (41–43). The activation of Vα14*i*NKT cells by *Sphingomonas* GSL is independent of TLR mediated APC activation and IL-12 (41, 42), indicating that these glycolipids stimulate Vα14*i*NKT cell TCRs directly. Moreover, CD1d tetramers loaded with *Sphingomonas* GSL detect the majority of *i*NKT cells, and these reactive cells are absent in Jα18 deficient mice and CD1d deficient mice (41–43). Importantly, the *i*NKT cell response to *Sphingomonas* GSLs is conserved between mice and humans (41, 42). Jα18 deficient mice and CD1d deficient mice have more bacteria in their livers and lungs after *S. yanoikuyae* and *S. capsulata* infection than do wild type mice (41, 42). These results show that *Sphingomonas* GSLs are bacterial antigens that can stimulate *i*NKT cell TCR, suggesting that recognition of microbial antigens may contribute to the host's protection against microbial pathogens. This is the first microbial antigen that has been shown to stimulate the majority of *i*NKT cells. Considering that *Sphingomonas* spp. are found in the ocean, they might have been in the marine sponge

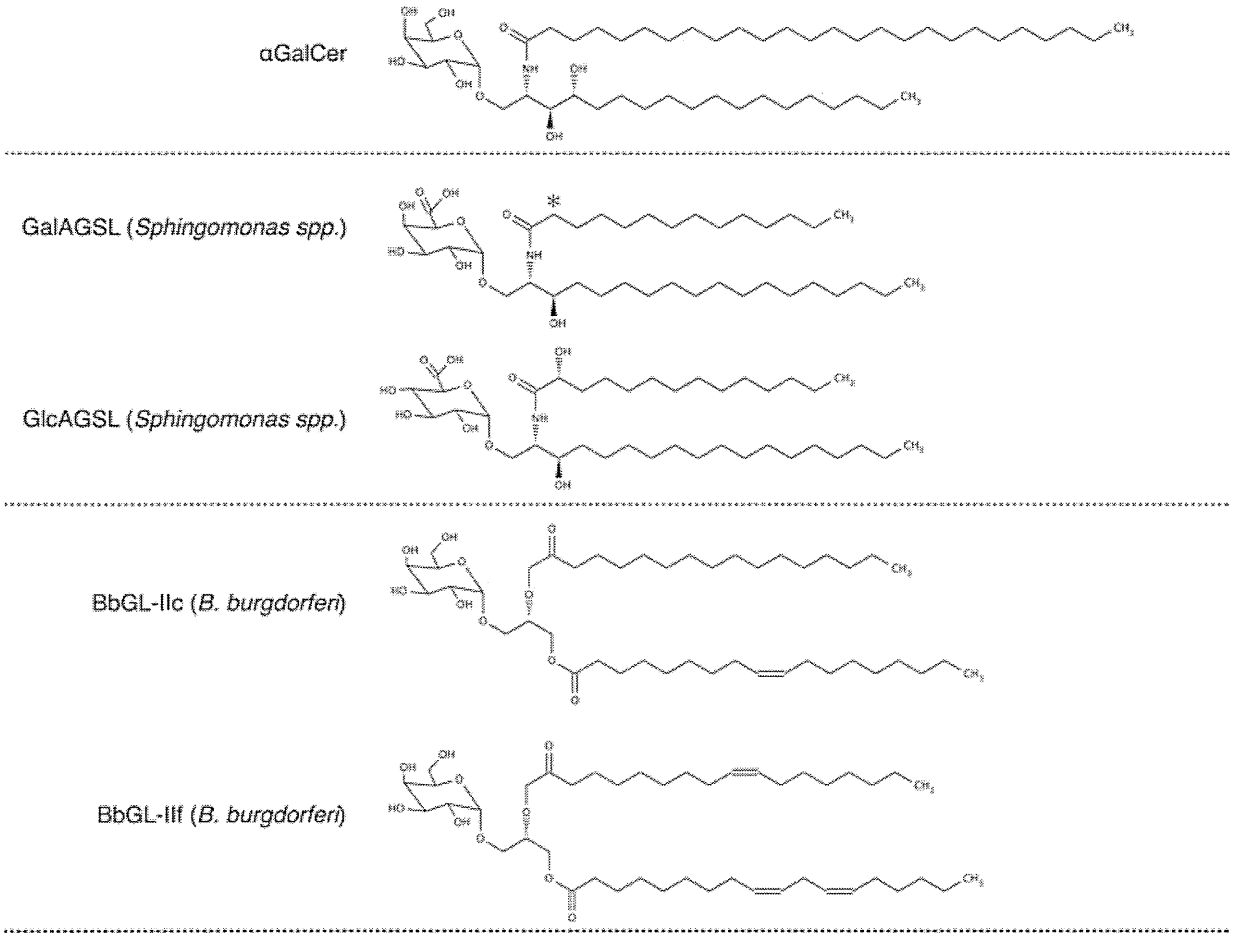

Fig. 5. Structures of bacterial glycolipid antigens recognized by *i*NKT cells. The structures of αGalCer (top), GalAGSL and GlcAGSL from *Sphingomonas* spp. (middle), and the *B. burgdorferi* glycolipids (BbGL)-IIc and BbGL-IIf (bottom) are shown. The asterisk indicates that the 2-hydroxyl on the acyl chain of GalAGSL is sometimes present in bacteria.

from which the original version of αGalCer was isolated. However, *Sphingomonas* is not highly pathogenic to humans. Also, GSLs are limited to *Sphingomonas* spp. and related bacteria. It remains unknown if pathogenic microbes have antigens for *i*NKT cells.

Natural killer T cells recognize bacterial diacylglycerol glycolipids

More recently, we found that *i*NKT cells recognize glycolipids from *B. burgdorferi*, the causative agent of Lyme disease (49). *B. burgdorferi* has two glycolipids: BbGL-I and BbGL-II. BbGL-I is a cholesterol-containing glycolipid and BbGL-II is an α-galactosyl DAG (50). BbGL-II, but not BbGL-I, binds to CD1d and stimulates *i*NKT cells (49). BbGL-II purified from *B. burgdorferi* contains a mixture of several different fatty acids, a palmitic acid ($C_{16:0}$) and an oleic acid ($C_{18:1}$) being the most common (50). In a test of several chemically synthesized variants of BbGL-II, BbGL-IIc, which contains an oleic acid in the *sn-1* position and a palmitic acid in the *sn-2* position (Fig. 5), was found to be the most potent antigen for mouse *i*NKT cells (49). BbGL-IIc, but not other variants of BbGL-II, induces *i*NKT cell proliferation *in vitro* and intracellular IFNγ expression by *i*NKT cells *in vivo* (49). Furthermore, BbGL-IIc induced *i*NKT cell activation occurs independently of MyD88 and TRIF signaling (49). These results show that BbGL-IIc is a bacterial antigen for the mouse *i*NKT cell TCR. BbGL-II compounds also stimulate human *i*NKT cells to release cytokines. Interestingly, BbGL-IIf, which contains linoleic acid ($C_{18:2}$) in the *sn-1* position and oleic acid in the *sn-2* position, has been found to be the most potent antigen for human *i*NKT cells (49). Data from another study suggest that the different *i*NKT cell responses to *Borrelia* glycolipids are due to a difference between human and mouse CD1d molecules (51). These

studies show that *i*NKT cell TCR detects DAGs, another category of glycolipid, in addition to glycosphingolipids. Moreover, DAG antigen induced *i*NKT cell activation is dependent on acyl chain length and saturation (49).

Structural basis of bacterial antigen recognition by the invariant T cell antigen receptor of natural killer T cells

The TCR of *i*NKT cells recognizes *Sphingomonas* GSL and *B. burgdorferi* DAG as well as αGalCer. Although the structures of these bacterial antigens are similar to that of αGalCer (Fig. 5), there are several small structural differences. DAG belongs to a different category of glycolipid than do αGalCer and *Sphingomonas* GSL. Also, the bacterial antigens are less potent than αGalCer. What determines the antigenic potency of these glycolipids? To address this point, crystal structures of mouse CD1d in complex with *Sphingomonas* GalAGSL or *B. burgdorferi* DAG were determined (51, 52). GalAGSL binds to mouse CD1d similarly to αGalCer. Between the α1 and α2 helices, the CD1d molecule has two pockets (A′ and F′) which accommodate the lipid tails of antigens (Fig. 6a, b) (6, 7). The fatty acid and sphinganine tails of GalAGSL extend into the A′ and F′ pockets, respectively (52). However, because of an alternative hydrogen-bonding interaction, the sphinganine tail of GalAGSL, which lacks 4-OH, is more deeply inserted into the F′ pocket (52). The sugar head group of GalAGSL is present in the center of the binding groove at the CD1d surface where an incoming TCR recognizes antigens (Fig. 6b, c), but it shows a slight lateral shift compared to αGalCer (52). These differences are thought to cause the difference in antigenic potency between *Sphingomonas* GalAGSL and αGalCer. The binding of *B. burgdorferi* galactosyl DAG is more flexible than that of *Sphingomonas* GalAGSL or αGalCer. The *sn-1* linked oleic acid and the *sn-2* linked palmitic acid of BbGL-IIc are inserted into the A′ and F′ pockets, respectively (51). The glycerol moiety of BbGL-IIc is tilted toward the α1 helix of the CD1d molecule, and the galactose of BbGL-IIc is pointed upward and away from the α2 helix of CD1d. These differences result in the loss of important hydrogen bonding interactions with the amino acids in the α2 helix that are present in the case of αGalCer (51). BbGL-IIf, which is not antigenic for mouse *i*NKT cells, binds to mouse CD1d in the opposite orientation with the *sn-1* linked linoleic acid in the F′ pocket and the *sn-2* linked oleic acid in the A′ pocket (51). Therefore, the lipid backbone of BbGL-IIf is rotated 180° inside the CD1d groove relative to that of BbGL-IIc, which leads to a dramatic repositioning of the galactose of BbGL-IIf (51). These results show that the fatty acid moieties also play an important role in stimulating *i*NKT cell TCR by determining the orientation of the sugar.

More recently, the crystal structures of two mouse ternary complexes were determined: CD1d-GalAGSL-*i*NKT TCR and CD1d-BbGL-IIc-*i*NKT TCR (53). These bacterial antigens and αGalCer bind to CD1d in different ways, as explained above (53). Surprisingly, these glycolipids are orientated in almost the same position above the CD1d binding groove when the TCR is bound (53). These data demonstrate that the *i*NKT cell TCR induces conformational changes in both microbial antigens and CD1d to adopt a conserved binding mode.

Natural killer T cells prevent arthritis and carditis after *Borrelia burgdorferi* infection

Natural killer T cells expressing an invariant T cell antigen receptor recognize a glycolipid from *B. burgdorferi*; however, do these cells play a protective role against *B. burgdorferi* infection? It was previously reported that CD1d deficient mice have increased bacterial burden and joint inflammation after syringe infection with *B. burgdorferi* (54). However, CD1d deficient mice lack not only *i*NKT cells, but also NKT cells with diverse TCRs. Moreover, CD1d has been shown to have a signaling function independent of CD1d dependent NKT cells (55, 56). To determine if *i*NKT cells play a role in the response to *B. burgdorferi*, Jα18 deficient mice were infected using *B. burgdorferi* infected ticks, the natural route of infection. The Jα18 deficient mice exhibited more severe and prolonged joint inflammation compared to wild type mice (57). Jα18 deficient mice had a reduced ability to clear bacteria from infected tissues such as the bladder, ears, heart and joints (57). In the early phase of *B. burgdorferi* infection, *i*NKT cells, but not conventional T cells, are activated and express intracellular cytokines including IFNγ (57). *i*NKT cells inhibit carditis after *B. burgdorferi* infection by accumulating in the heart (58). After *B. burgdorferi* infection, IFNγ expression increases in wild type mice, but not in Jα18 deficient mice, and IFNγ receptor α chain deficient mice have higher bacterial burdens and increased inflammation in the heart compared to control mice (58). Furthermore, IFNγ treatment enhances *B. burgdorferi* uptake by macrophages (58). Collectively, these results show that *i*NKT cells play an important role in the clearance of bacteria and the prevention of chronic inflammation in the joints and heart in *B. burgdorferi* infection, suggesting that recognition of bacterial antigens by *i*NKT cell TCR contributes to the response to certain microbial pathogens.

Natural killer T cells in the pathogenesis of infection-induced autoimmune disease

Natural killer T cells expressing an invariant T cell antigen receptor contribute to the clearance of bacteria after

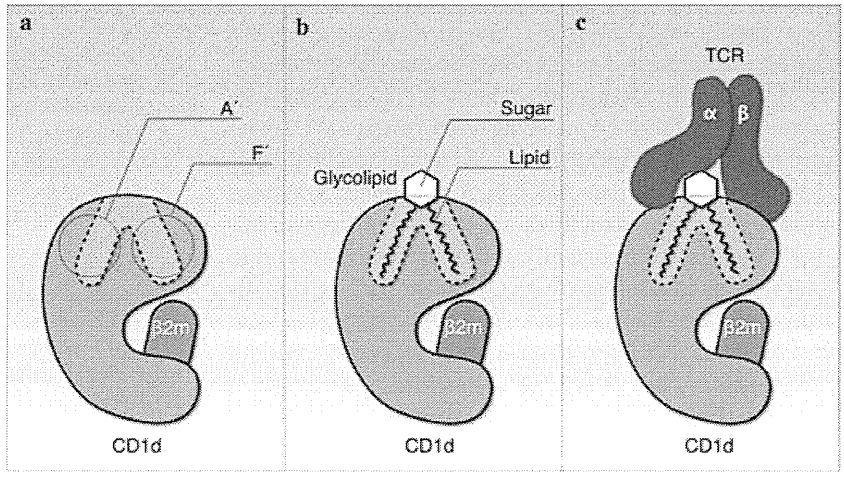

Fig. 6. Recognition of a glycolipid antigen bound to CD1d by *i*NKT cell TCR. (a) The structure of CD1d molecule. The extracelluar domains of CD1d bind to β2 microglobulin (β2m). The CD1d molecule has two pockets (A′ and F′). (b) A′ and F′ pockets accommodate the lipid tails of a glycolipid antigen. The sugar head group of the antigen is present in the center of the binding groove at the CD1d surface. (c) *i*NKT cell TCR recognizes the antigen and CD1d. Its α chain makes contact with the antigen.

Sphingomonas infection. However, wild type mice, but not *i*NKT cell deficient mice, have been shown to die after *S. capsulata* infection when a high dose of bacteria has been inoculated (42). This result suggests that *i*NKT cell activation by microbes can lead to severe inflammation in some cases. Recent studies have indicated that the *i*NKT cell response to *Sphingomonas* spp. is important in the pathogenesis of PBC, an autoimmune disease characterized by the destruction of small bile ducts in the liver. PBC patients express antibodies against mitochondrial PDC-E2 in serum (45). Interestingly, *N. aromaticivorans*, a member of the *Sphingomonodaceae* family found in human intestines, also expresses PDC-E2 (45). Serum from PBC patients reacts with *N. aromaticivorans*, but not with *E. coli* (45). Mice infected with *N. aromaticivorans* express antibodies against PDC-E2 and develop chronic inflammation in the small bile duct mediated by autoreactive T cells, *i*NKT cells being required in this process (59). These results indicate that *i*NKT cells play an important role in PBC pathogenesis.

Glycolipid induced activation of natural killer T cells augments the antimicrobial response

When *i*NKT cells are activated by αGalCer or its analogues, they stimulate many other cells, including APCs, NK cells, B cells and conventional T cells (1–4). Glycolipid mediated *i*NKT cell activation induces protective responses against various microbial pathogens including bacteria, fungi, parasites and viruses (1–4). For example, αGalCer treatment has a positive effect during certain microbial infections. In mouse pneumonia models with *P. aeruginosa* and *S. pneumoniae*, αGalCer treatment induces rapid clearance of bacteria from the lungs by activating alveolar macrophages and increasing neutrophil recruitment to the lungs, respectively (11, 60). In a urinary tract infection model with *E. coli*, *P. aeruginosa*, and methicillin resistant *Staphylococcus aureus*, αGalCer treatment enhances antibacterial effects (61).

α–galactosylceramide treatment has also been shown to be protective in mice infected with intracellular fungi and bacteria. During *C. neoformans* infection, αGalCer treatment enhances clearance of fungi from the lungs and spleen through an enhanced Th1 response (62). When mice infected with *L. monocytogenes*, an intracellular Gram-positive bacterium, are treated with αGalCer, bacterial numbers in the liver, spleen and peritoneal cavity decrease compared to control mice (63). *i*NKT cells stimulated by αGalCer enhance the killing of *L. monocytogenes* in macrophages with an increased respiratory burst (63). Similarly, in *M. tuberculosis* infected mice, αGalCer treatment prolongs survival and decreases the bacterial burden and tissue injury in the lungs (64). Furthermore, a combination of αGalCer and isoniazid, a first line antibiotic for tuberculosis, reduces bacterial numbers in the spleen and lungs in mice significantly more than does isoniazid alone (65). Human *i*NKT cells have also been shown to have lytic activity involving granulysin (an antimicrobial peptide) against *M. tuberculosis* infected APCs, and this is greatly enhanced by αGalCer (22). These data suggest that combining glycolipids and antibiotics may be an effective treatment for some infectious diseases in humans.

CONCLUSIONS

Natural killer T cells expressing an invariant T cell antigen receptor recognize glycolipid antigens by their invariant TCR; however, natural antigens recognized by this receptor were not identified for many years. Recent studies have shown that *i*NKT cells recognize glycolipids from microbes such as *Sphingomonas* spp. (41–43) and

B. burgdorferi (49), suggesting that the *i*NKT TCR detects certain microbes. The crystal structures of two ternary complexes of mouse CD1d-bacterial glycolipid-*i*NKT TCR have revealed that the *i*NKT TCR recognizes bacterial glycolipids by inducing conformational changes in antigens and CD1d to adopt a conserved binding mode (53). We speculate that *i*NKT TCR recognizes microbial glycolipids whose structures are similar to known microbial antigens. Importantly, *i*NKT cells also respond to microbes via inflammatory cytokines and/or endogenous antigens in the absence of microbial glycolipids. However, in some cases, *i*NKT cells participate in the pathogenesis of inflammatory diseases (28, 59). Therefore, it is important to clarify the mechanisms that initiate and regulate *i*NKT cell mediated inflammatory responses. Furthermore, an important future goal of *i*NKT cell research is the identification of endogenous antigens for these cells. Although it has been reported that one glycolipid is the endogenous antigen that is responsible for *i*NKT cell development (66), later studies have disputed this (67–69). More studies are needed to identify the endogenous antigen for *i*NKT cells.

Many mouse studies have shown that glycolipid mediated *i*NKT cell activation augments antimicrobial responses in various microbial infections (2, 4, 9, 10). Moreover, recent studies indicate that *i*NKT cell antigens are useful adjuvants for vaccines against microbial pathogens such as influenza virus (70–74), malaria (75, 76), HIV (76–78) and HSV-2 (79). Positive results have been reported from several clinical trials of tumor immunotherapy with αGalCer pulsed APCs and *in vitro* expanded *i*NKT cells (80, 81). These data indicate that *i*NKT cell glycolipid antigens may also be useful for new antimicrobial therapies and vaccines.

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A possible relationship of natural killer T cells with humoral immune response to 23-valent pneumococcal polysaccharide vaccine in clinical settings

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ABSTRACT

Pneumococcal polysaccharide vaccine (PPV), a type-2 thymus-independent antigen, induces the activation of B cells by directly triggering their antigen receptors. Although this type of antigen generally does not undergo class switching from IgM to IgG, PPV has been known to induce IgG2 in vaccinated subjects, which suggests the possible involvement of certain innate immune lymphocytes supporting the activation of B cells and their class switching. In the present study, we addressed the possibility that natural killer (NK) T cells are involved in Ab production caused by PPV. We measured serum levels of IgG against pneumococcal capsular polysaccharides and the numbers of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ double negative (DN) invariant NKT (iNKT) cells and $CD3^+CD56^+$ NKT cells in the peripheral blood before and after PPV injection. IgG was increased after PPV injection, peaking at 4 weeks after injection in serotypes 6B, 19F and 23F and at 3 months in serotype 14. Low responders, whose serum concentrations of IgG peaked at less than double their original levels, constituted 16%, 13%, 13% and 16% of vaccinated subjects with regard to serotypes 6B, 14, 19F and 23F, respectively. A significant positive correlation was detected between an increase in DN iNKT cells and the elevation of anti-serotype 14 IgG; in serotype 19F, DN iNKT cells were more markedly increased in responders than in low responders. These results suggest that DN iNKT cells may be involved in IgG production caused by vaccination against pneumococcal capsular polysaccharides.

1. Introduction

Streptococcus pneumoniae is a major bacterial agent which causes community-acquired pneumonia as well as other invasive diseases, such as bacteremia and meningitis, which arise as complications of pneumonia in 15–30% of cases [1]. The incidence rate of pneumococcal bacteremia is 18 to 30 per 100,000 in the general population, but can be as high as 56 to 83 per 100,000, especially in people aged 65 years or over in the USA [2–5]. In Japan, pneumonia is the fourth leading cause of death, and *S. pneumoniae* is a leading causative agent of pneumonia, being detected in 23% of community-acquired pneumonia cases [6]. This bacterium is also frequently detected as an etiologic agent in secondary pneumonia arising as a complication of the flu [7–9]. Morens and co-workers have demonstrated that the majority of deaths in the 1918–1919 influenza pandemic resulted directly from secondary bacterial pneumonia caused by common upper respiratory-tract bacteria, among which *S. pneumoniae* was most frequently detected in autopsy lung samples [10].

To prevent these pneumococcal diseases, 23-valent pneumococcal polysaccharide vaccine (PPV) is used for people aged 65 years or older and younger people with certain risk factors such as chronic cardiopulmonary diseases [11]. PPV is a type 2 thymus-independent (TI-2) antigen, which does not require helper T cells for the activation of B cells [12]. While thymus-dependent (TD) antigens activate B cells *via* engagement of CD40 by CD40L

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during cognate interaction with helper T cells, TI-2 antigens directly trigger surface immunoglobulin for the activation of B cells [13]. These different types of antigen produce distinct humoral immune responses: TD-antigens undergo class switching from IgM to IgG, which causes affinity maturation of Ab and induces memory B cell response; TI-2 antigens, on the other hand, do not [14]. Although PPV is a TI-2 antigen, Barrett and Ayoub [15] have found that it induces the restriction of production of IgG2 specific for pneumococcal polysaccharides. Snapper and co-workers [16] have reported that interferon (IFN)-γ contributes to Ab class switching to IgG3 in mice, which corresponds to IgG2 in humans, after PPV administration [15]. These findings suggest that a certain group of innate immune cells may be involved in the activation of B cells and Ab class switching caused by PPV.

Natural killer (NK) T cells, which express both αβ T cell antigen receptors and NK cell markers, have been identified as a novel lymphocyte population that acts in the innate stages of immune responses [17]. A major subset of NKT cells is the invariant NKT (iNKT) cells, which possess an extremely limited repertoire with antigen receptors consisting of Vα14-Jα18 in mice and Vα24-Jα18 in humans [18]. These cells recognize glycolipid antigens, such as α-galactosylceramide (α-GalCer), in the context of CD1d molecules on dendritic cells [19], which leads to the rapid production of IFN-γ and IL-4 [20,21]. iNKT cells are concentrated in the thymus, liver and bone marrow in mice [20,22] and occur at a rate of approximately 0.05% in human peripheral blood [23]. In our previous studies using a mouse model [24], iNKT cells were observed to play a critical role in neutrophilic inflammatory responses to and host defense against pneumococcal infection through production of IFN-γ. Interestingly, Kobrynski and co-workers have demonstrated that Ab production after PPV injection was completely abrogated in mice lacking iNKT cells [25]. These earlier observations raised the possibility that iNKT cells may contribute to Ab production and class switching caused by the administration of PPV.

In the present study, to address this possibility in a clinical setting, we analyzed the relationship between serum concentrations of Ab against pneumococcal capsular polysaccharides and the number of $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ double negative (DN) iNKT cells in the peripheral blood of subjects who received PPV administration. We found that DN iNKT cell counts increased, and that this increase was positively correlated with the production of IgG against a certain serotype of *S. pneumoniae*.

2. Materials and methods

2.1. Subjects

Fifty-five outpatients with chronic respiratory diseases were vaccinated with 0.5 ml of PPV (Pneumovax®, Banyu Pharmaceutical Co., Tokyo, Japan), intramuscularly, at the Department of Respiratory Diseases, Katta General Hospital, Shiroishi-shi, Miyagi, Japan between July 2006 and August 2008 after giving informed consent. The PPV23 contained 25 μg each of 23 different types of pneumococcal polysaccharide antigen (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). Serum samples were collected prior to vaccination and at 2 weeks, 4 weeks, 3 months, 6 months and 1 year after vaccination. The average age of these subjects was 74.4 years (range 61–88 years); 67.3% of subjects were male, 43.6% were smokers, and 10.9% were receiving glucocorticoid therapy. The most common chronic respiratory diseases among these patients were chronic obstructive pulmonary disease, bronchial asthma, bronchiectasis and old pulmonary tuberculosis (Table 1). This study was approved by the institutional ethics committees of Tohoku University, Sendai, Japan (#2005-233) and Katta General Hospital. We also paid the utmost attention to ensure that personal information was handled in compliance with our institutions' guidelines.

Table 1
Clinical characteristics of all subjects ($n = 55$).

	Number (%)
Males	37 (67.3)
Smoking	24 (43.6)
Alcohol abuse	1 (1.8)
Underlying diseases	
COPD	20 (36.4)
Bronchial asthma	13 (23.6)
Bronchiectasis	2 (3.6)
Old pulmonary tuberculosis	6 (10.9)
Chronic cardiovascular diseases	5 (9.1)
Immunosuppressive conditions	1 (1.8)
Chronic renal failure	1 (1.8)
Chronic liver diseases	1 (1.8)
Diabetes mellitus	8 (14.5)
Treatment with glucocorticoids	6 (10.9)
Home oxygen therapy	6 (10.9)

Mean age (yr) ± SD = 74.4 ± 6.6.

2.2. Measurement of anti-pneumococcal capsular polysaccharide Ab

Serotype-specific antibodies against 6B, 14, 19F and 23F (American Type Culture Collection, Manassas, VA, USA) were measured by means of a third-generation Enzyme-Linked Immunosorbent Assay (ELISA) as described previously [26] after absorption of non-specific antigens to cell wall polysaccharide (CWP: Statens Serum Institute, Copenhagen, Denmark) and serotype 22F (American Type Culture Collection). In brief, microtiter plates (MICROLON: Greiner Bio-One, Frickenhausen, Germany) were coated individually with 100 μl of a polysaccharide antigen: either 5 μg/ml of 6B, 2.5 μg/ml of 14, 5 μg/ml of 19F or 2.5 μg/ml of 23F, in PBS. After five hours of incubation at 37 °C, these plates were stored at 4 °C until use, which occurred within 6 months. Prior to testing, the sera from our patients and U.S. anti-pneumococcal reference serum [89-SF: kindly provided by Dr. Milan S. Blake (Food and Drug Administration, Silver Spring, MD, USA)] were also stored at −80 °C. Serum samples and 89-SF were diluted with an absorption buffer of 0.05% Tween-20 PBS to 1:50 and 1:100, respectively, and incubated at room temperature for 30 min. Next, serial two-fold dilution of these sera to 1:51200 were performed arbitrarily; the resulting solutions were added to the wells and incubated at 37 °C for 1 h. After the microtiter plates were washed, a detection antibody, consisting of AP-conjugated goat anti-human IgM or IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted to 1:2000, was added to each well. *p*-nitro phenyl phosphate (Sigma–Aldrich, St. Louis, MO, USA) was dissolved with 1 mol/l of diethanolamin (Sigma–Aldrich) to a concentration of 1 mg/ml as a substrate solution. Then, after the plates were washed again, this substrate was added to the wells and incubated at room temperature. Sodium hydroxide was added at 3 M to stop the enzyme reaction, and the absorbance values were detected at 405 nm as well as at 600 nm for reference. The concentrations of IgM and IgG Abs were calculated on the basis of a reference standard based on the 89-SF absorbance and expressed as μg/ml.

2.3. Flow cytometric analysis of PBMCs

Peripheral blood mononuclear cells (PBMCs) were collected from patients before vaccination and at 2 weeks, 4 weeks, 3 months and 6 months after vaccination. After Fc receptors on the cell surface were blocked, PBMCs were stained with FITC-anti CD3 [Clone: UCHT1 (eBioscience, San Diego, CA, USA)] and PE-anti CD56 [Clone:

B159 (BD Biosciences, Franklin Lakes, NJ, USA)] mAbs and PE-α-galactosylceramide (α-GalCer)-conjugated CD1d tetramer. These cells were also stained with APC-anti-CD4 and -CD8 mAbs [Clones: RPA-T4 and RPA-T8 (eBioscience), respectively]. Isotype control IgG (eBioscience) for each Ab and PE-α-GalCer-unconjugated CD1d tetramer were used as references. Flow cytometric analysis was performed using a Cytomics FC500 cytometry system (Beckman Coulter, Fullerton, CA, USA). The number of NKT cells (/μl) was calculated as follows: white blood cell (WBC) counts (100/μl) × % of lymphocytes in WBC/100 × % of NKT cells in lymphocytes. The WBC counts and % of lymphocytes were measured in blood samples collected from the patients during routine examinations.

2.4. Statistical analysis

Ab concentrations in sera, fold increases after vaccination and number of NKT cells in peripheral blood are expressed as geometric means. The concentrations of serum Ab and degrees of change in NKT cell counts during the first 2 weeks after vaccination were compared between responders and low responders using the Mann–Whitney *U*-test. The concentrations of serum Ab between pre- and peak levels were compared using Wilcoxon *t*-test. The correlation between the degree of change from pre-vaccination to peak levels of anti-pneumococcal IgG and the degree of change in NKT cell counts during the first 2 weeks post-vaccination was tested using Spearman's correlation test. A p value less than 0.05 was considered significant.

3. Results

3.1. Serum levels of anti-pneumococcal Ab after vaccination

Initially, we measured the concentrations of IgM anti-pneumococcal Ab against serotypes 6B, 14, 19F and 23F in 15 subjects at various time intervals after pneumococcal vaccination. As shown in Fig. 1A, the pre-vaccination levels of IgM Ab were 0.91, 0.59, 1.04 and 0.26 μg/ml for serotypes 6B, 14, 19F and 23F, respectively, and these levels were not altered during the six months post-vaccination.

Next, we measured the concentrations of IgG anti-pneumococcal Ab against the same serotypes in 55 subjects. As shown in Fig. 1B, in contrast to IgM Ab, IgG Ab began to increase during the second week, reached its peak at the fourth week for serotypes 6B, 19F and 23F and at the third month for serotype 14, then decreased one year after vaccination. For all the serotypes, the peak values were significantly higher than the values measured before vaccination (1.60 *vs.* 4.53, 3.04 *vs.* 12.87, 2.98 *vs.* 7.73 and 1.69 *vs.* 6.32 μg/ml for serotypes 6B, 14, 19F and 23F, respectively). One year post-vaccination, IgG levels had decreased from the peak levels by 12.3%, 37.8%, 25.2% and 41.5% for serotypes 6B, 14, 19F and 23F, respectively.

3.2. Responders and low responders

The individuals who received PPV administration were divided into two groups based on their responsiveness, *i.e.* responders and low responders. Here, we defined responders as individuals whose peak IgG levels were more than twice their IgG levels before vaccination, and low responders as individuals whose serum IgG concentrations were less than 2 μg/ml before vaccination and whose peak IgG levels were less than twice their IgG levels before vaccination. By these definitions, 62%, 62%, 45%, and 65% of the 55 vaccinated individuals were responders, and 16%, 13%, 13%, and 16% were low responders, with regard to the serotypes 6B, 14, 19F and 23F, respectively. As shown in Table 2, for all serotypes, peak IgG levels were significantly higher than IgG levels measured before

Table 2
Serotype-specific antibody levels in responders and low responders.

Serotype	Time point	Geometric mean concentrations (μg/ml) (95% CI)			Geometric mean increase from pre-vaccination to peak concentration (*n*-fold) (range)		
		All subjects (n = 55)	Responders[a]	Low responders[b]	All subjects (n = 55)	Responders[a]	Low responders[b]
6B	Pre	1.60 (1.20–2.14)	1.08 (0.76–1.52)	1.33 (1.10–1.60)			
	Peak	4.53 (3.44–5.95)**	4.56 (3.04–6.84)**	2.02 (1.57–2.59)*#	2.83 (0.89–78.89)	4.24 (2.00–78.89)	1.52 (0.11–1.92)
14	Pre	3.04 (1.96–4.70)	2.20 (1.38–3.50)	0.74 (0.35–1.60)			
	Peak	12.87 (8.46–19.59)**	21.14 (13.88–32.19)**	1.11 (0.52–2.37)$	4.24 (0.68–120.18)	9.61 (2.11–120.18)	1.49 (0.86–1.93)
19F	Pre	2.98 (2.20–4.03)	2.04 (1.41–2.95)	1.06 (0.86–1.30)			
	Peak	7.73 (5.70–10.49)**	11.23 (7.55–16.72)**	1.51 (1.18–1.93)$	2.60 (0.81–49.48)	5.51 (2.03–49.48)	1.43 (1.09–1.90)
23F	Pre	1.69 (1.18–2.43)	1.43 (0.95–2.15)	0.61 (0.35–1.06)			
	Peak	6.32 (4.18–9.55)**	8.96 (5.44–14.76)**	0.83 (0.49–1.40)$	3.73 (0.97–60.62)	6.25 (2.03–60.62)	1.36 (0.97–1.67)

*p < 0.05, **p < 0.01, compared with pre-vaccination level; #p < 0.05, $$p$ < 0.01, compared with peak level in responders.
[a] Responders are 34, 33, 26 and 36 subjects for serotypes 6B, 14, 19F and 23F, respectively.
[b] Low responders are 9, 8, 10 and 9 for serotypes 6B, 14, 19F and 23F, respectively.

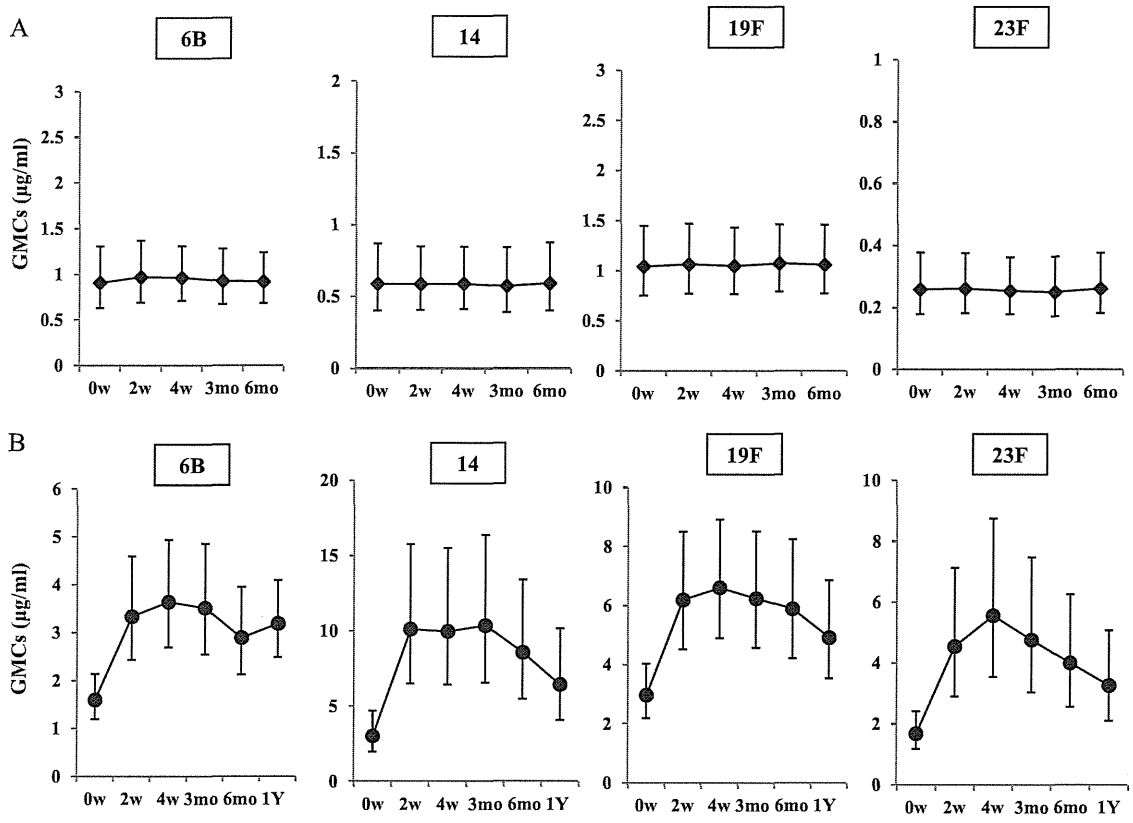

Fig. 1. Serum levels of anti-pneumococcal polysaccharide Abs after PPV injection. Concentrations of IgM (A: $n=15$) and IgG (B: $n=55$) Abs against each serotype of pneumococcal capsular polysaccharide in sera were measured at indicated time points after PPV administration. Data are shown as the geometric mean concentrations and 95% confidence intervals. GMCs, geometric mean concentrations; 0w, pre-vaccination; 2w, 2 weeks; 4w, 4 weeks; 3 mo, 3 months; 6 mo, 6 months; 1y, 1 year post-vaccination.

vaccination in the responder group, whereas no such significant increase in IgG concentration was observed in the low responder group, except for serotype 6B [pre-vaccination: 1.33 (95% CI was within 1.10–1.60) *vs.* peak: 2.02 (95% CI was within 1.57–2.59) ($n=9$, $p<0.05$)].

3.3. Alteration in the number of NKT cells in the peripheral blood after pneumococcal vaccination

We analyzed the number of NKT cells in the peripheral blood before vaccination and 2 weeks, 4 weeks, 3 months and 6 months after vaccination in 24 individuals, in whom the surface antigens on lymphocytes could be tested. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into CD4$^+$CD8$^-$ (CD4$^+$ iNKT), CD4$^-$CD8$^+$ (CD8$^+$ iNKT) and CD4$^-$CD8$^-$ (double negative: DN iNKT) subsets. As shown in Fig. 2, iNKT cell subsets did not show significant elevation in their cell count at any time point after vaccination, although increased iNKT cell counts were observed during the first two weeks in 11 or 12 individuals (data not shown).

3.4. NKT cell counts and serum levels of anti-pneumococcal Ab

In order to address the possible role of NKT cells in the humoral response to the pneumococcal vaccine, we analyzed the relationship between the degree of change in NKT cell counts during the first 2 weeks post-vaccination and the degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to their peak. As shown in Fig. 3, a significant positive correlation was detected between increases in DN iNKT cells and increases in anti-serotype 14 IgG, and there were tendencies toward positive correlations between changes in CD8$^+$ iNKT and DN iNKT cell counts and increases in anti-serotype 19F IgG levels ($p=0.069$ and 0.067, respectively), and between changes in DN iNKT cell counts and increases in anti-serotype 6B and 23F IgG levels ($p=0.062$ and 0.082, respectively). By contrast, CD4$^+$ iNKT, CD8$^+$ iNKT and CD3$^+$CD56$^+$ cells showed neither a positive nor a negative correlation with changes in the serum levels of anti-pneumococcal IgG in all of the serotypes except for 19F in CD8$^+$ iNKT and CD3$^+$CD56$^+$ cells.

Finally, we compared changes in DN iNKT cell counts between responders and low responders, because these cells showed a tendency toward a positive correlation with Ab responses to PPV. As shown in Fig. 4, in serotype 19F, the increase in DN iNKT cells was significantly more marked in responders than in low responders. This tendency was also observed in serotypes 6B, 14 and 23F, although it was not statistically significant.

4. Discussion

In the present study, serum levels of anti-pneumococcal IgG increased after pneumococcal vaccination, peaking in the fourth week for serotypes 6B, 19F and 23F and in the third month for serotype 14; in 45–65% of vaccinated subjects, these levels increased more than two-fold. There were also low responders, however, producing smaller quantities of anti-pneumococcal Ab; these constituted 16%, 13%, 13% and 16% of our 55 subjects for serotypes 6B, 14, 19F and 23F, respectively. Of the low responders, 15 showed a low response to one of the four serotypes examined, nine showed a low response to two serotypes, and one showed a low response to three serotypes, indicating that 45% of our 55 subjects were low responders for at least one serotype. Although there is no standardized definite on of a low responder, our results appear

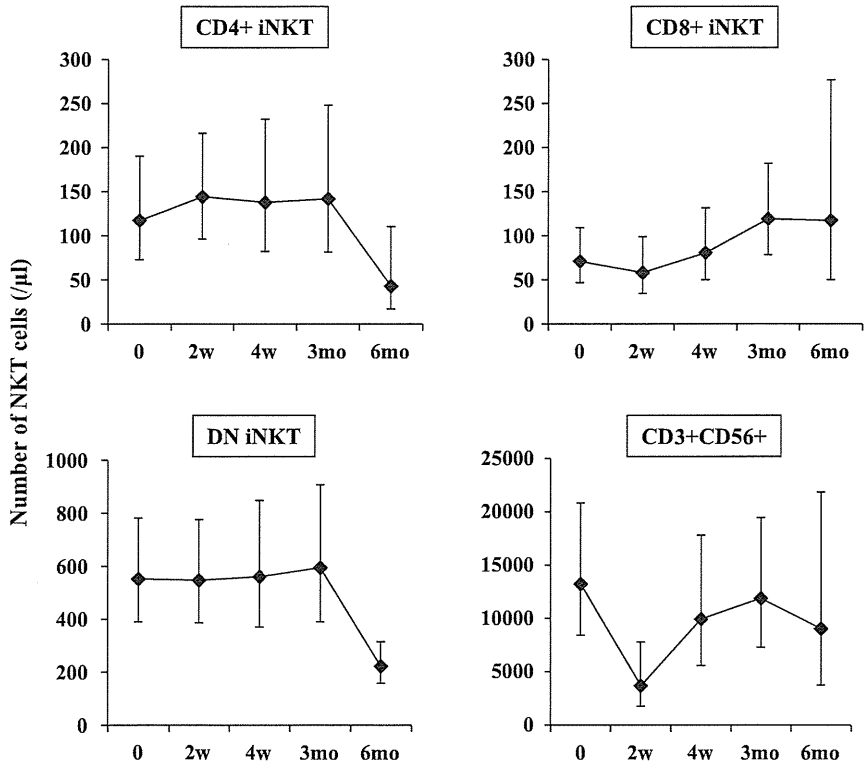

Fig. 2. NKT cells in the peripheral blood after PPV injection. Number of NKT cells in the peripheral blood was examined before PPV administration and 2 weeks, 4 weeks, 3 months and 6 months after PPV administration in 24 individuals. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into $CD4^+CD8^-$ ($CD4^+$ iNKT), $CD4^-CD8^+$ ($CD8^+$ iNKT) and $CD4^-CD8^-$ (double negative: DN iNKT) subsets. Data are shown as the geometric means and 95% confidence intervals in each NKT cell subset.

to be in accordance with those of previous investigations, which indicate that 16–31% of vaccinated subjects are low responders, whose anti-pneumococcal Ab levels increase less than two-fold for two among four to seven analyzed serotypes [27–29].

Previous studies have shown NKT cells to be involved in immune responses to TI-2 antigens, as a possible source of the secondary stimulatory signal for B cell activation [25] as well as in protection against pneumococcal infection [24]. These earlier observations suggest that NKT cells may play a certain role in the clinical effects of anti-pneumococcal vaccination. In agreement with this possibility, in the present study, a significant positive correlation was detected between changes in the number of DN iNKT cells, though not of $CD4^+$ iNKT cells, and increases in Ab levels against serotype 14 antigen. Moreover, the increase in DN iNKT cells was more marked in responders than in low responders, and this difference was statistically significant for serotype 19F. However, the positive correlation between DN iNKT cells and Ab levels and the difference in DN iNKT cells between responders and low responders were not significantly detected in other serotypes, although there were such tendencies with lower *p* values. The increase of study subjects would help in making these differences statistically significant. In addition, there is a possibility that the increase of DN iNKT cell number in responders may be due to overall immune activation of these individuals in response to vaccine, rather than selective effect on NKT cells. This may not apply to our case, because there was no tendency of difference between low responders and responders in other NKT cell subsets (data not shown).

$CD4^+$ and DN iNKT cells are major subsets in humans, both of which secrete large amounts of IFN-γ upon stimulation [21]. Yet these subsets differ in their secretion of such Th2 cytokines as IL-4, IL-5 and IL-13, and in their expression of chemokine receptors, integrins and NK receptors [21,30–32]. Galli and co-workers have demonstrated that iNKT cells promote immunoglobulin production by B cells, an activity that is more potent in $CD4^+$ iNKT cells than in DN iNKT cells [33]. The same group has also reported that activated human iNKT cells directly support the proliferation of and immunoglobulin production by naive and memory B cells. All these experiments were conducted *in vitro*, however, and frequent stimulation of iNKT cells during culture has been reported to cause a shift in their cytokine profile toward a Th2-dominant condition [34], raising the possibility that cultured NKT cells are not always equivalent to those in circulation *in vivo*. In the present clinical study of individuals receiving PPV, the relationship between iNKT cells and Ab production does not seem to be identical between $CD4^+$ and DN iNKT cells. Taken together, the data suggest that these subsets play distinct roles in Ab production by B cells after PPV administration. Further investigation is necessary to define the precise mechanism by which this occurs.

On the other hand, only a limited subset of NKT cells expressing NK cell markers, such as CD56 or CD161, is reactive to α-GalCer-loaded CD1d tetramer [31]. Therefore, $CD3^+CD56^+$ NKT cells, described as NKT-like cells, are distinguished from iNKT cells by certain characteristics, including the differences in their cytokine production profiles and their TCR αβ chains [18]. Our results suggest that iNKT cells rather than NKT-like cells may be particularly involved in IgG production caused by pneumococcal capsular polysaccharides, because no correlation was observed between $CD3^+CD56^+$ NKT cell count and Ab response.

To the best of our knowledge, the current study is the first report presenting clinical data that suggests a possible relationship between the activation of iNKT cells and Ab responses after PPV administration. The increase in DN iNKT cell count seems to be particularly correlated with serotype-specific IgG production, suggesting a higher contribution from DN iNKT cells than from other subsets. The population size in this study was limited, and the

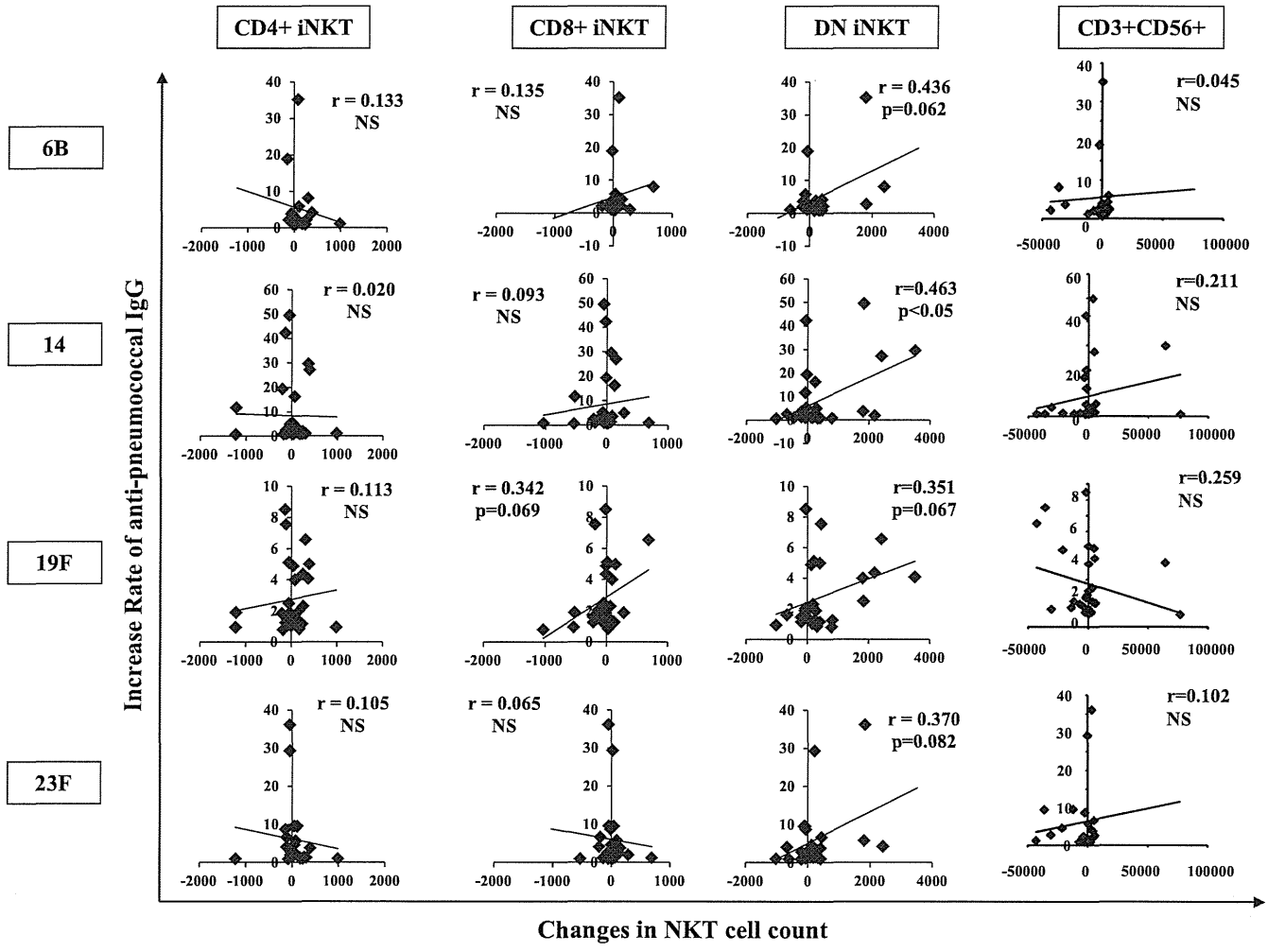

Fig. 3. Relationship between NKT cell counts and anti-pneumococcal IgG. Relationship between changes in NKT cell counts during the first 2 weeks post-vaccination and degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to peak. Each symbol indicates the relationship for one subject. *R* and *P* values and number of subjects in each analysis are shown.

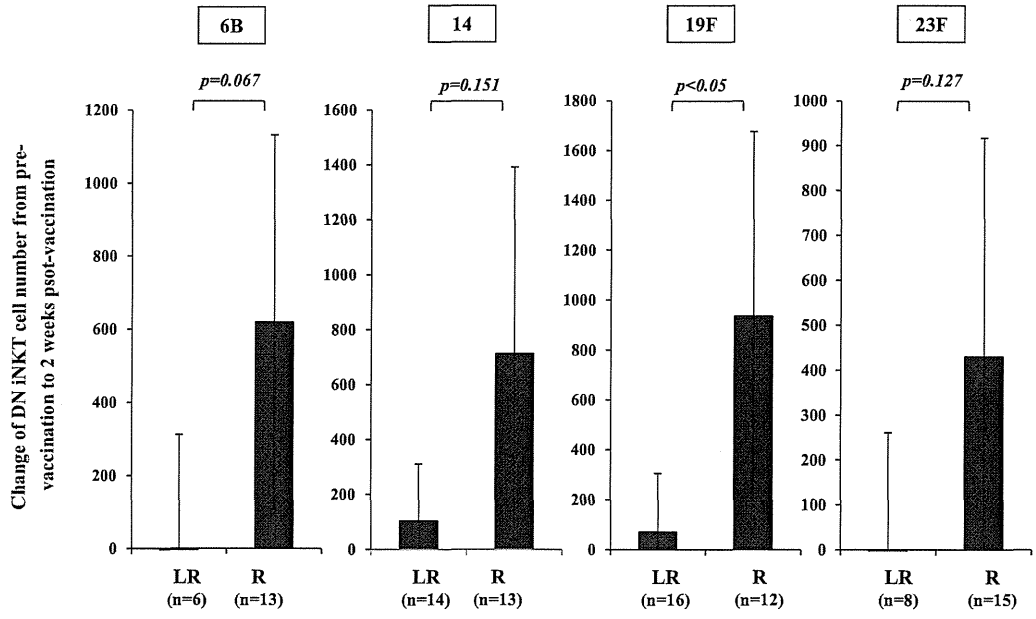

Fig. 4. Changes in DN iNKT cell counts in responders and low responders. Degree of change in DN iNKT cell count during the first 2 weeks after vaccination was compared between responders and low responders for each serotype. Data are expressed as the arithmetic means and 95% confidence intervals of indicated number of subjects. LR, low responders; R, responders.

enrolled subjects were aged (74.4 ± 6.6 years) and had underlying diseases that affected their immune condition. In these respects, there are some limitations in interpreting the results. At present, it remains to be elucidated how iNKT cells are involved in humoral immune responses to pneumococcal capsular polysaccharides in the clinical setting, but further investigations are already under way in our laboratory to define the precise mechanism underlying the relationship between iNKT cells and Ab responses.

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