in apoptotic Bε cells that was not observed in the Bγ cells (Fig. 4 D, middle). Apoptosis of Bε cells was abrogated by the addition of anti–IL-21, a treatment that had no significant effect on Bγ cells (Fig. 4 D, right).

Bmf-induced Bε cell apoptosis

To understand the molecular mechanisms underlying IL-21–induced IgE suppression, we performed DNA microarray analyses to compare gene expression between Bε and Bγ cells. The DNA microarray data were deposited in the Center for Information Biology Gene Expression database (CIBEX; http://cibex.nig.ac.jp/) under accession number CBX15. The proapoptotic Bmf gene (34) was dramatically up-regulated in Bε cells, a finding that was confirmed by RT-PCR (Fig. 5 A). No significant difference in the expression of IL-21R, Bcl-2, or γc was detected (Fig. 5 A), suggesting that elevated Bmf gene expression in Bε, but not in Bγ, cells may account for their differential sensitivity to IL-21–mediated apoptosis.

To investigate whether the Bmf expressed in Bε cells is functional in its proapoptotic activity, Bmf cDNA was isolated from Bε cells and used to prepare several mutants of enhanced GFP–fused Bmf. These mutations included an A69P mutation in the dynein light chain 2 binding motif and an L138A mutation in the BH3 domain. These Bmf mutants were transfected into Baf3 cells. Upon IL-3 deprivation, mock transfectants underwent apoptosis. Transfection with WT Bmf or Bmf-A69P to Baf3 cells also significantly augmented apoptosis (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). However, reduced apoptosis was seen in Baf3 cells transfected with BH3 mutants, such as Bmf-L138A or Bmf-A69P/L138A (Fig. S3), indicating that Bmf in Bε cells is functional and the BH3 domain of the protein is important for mediating its proapoptotic activity.

Based on the understanding of proapoptotic activity of Bmf expressed in Bε cells, we investigated the formation of Bmf–Bcl-2 complexes in Bε cells after activation with IL-21. Bmf in Bε cells faintly binds to Bcl-2 in unstimulated cells (Fig. 5 B, left). However, when Bε cells were stimulated with IL-21, the formation of Bmf–Bcl-2 complexes was significantly augmented (Fig. 5 B, right).

BCG-mediated IL-21 induction in human Vα24 NKT cells

To determine how widespread our findings are, we investigated whether IL-21 and Vα24 NKT cells are required for the BCG-mediated suppression of human IgE responses. When human PBMCs were stimulated with α-GalCer or BCG, a significant up-regulation of IL-21 mRNA was detected by quantitative PCR (Fig. 6 A). The BCG-induced up-regulation of IL-21 mRNA was effectively suppressed by blocking with antibodies against CD1d, IL-12p40/p70, or both (Fig. 6 B), indicating that the CD1d-restricted NKT cell–dependent suppression of IgE responses observed in mice also operates in the human immune system. IL-21 mRNA expression by anti-CD1d and anti–IL-12 treatment was significantly reduced but was not as effective as in the mouse Vα14 NKT cell system (Fig. 3 D), perhaps suggesting a significant contribution of human conventional $CD4^+$ T cells (Fig. S2 B).

To evaluate in vivo responses, we inoculated BCG into healthy volunteers and examined IL-21 mRNA levels in PBMCs 1 wk later. There was a significant up-regulation of IL-21 mRNA levels in five out of six individuals (Fig. 6 C), and, furthermore, IL-21 suppressed IgE production by human Bε cells (Fig. 6 D, left). As expected, the addition of BCG-stimulated, but not control, PBMCs significantly inhibited IgE production (Fig. 6 D, right).

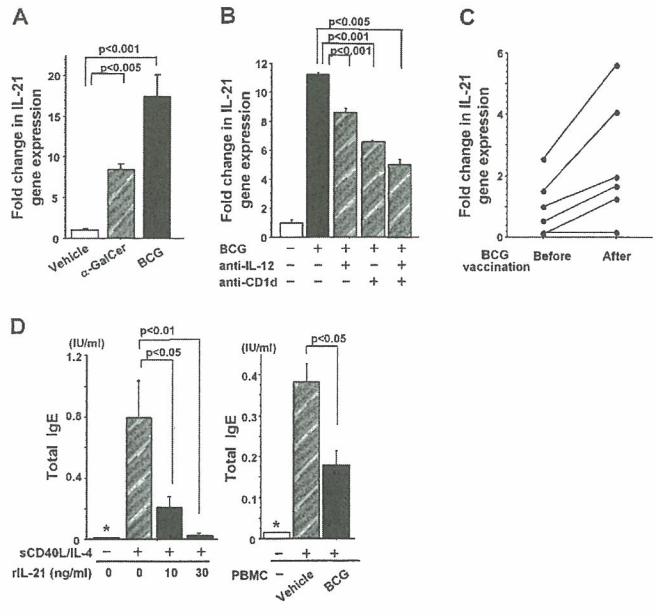

Figure 6. IL-21 mRNA expression and IL-21–induced IgE suppression in humans. (A) IL-21 mRNA expression in PBMCs. 10^6 human PBMCs were stimulated with 100 ng/ml α-GalCer or 50 μg/ml BCG and examined for IL-21 expression by quantitative real-time PCR with Taqman probes. The data are representative of five donors. (B) IL-12 and CD1d are required for IL-21 expression. 10^6 PBMCs were stimulated in vitro with 50 μg/ml BCG in the presence of 10 μg/ml anti-CD1d and/or anti–IL-12p40/p70 mAb. Representative data from five donors are shown. (C) IL-21 mRNA expression in PBMCs. Healthy volunteers were inoculated intradermally with BCG (two drops of 26.7 mg/ml of BCG emulsion per person). In A–C, the data for IL-21 expression were normalized to 18S ribosomal RNA expression, and relative expression levels are shown. Statistical analysis was performed using a matched pairs *t* test in C. (D) Suppression of IgE production. Left, suppression of IgE production by IL-21. 2×10^5 human B cells were cultured with sCD40L and IL-4 in the presence of human IL-21 for 14 d. Right, suppression of IgE production by BCG-activated human PBMCs. 10^5 Bε cells were co-cultured with 10^5 PBMCs, sCD40L, and IL-4 in the presence of 50 μg/ml BCG for 14 d. Total IgE was measured by ELISA. Values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the IgE levels are below the detection limit for total IgE (<0.014 IU/ml). Data shown are representative of three donors. Results were expressed as a fold difference in human IL-21 gene expression relative to a control sample (vehicle) after being normalized with 18S ribosomal RNA expressions in each sample.

DISCUSSION

It is widely accepted that the mechanistic basis of the hygiene hypothesis for suppression of IgE responses is an increase in the Th1/Th2 ratio (12). However, in reality, the Th1 response exacerbates allergic reactions, as human asthma is associated with the production of IFN-γ, a cytokine that appears to contribute to the pathogenesis of the disease (35). Furthermore, the adoptive transfer of allergen-specific Th1 cells causes severe airway inflammation (36). Thus, a shift in the Th1/Th2 ratio alone cannot explain all of the immunological findings observed in allergic diseases (1). Furthermore, there are several studies suggesting that BCG vaccination has little or no effect on the development and prevalence of allergic diseases (37, 38). Therefore, it is necessary to better understand the precise mechanism of IgE suppression in BCG-treated animals or humans.

In this study, neither a Th1/Th2 imbalance nor an involvement of regulatory T cells was observed in response to BCG treatment (Fig. 1). Instead, we demonstrated that IL-21–induced Bε cell apoptosis is the mechanism responsible for BCG-mediated suppression of IgE production (Figs. 1, 3, and 5). Because the human IL-21 responses to BCG vaccination were heterogeneous (Fig. 6 C), it seems likely that the magnitude of the response in each individual could cause different degrees of BCG-induced IgE suppression and might be prognostic.

Previous studies have indicated that IL-21 is preferentially expressed by activated $CD4^+$ T cells (20), the results that are partially in agreement with the present data, as half of peripheral Vα14 NKT cells are $CD4^+$ (39, 40). Interestingly, upon anti-CD3 mAb stimulation, Vα14 NKT cells, but not conventional T cells, preferentially expressed IL-21 (Fig. S2 A), similar to the results with BCG (Fig. 3 B). Therefore, the major IL-21 producers in response to BCG in mice are Vα14 NKT cells.

It has been proposed that, for full activation of Vα14 NKT cells to produce IFN-γ, two signals are required: one CD1d-dependent and the other TLR-mediated IL-12–dependent signals (31). In agreement with this, IL-21 expression by BCG-activated Vα14 NKT cells was significantly inhibited by blocking with antibodies to IL-12 and/or CD1d (Fig. 3 D). Therefore, it is likely that Vα14 NKT cells recognize endogenous antigens presented by CD1d molecules but require IL-12 signals to produce IL-21. Nevertheless, it is still possible that glycolipid BCG components such as phosphatidylinositol mannoside may directly stimulate Vα14 NKT cells to produce IL-21 in a CD1d-dependent manner (41, 42).

In terms of the receptors on DCs that are required for BCG recognition and signal transduction, we showed in this study that BCG-induced IL-12 production is IRAK-4 and MyD88 dependent (Fig. 2 E and Fig. S1). These results in mice are consistent with a recent report indicating that BCG cannot induce IL-12 or IFN-γ production by PBMCs from IRAK-4–deficient patients (43). In addition, it has been reported that BCG enhances NF-κB–dependent gene transcription through the activation of phosphatidylinositol 3 kinase and c-Jun N-terminal kinase cascades (44). The activated NF-κB is then liberated for nuclear translocation and transactivates a variety of immune response genes, including IL-12.

In contrast to a previous report that implicated TLR2 and TLR4 in the recognition of mycobacterial antigens (32), we could not identify any involvement of these receptors in IL-12 production by BCG-stimulated BM-DCs (Fig. 2, C and D). In agreement with our findings, it has recently been reported that TLR2/4 double KO mice infected with live BCG have normal adaptive immune responses and survived as long as WT mice (45). As whole BCG contains multiple components including mycobacterial glycolipids, proteins, and DNA, several receptors that use IRAK-4 and MyD88 as the signal transducer appear to be involved in the complex recognition of BCG.

In IL-21R–deficient mice, the level of circulating IgE is high, whereas that of IgG1 is low (23, 46). Similarly, in human B cells, IL-21 inhibits IgE production and stimulates IgG4 (analogous to mouse IgG1) production (19). These results suggest that IL-21 differentially regulates IgE and IgG1 (IgG4 in humans) class switching. In fact, Suto et al. (18) reported that IL-21 specifically suppresses IgE production by inhibiting germ line Cε transcripts. Our present findings do not exclude this possibility. IL-21 has also been reported to induce apoptosis in resting and activated B cells by reducing the expression levels of apoptosis-related genes (25, 26). However, in this report, we have shown that IL-21 selectively induces apoptosis in Bε, but not Bγ, cells (Fig. 4 D). Thus, our findings that BCG-activated IL-21–expressing Vα14 NKT cells suppressed IgE production even after class switching (Fig. 4 C) suggests that the role of IL-21 on Bε cells is to control cell growth and viability, rather than to regulate the differentiation and maturation of these cells.

We found that expression of a proapoptotic gene, Bmf, was significantly higher in Bε cells than in Bγ cells (Fig. 5 A). Under physiological conditions, Bmf, which is a BH3 domain–only Bcl-2 family member that inhibits Bcl-2 function and accelerates apoptosis, binds to myosin V motors via the dynein light chain 2 domain of Bmf (34). In response to certain cellular damage signals, Bmf is supposed to be released from the myosin V motors and trigger apoptosis (34). Because Bmf from Bε cells induced apoptosis and a mutation in the BH3 domain of Bmf failed to induce apoptosis (Fig. S3), we confirmed that Bmf expressed in Bε cells is functional, and that the BH3 domain is important for the binding to Bcl-2 and is essential for its proapoptotic activity. In fact, the binding of Bmf with Bcl-2 was up-regulated by IL-21R signaling (Fig. 5 C). Therefore, BCG-mediated Bε cell apoptosis is due to the augmented formation of Bmf–Bcl-2 complexes generated by IL-21R signaling in Bε cells.

Finally, we defined the mechanism of BCG-induced IL-21–dependent suppression of IgE production in humans (Fig. 6). In a broader context, these findings may explain the mechanisms underlying the BCG-mediated suppression of allergic diseases and the epidemiological data indicating a reduction in the morbidity of allergic diseases in patients who

have been infected with *Mycobacterium tuberculosis*. Interestingly, IL-21–mediated B cell responses in C57BL/6 mice differ from those in BALB/c mice (26), suggesting that there is a genetic polymorphism with respect to the outcome of IL-21 signaling in B cells. In fact, a recent report indicated that polymorphisms in the IL-21R gene locus differentially affect serum IgE levels in humans (47). In this study, consistent with our data, the levels of IL-21 expression induced by BCG stimulation varied among the individuals examined (Fig. 6 C). These results suggest that the response to BCG in humans is dependent, at least in part, on genetic background. The specific genes responsible for the heterogeneity in BCG-mediated IL-21 production have not been identified. However, this observation may be applied to the development of diagnostic or therapeutic strategies in which the levels of IL-21 expression are used to evaluate the efficacy of BCG treatment, or in determining the potential benefit of therapy using bacterial products such as CpG for allergic diseases.

MATERIALS AND METHODS

Mice. 7–10-wk-old female BALB/c mice were purchased from Japan CREA Inc. Vα14 NKT-deficient (Vα14 NKT KO) mice on a BALB/c background (48), IRAK-4 KO (49), TLR2 KO, TLR4 KO, and MyD88 KO mice (50, 51) have been described. TLR2 and TLR4 double KO mice were generated by breeding. Mice were kept under specific pathogen-free conditions, maintained on an OVA-free diet, and treated in accordance with the guidelines for animal care at RIKEN Research Center for Allergy and Immunology.

Allergic sensitization and BCG. Allergic epicutaneous sensitization was performed as described previously (27). In brief, a 1-cm^2 sterile patch infused with 100 μl of PBS solution with or without 100 μg OVA (grade V; Sigma-Aldrich) was placed on the shaved back of mice and fixed in place with a bio-occlusive dressing and an elastic bandage. Patches were left on for 48 h and removed. The sensitization course was repeated at the same skin site every week for 4 wk. For BCG vaccination, mice were given a weekly i.p. injection of BCG (500 μg/mouse) or PBS at the time of OVA sensitization. The attenuated BCG (strain Tokyo) was purchased from the Japan BCG Laboratory.

Flow cytometry. Cells were stained with antibodies after adding 2.4G2 (BD Biosciences) for Fc blocking. The following antibodies were used: FITC–anti-CD19 (1D3), FITC–anti-IgE (R35-72), APC–anti-IgG1 (X59), FITC–anti-TCRβ (H57-597), APC–anti–IL-12p40/70 (C15.6), and PE–anti-CD11c (HL3; BD Biosciences). PE-conjugated α-GalCer–loaded CD1d tetramer (α-GalCer/CD1d tetramer) was prepared as described previously (52). For intracellular staining, BM-DCs were fixed and permeabilized with BD Cytofix and Cytoperm kits after staining with PE–anti-CD11c. They were then stained with APC–anti–IL-12p40/70. FACS analysis of at least 10,000 cells and cell sorting were performed with a FACSCalibur (BD Biosciences) with FlowJo software (TreeStar) or with a MoFlo cell sorter (DakoCytomation).

Cell preparations and cultures. 2×10^6 BM-DCs obtained by culturing BM for 6 d with 10 ng/ml GM-CSF were further cultured in the presence or absence of BCG, CpG, LPS (Invivogen), PGN from *Escherichia coli* (Invivogen), or 10 μg/ml anti-CD3 mAb (2C11; BD Biosciences) for 48 h at 37°C. For blocking experiments, mAb against CD1d or IL-12p40/p70 (clones 1B1 and C17.8, respectively; BD Biosciences), or an isotype control was added at a concentration of 20 μg/ml after 2.4G2 treatment. $TCR\beta^+$ cells or Vα14 NKT cells with a purity of >98% were obtained from liver MNCs (52) using an Auto MACS (Miltenyi Biotec) after staining with FITC–anti-TCRβ and sorting with anti-FITC magnetic beads (Miltenyi Biotec). Vα14 NKT cells were then isolated from $TCR\beta^+$ cells by MoFlo using PE–α-GalCer/CD1d tetramer. Conventional T or $CD4^+$ T cells were isolated from an α-GalCer/CD1d $tetramer^-$ fraction of $TCR\beta^+$ liver MNCs. Bε and Bγ cells generated from splenic $CD19^+$ cells in the presence of 10 μg/ml sCD40L (ALX-850-075; Qbiogene) and 20 ng/ml of recombinant IL-4 (PeproTech) for 3 d (33) were cultured for 30 h for the apoptosis assay or for an additional 5 d to investigate IgE responses.

ELISA. Cytokines (IL-12p70 and IL-6) and Ig subclasses (IgG1, IgG2a, and IgE) were measured by ELISA using kits or sets of antibodies (BD Biosciences) according to the manufacturer's protocol. Specific antibodies were also measured as described previously (7).

RT-PCR. Total RNA was extracted by RNAeasy (QIAGEN), and cDNA was synthesized with random primers after DNase treatment. The following RT-PCR primer sets were used for mouse genes: IL-21, 5′-CCCTTGTCTGTCTGGTAGTCATC-3′ and 5′-ATCACAGGAAGGGCATTTAGC-3′; IgE (Cε), 5′-AGGAACCCTCAGCTCTACCC-3′ and 5′-GCCAGCTGACAGAGACATCA-3′; mIL-21R, 5′-TGTCAATGTGACGGACCAGT-3′ and 5′-CAGCATAGGGGTCTCTGAGG-3′; γc, 5′-GTCGACAGAGCAAGCACCATGTTGAAACTA-3′ and 5′-GGATCCTGGGATCACAAGATTCTGTAGGTT-3′; Bmf, 5′-CAGACCCTCAGTCCAGCTTC-3′ and 5′-CGTATGAAGCCGATGGAACT-3′; Bcl-2, 5′-GGTGGTGGAGGAACTCTTCA-3′ and 5′-CATGCTGGGGCCATATAGTT-3′; and HPRT, 5′-AGCGTCGTGATTAGCGATG-3′ and 5′-CTTTTATGTCCCCCGTTGAC-3′. The numbers of PCR cycles were as follows: 30 for HPRT; 35 for IgE, γc, and IL-21R; 40 for IL-21 and Bmf; and 45 for Bcl-2. The amounts of cDNA were standardized by quantification of the housekeeping gene HPRT using primers for mouse samples. The human IL-21 mRNA levels were quantified by real-time quantitative PCR on the ABI Prism 7000 sequence detection system (Applied Biosystems) by using TaqMan assay kits and TaqMan Gene Expression Assays (primers and TaqMan probes).

Electrophoretic mobility shift assay. 2×10^6 BM-DCs were stimulated with 50 μg/ml BCG or 1.0 μM CpG-B for the indicated periods. Nuclear extracts were prepared and used for Gel Shift Assay Systems (Promega) as described previously (50).

Bε cell–derived Bmf and its mutants. cDNAs encoding bmf were amplified from Bε cells by PCR using primers 5′-CCGAATTCGGATGGAGCCACCTCAGTGTGT-3′ and 5′-GCGGCCGCCTGCATTCCTGGTGATCCAT-3′ (EcoRI and NotI sites for cloning are underlined). The amplified products were cloned using the pGEM-T Easy Vector System (Promega). Mutant cDNAs were generated by PCR using point-mutated primer pairs.

Immunoprecipitation and Western blotting. Interaction of Bmf with Bcl-2 in Bε cells was detected by immunoprecipitation with anti–Bcl-2 mAb (clone 7; BD Transduction Laboratories) and subsequent immunoblotting with anti-Bmf rabbit antibody (Cell Signaling). The protein levels were visualized by ECL (GE Healthcare) using horseradish peroxidase–conjugated Protein A/G (Pierce Chemical Co.).

Human studies. All human specimens were obtained under informed consent. The protocol for the human research project has been approved by the Ethics Committee of Chiba University and RIKEN, and conformed to the provisions of the Declaration of Helsinki in 1995. 10^8 PBMCs from healthy volunteers were prepared by Ficoll-Paque density gradient centrifugation and used for the cultures. Human recombinant IL-21 was purchased from BIOSOURCE Inc. Human total IgE was measured with a sensitive immune assay (GE Healthcare).

Statistical analysis. Statistical analyses were performed using the Student's *t* test or matched pairs *t* test. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 provides data demonstrating that MyD88 signaling in DCs is required for BCG-induced activation. Fig. S2 contains data demonstrating IL-21 mRNA expression by NKT cells, $CD4^+$ T cells, and $CD8^+$ T cells of murine and human origin. Fig. S3 provides the data indicating proapoptotic activity of Bε cell–derived Bmf and functional domain analysis using mutant Bmf in Baf3 cells. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062206/DC1.

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Side-by-side comparison of automatic pollen counters for use in pollen information systems

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Background: Recent effort to build an unmanned pollen monitoring network in Japan has led to new developments in automatic pollen counters. In-the-field performance tests of these automatic counters have not been reported.

Objective: To characterize recently developed automatic pollen counters, with a view of using their data in pollen information systems.

Methods: We performed side-by-side comparisons between 2 recently developed automatic pollen counters and 2 reference samplers at 2 sites during the 2005 pollen season.

Results: Both automatic counters were found to have similar overall performance in terms of their correlations with the reference samplers. The linear correlation coefficient between the hourly values of the counters and one of the reference samplers was larger than 0.8 at both sites for both counters. Although these results are encouraging, our analysis also points to weaknesses of the investigated automatic counters in the areas of pollen discrimination, minimum measurable concentration, and calibration. Both counters were found to be affected by large concentrations of particulate matter, although the conditions and extent to which the particulate matter disrupted the measurements differ for the 2 sensors. The effect of particulate matter is particularly noticeable at the start and end of the pollen season, that is, when pollen concentration is low relative to particulate matter concentration. Further, it was found that one of the automatic counters could not differentiate snow particles from pollen grains.

Conclusions: The tested automatic pollen counters had good overall performances, but weaknesses in the areas of pollen discrimination, minimum measurable concentration, and calibration still have to be addressed for these counters to find widespread use in the allergy community.

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INTRODUCTION

Monitoring of airborne pollen has recently attracted much attention because of its potential contribution to both allergen avoidance measures by providing individuals with allergy with pollen alerts[1] and the evaluation of cross-pollination between genetically modified crops and their wild relatives.[2,3] Automatic pollen monitoring has only been introduced recently in Japan in an attempt to provide the general public with alerts for Japanese cedar (*Cryptomeria japonica*) and hinoki cypress (*Chamaecyparis obtusa*) pollen. The cedar and cypress tree species are major sources of airborne pollen, carrying potent allergens that have been reported to be the main cause of pollinosis in Japan.[4] Today, it is estimated that more than 1 in 10 Japanese citizens has pollinosis. Further, the cedar and cypress pollen share a common antigen (70% of Japanese patients with cedar pollen allergy also developed cypress pollinosis) and have their season shifted in time but overlapping so that the pollen season of the combined cedar and cypress pollen is unusually long. The cedar pollen season starts in February and ends in the beginning of April, whereas the cypress pollen season starts in March and can last until the beginning of May, thus making a pollen season of approximately 12 weeks, twice as long as the typical 6-week ragweed pollen season. Thus, cedar and cypress allergy patients are exposed to pollen for a long time, making the development of a pollen alert system desirable. Current pollen alerts are generated by information systems that use the data collected by a network of automatic pollen counters as one of their model inputs to compute pollen forecasts.[5–7] Besides the application in allergy prevention, the spread of transgenes through pollen of genetically modified crops needs to be monitored to evaluate the impact of genetically modified crops, which could lead to disruption of natural habitats.[2,3] The environment evaluation of genetically modified crops requires detailed data on pollen dispersal that can only be collected with an automatic network of pollen counters.

Automatic pollen counters that are widely used in Japan include the KH3000 (Yamato, Yokosuka, Japan),[8] Kowa (Hamamatsu, Japan),[9] and NTT (Tokyo, Japan)[10] counters. Recently, a new pollen counter developed by Shinyei Corporation (Kobe, Japan) was introduced on the market and has triggered much interest because of its new pollen discrimination principle and its low cost. The design of the new automatic counter is based on the design of the standard particle counter in which a defined volume of air is circulated through a fine pipe that is intersected by a laser beam. When

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a particle passes through the laser beam, a scattered signal is detected, the intensity of which is related to particle size and optical index. The measured intensity of the scattered light can be related to particle size. In addition to the scattered intensity, the Shinyei counter includes a measure of the change in the polarization state of scattered light, which is known to be related to the particle shape and its internal structure. Pollen grains generate intensity and polarization signals that are different from those of nonpollen particles, so that pollen can be recognized from these 2 measures. The application of this pollen recognition principle may lead to some errors, because it was recognized that under certain circumstances (types of particulate matter) some overlapping between the scattered intensity and polarization values of pollen and particulate matter takes place. The KH3000 counter uses the spherical shape of pollen grains to discriminate them from other particles (the nearly perfect spherical shape of pollen is not found in particulate matter that lacks a biological origin such as sand and soot). To discriminate spherical particles from others, 2 laser beams and their respective detectors are used to measure the intensity of the scattered light from the same particle but measured at 2 different incident angles. If the particle is spherical and homogeneous, the 2 intensities will be the same. The existing automatic counters together with their main characteristics are listed in Table 1. Extensive comparisons against reference samplers, such as the gravimetric Durham sampler and the volumetric Burkard sampler (Burkard Manufacturing Co, Rickmansworth, England), remain scarce, resulting in poor characterization of actual counter performance in the field and, therefore, in ambiguity as to how the data should be used in a pollen information system. The new counter introduced by Shinyei and the lack of data on actual performance of the available counters prompted us to conduct our own side-by-side comparison.

MATERIALS AND METHODS

During the cedar and hinoki pollen season of 2005, we conducted extensive side-by-side comparisons between the KH3000 and Shinyei automatic pollen counters and the Durham and Burkard reference pollen samplers. Our measurement campaign was performed at 2 different sites, namely, on the roof of a 4-story building in the Chiba University Campus (140.133° east and 35.602° north) and on a 5-story building of the Akita Health Institute (140.898° east and 39.717° north). The measured items together with the measurement periods are given in Table 2. Although the Burkard samplers used at both sites were of a different type, they were of a similar design except for the vacuum source, which uses a fan in the old version (7-day spore trap) and a mechanical pump in the new version (SporeWatch trap). The pollen counts on the Melinex tape used in the Burkard samplers and on glass slides used in the Durham samplers were performed under a microscope at a magnification of ×400 by trained staff. Both cedar and hinoki pollen grains were counted, because the automatic counters do not distinguish these pollen species, which are similar in size and shape. Durham daily counts were only available at the Chiba site and were determined by observing an area of 1 cm^2 (in this case, cedar and hinoki were counted separately). For the Burkard counts, the observed areas of the Chiba data and the Akita data were 0.5 × 5 mm^2 and 2 × 14 mm^2, respectively. The KH3000 counters were operated with a sand gravimetric trap, which is thought to filter out yellow sand particles originating from deserts in China and Mongolia and sporadically blowing across Japan. The actual performance of the sand trap with regard to discrimination between pollen and sand has not been reported to our knowledge. The recently developed Shinyei pollen counter[11] exists in 2 versions: the original version, referred to as Shinyei, which has to be placed in a weather instrument shelter, and a modified version, referred to as NTT-Shinyei, which has a higher flow rate and is protected by an all-weather casing. The measurement results obtained with the original version of the NTT counter[10] are not shown because the NTT counter was updated to the NTT-Shinyei counter. The KP1000 Kowa counter[9] was found to be difficult to operate during a long period (we have had experience during 2 cedar pollen seasons) because of high running cost and repeated failures, so we decided not to include data from this counter in our study.

RESULTS

Figure 1 shows the variation in time of the daily deposition count and the average daily concentration for the Chiba site. The deposition count was determined by using a Durham sampler and counting separately cedar and hinoki pollen grains. The average daily concentration was obtained by averaging hourly concentrations measured by the automatic pollen counters. These counters cannot distinguish cedar from hinoki pollen grains and thus should be compared with observations of the total count of cedar and hinoki pollen grains. As shown in Figure 1, the hinoki pollen contribution to deposition counts was significant from the middle of March and predominant at the end of March. For the Akita

Table 1. Main Characteristics of the Most Widely Used Automatic Counters and the Burkard Reference Sampler

Counter	Measurement principle	Pollen discrimination	Flow rate, L/min
Burkard	Impactor and microscopy	Microscope observation	10
KH3000	Scattering from 2 beams	Spherical shape	4.1
Kowa	Scattering and fluorescence	Size and fluorescence	4.0
Shinyei	Scattering and polarization	Size and shape	0.9 (original), 2.2 (NTT-Shinyei)
NTT	Scattering	Size	30

Table 2. List of Instruments and Their Period of Operation at Both Measurement Sites

Site (period of operation)	Durham	Burkard	KH3000	Shinyei
Chiba (1/24/05–4/24/05)	C	3/25–3/30 (SporeWatch, 1-hour sampling)	C (with sand filter)	C (NTT-Shinyei)
Akita (3/28/05–4/25/05)	NA	C (7-day sampler, 2-hour sampling)	C (with sand filter)	C (Shinyei original)

Abbreviations: C, complete dataset; NA, dataset not available.

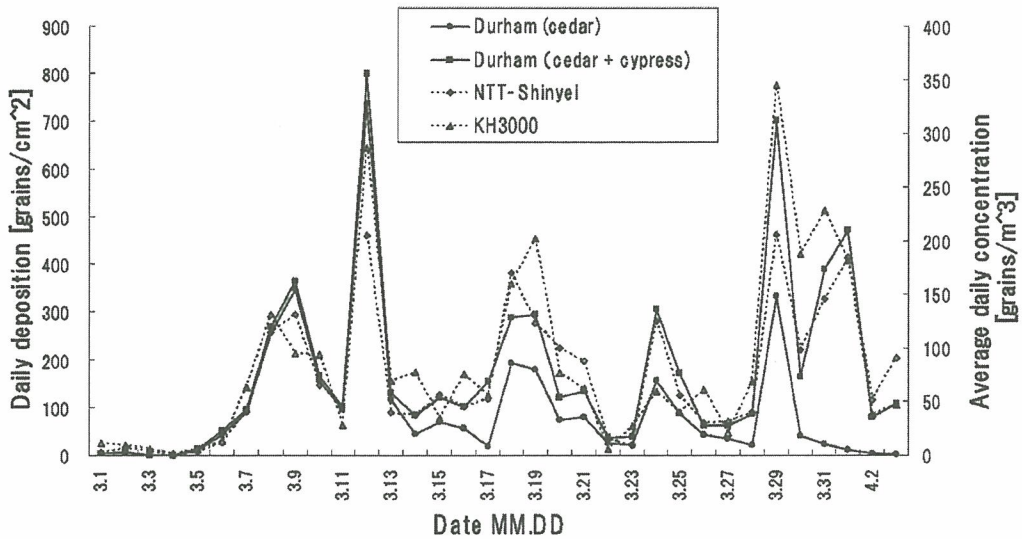

Figure 1. Comparison between daily deposition and daily average concentration of pollen at the Chiba site for the complete pollen season. The daily deposition counts for the cedar and hinoki total and for the cedar only are shown to illustrate the contribution of hinoki to the total count of pollen. Average daily concentrations were computed from hourly concentration values measured by automatic counters.

site, hinoki pollen contribution is known to be negligible during the entire pollen season because of the quasi-absence of the hinoki species in this area. From Figure 1 it is clear that the variations in daily deposition counts determined by the Durham reference sampler are well reproduced by the variations in the daily average concentrations of the automatic counters. Note that the relation between deposition and concentration depends on local factors, such as irregular topography and micrometeorological conditions, and is therefore varying over space and time. In our comparison, we assume the average over a day of these factors to be constant. The linear correlation coefficients between the Durham counts and the concentrations of both automatic counters were higher than 0.9, strongly suggesting that the automatic counters correctly approximate daily pollen variations.

F2 Figure 2 shows the pollen concentration time series as measured with the automatic counters and the reference sampler at both sites. Timing of the pollen bursts are well reproduced by the 2 automatic counters. This is further evidenced by the high values of the linear correlation coefficients between the counters and the reference sampler. For the Chiba data, both a Shinyei and a NTT-Shinyei counter were used and the correlations were computed on hourly concentrations. For the Akita data, a Shinyei module was used and the correlations were computed on 2-hour average concentrations. At the Chiba site, the linear correlation coefficients were 0.89, 0.92, and 0.90 for the KH3000, Shinyei, and NTT-Shinyei counters, respectively. At the Akita site, the linear correlation coefficients were 0.83 and 0.81 for the KH3000 and Shinyei counters, respectively. It also appears from Figure 2 that the intensity of the burst is not always well determined by the automatic counters. All counters underestimated pollen concentrations, pointing to calibration problems in the automatic counters. Small variations in pollen concentrations (<100 grains/m^3) as measured by automatic counters did not compare well with those determined with the reference sampler. In the Akita time series of Figure 2b, we found a high correlation between a sleet episode recorded by the Japan Meteorology Agency on March 29 to 30 and high counts of the KH3000. During this sleet episode, no pollen was observed in the Burkard reference concentration series. This strongly suggests that the KH3000 counted snow particles as pollen grains. This phenomenon was not observed with the Shinyei counter.

In Figure 3 and Figure 4, we examine in more detailed F3,F4 measurement errors in pollen concentration determined by the automatic counters for the Akita series. The concentrations measured by the automatic counters were first corrected for bias introduced by calibration errors using a linear calibration curve between the reference and the automatic

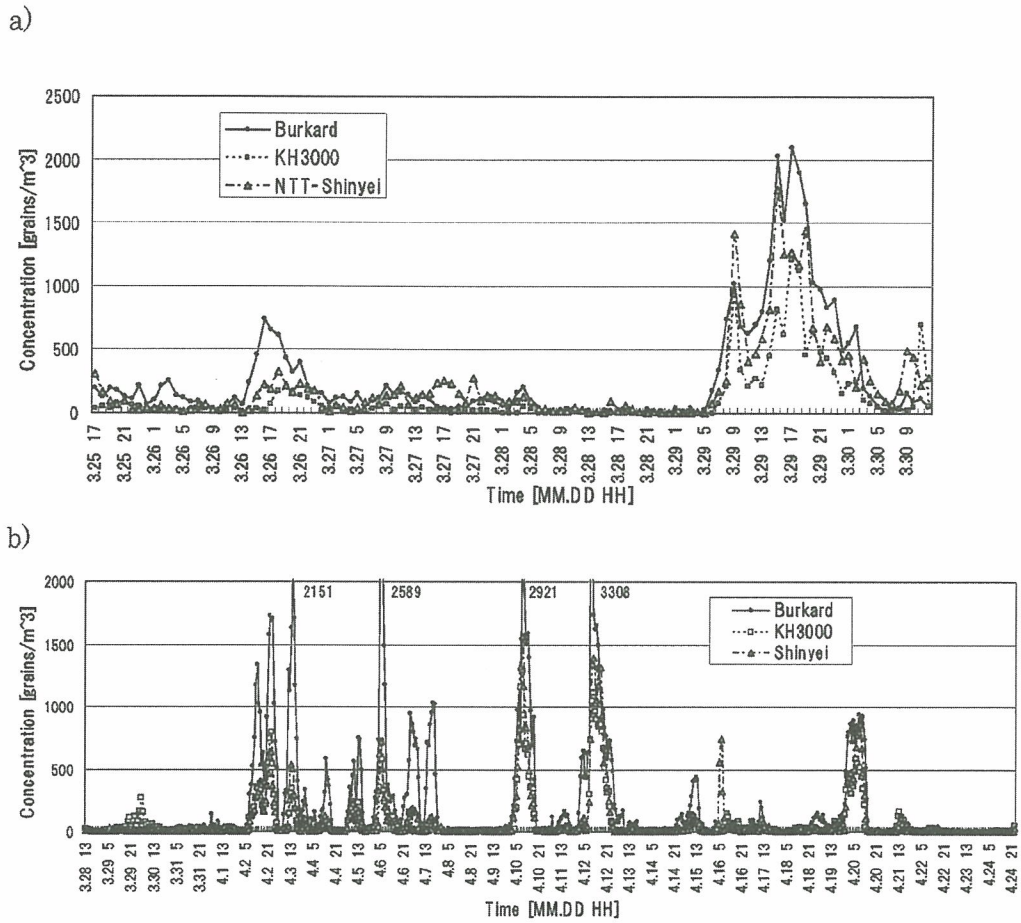

Figure 2. Comparison between pollen concentrations measured with a Burkard, KH3000, and Shinyei counter at the Chiba site (a) and the Akita site (b). In section b, the maximum value of the concentration axis was set to 2,000 grains/m^3 to make visible the variations for all counters (actual values for the Burkard counts that fall outside the plotted range are indicated on the right side of each peak). The 1-hour average and 2-hour average of pollen concentration are plotted for the Chiba site and the Akita site, respectively.

counters, and then the residuals were computed and used to derive the absolute errors between the automatic counters and the reference sampler. Figure 3a-b shows detailed time variations in the corrected concentrations together with the reference concentration for a low-concentration region and a high-concentration region of the Akita series, evidencing poor (good) correlation between the automatic counters and the reference sampler for the low (high) concentration region. Figure 4 shows the absolute errors in concentration measurement between the automatic counters and the reference sampler as a function of the averaged concentration. The curves indicated as "observed" refer to errors estimated from differences between the reference sampler and the automatic counters, whereas the curves indicated as "statistical" refer to errors estimated from the theory of statistical fluctuations. When counting airborne pollen grains, unavoidable statistical fluctuations in the observations result from the random nature of the observed process. These fluctuations are not related to any instrument error; that is, an estimate of the statistical fluctuations gives the lower limit to the measurement error for an ideal instrument. An estimate of the fluctuation error can be obtained from the SD of the observation distribution, which is known to follow a Poisson distribution in a counting experiment. The SD of a Poisson distribution is the square root of the mean of the counted events, which was used to compute the statistical errors of Figure 4. The difference in the statistical errors between the 2 automatic counters is explained by a difference in the sampled volumes (see the flow rate column of Table 1), that is, a difference in the number of counted pollen grains for the same concentration. For both automatic counters, the observed errors are found to be much larger than the statistical errors, pointing to the existence of instrumental errors. For concentration in the 0 to 50 grains/m^3, the error of the Shinyei counter is as large as the

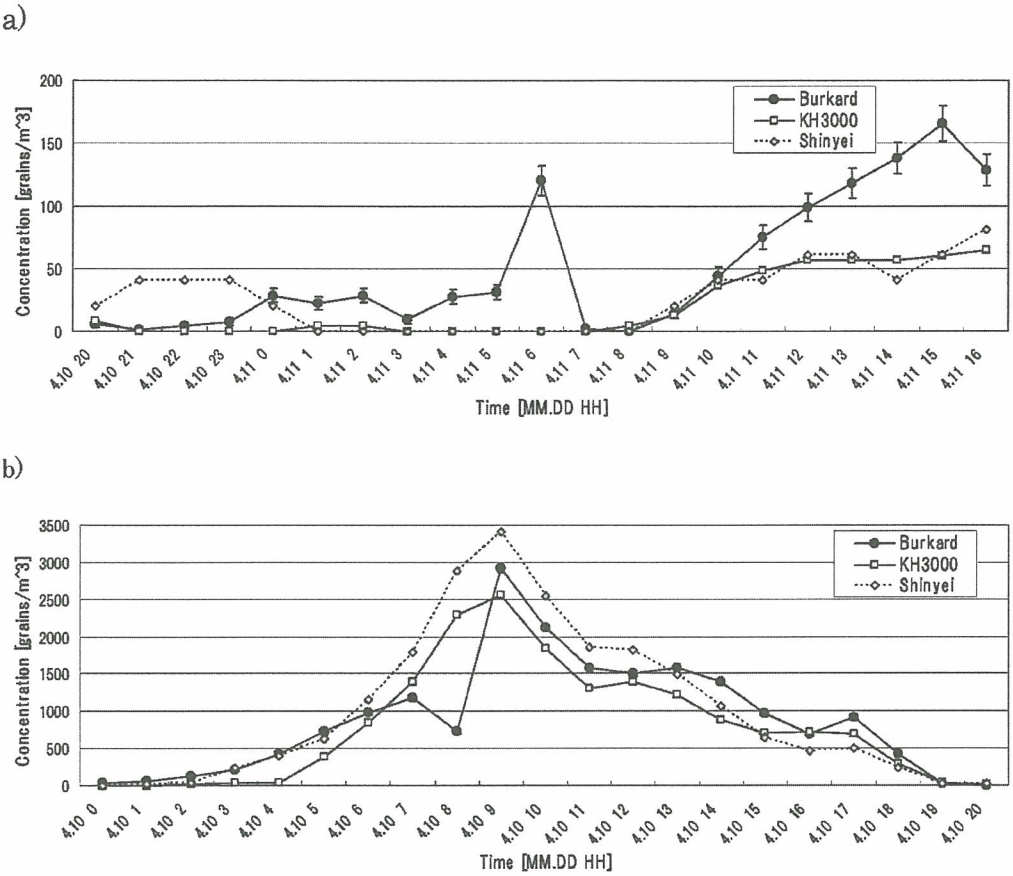

Figure 3. Detailed variations in pollen concentration with time at Akita as measured with a Burkard sampler and a KH3000 and Shinyei counter for a low-concentration region (a) and a high-concentration (b) region. The error bars of the Burkard series represent statistical uncertainty, which arises from statistical fluctuations in the pollen counts and were estimated as the square root of the counted pollen grains. (Note that the values of the error bars in section b are too small compared with the extent of the y-axis to be represented.)

measured concentration, indicating that data collected with this counter should be used for concentrations larger than 50 grains/m^3.

DISCUSSION

A side-by-side comparison conducted at 2 different sites between reference samplers and automatic pollen counters revealed some weaknesses of the investigated counters in the areas of pollen discrimination, minimum measurable concentration, and calibration. These imperfections rooted in the design of the investigated automatic counters directly affect the accuracy of the measured pollen concentration and limit their applications. Apart from changes in counter design, which fall outside the scope of this report, we would like to point out to a possible area of improvement. Because the 2 investigated automatic counters have been found to exhibit different characteristics in their ability to discriminate pollen from other particles, combining observations of both counters to filter out false peaks should lead to some improvements in the accuracy of these pollen counters when used simultaneously. Also some recommendations when setting up pollen counters in weather shelters (commonly used to protect instruments from sunshine and precipitation) may be useful. When operating a counter in weather shelter, the air should be sampled outside the shelter through a channel like the ones of the KH3000 or the NTT-Shinyei counters. Sampling inside the shelter could result in measurement errors, because pollen grains that are inevitably deposited inside the shelter are likely to be reemitted at a later time.

Pollen discrimination problems and calibration inaccuracies greatly hamper the use of automatic pollen counter data in a pollen forecasting system and should be addressed. The minimum measurable concentration of the investigated counters was found to be approximately 50 grains/m^3, a concentration that may be too high to provide useful information to patients with allergy. The value of the minimum concentration to be monitored in a pollen information system has to be debated and agreed on by the allergy community.

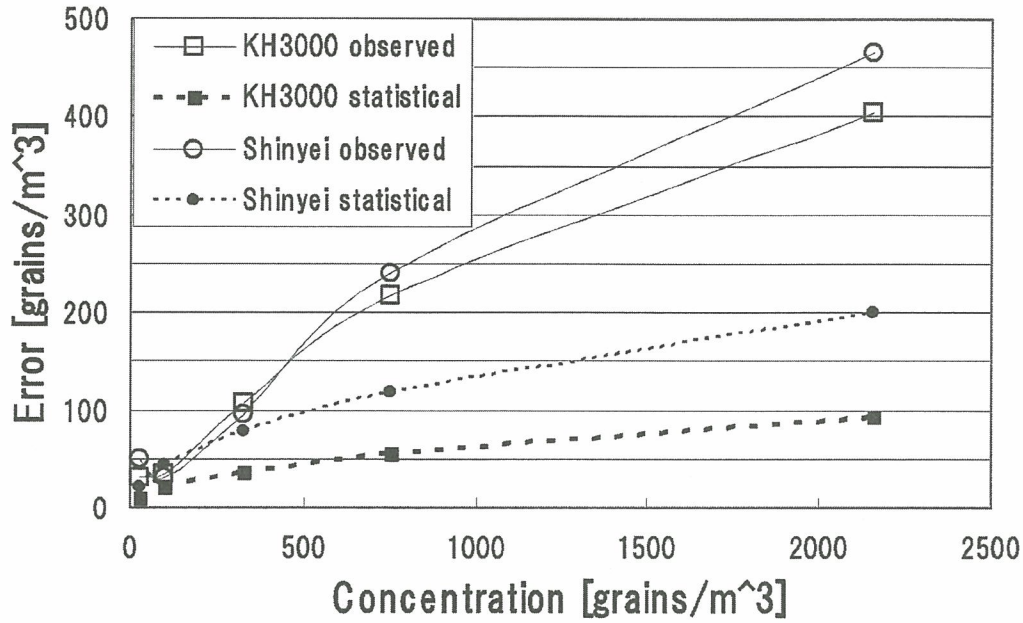

Figure 4. Error analysis of automatic pollen counters performed on the Akita dataset. Absolute errors are computed between the concentrations measured with the Burkard reference sampler and the automatic counters. The 2-hour average concentrations were used and errors computed in the following bins: 0 to 50, 50 to 150, 150 to 500, 500 to 1,000, and more than 1,000 grains/m^3.

For the automatic pollen counters to be successfully integrated in a pollen monitoring network, further developments in the pointed areas have to be made.

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A Phase I Study of *In vitro* Expanded Natural Killer T Cells in Patients with Advanced and Recurrent Non–Small Cell Lung Cancer

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Abstract

Purpose: Human Vα24 natural killer T (Vα24 NKT) cells bearing an invariant Vα24Jα Q antigen receptor are activated by a glicolipid ligand α-galactosylceramide (αGalCer; KRN7000) in a CD1d-dependent manner. The human Vα24 NKT cells activated with αGalCer and interleukin-2 have been shown to produce large amounts of cytokines, such as IFN-γ, and also exerting a potent killing activity against various tumor cell lines. We did a phase I study with autologous activated Vα24 NKT cell therapy.

Experimental Design: Patients with advanced or recurrent non–small cell lung cancer received i.v. injections of activated Vα24 NKT cells (level 1: $1 \times 10^7/m^2$ and level 2: $5 \times 10^7/m^2$) to test the safety, feasibility, and clinical response of this therapeutic strategy. Immunomonitoring was also done in all cases.

Results: Six patients were enrolled in this study. No severe adverse events were observed during this study in any patients. After the first and second injection of activated Vα24 NKT cells, an increased number of peripheral blood Vα24 NKT cells was observed in two of three cases receiving a level 2 dose of activated Vα24 NKT cells. The number of IFN-γ-producing cells in peripheral blood mononuclear cells increased after the administration of activated Vα24 NKT cells in all three cases receiving the level 2 dose. No patient was found to meet the criteria for either a partial or a complete response.

Conclusions: The clinical trial with activated Vα24 NKT cell administration was well tolerated and carried out safely with minor adverse events even in patients with advanced diseases.

A unique lymphocyte subpopulation, consisting of invariant natural killer T (iNKT) cells, is characterized by the coexpression of an invariant antigen receptor and natural killer (NK) receptors (1–4). Human iNKT cells express the invariant Vα24JαQ paired with the Vβ11 antigen receptor and are activated by a specific glycolipid antigen α-galactosylceramide (αGalCer) in a CD1d-dependent manner. CD1d is an HLA class Ib antigen-presenting molecule, which is well conserved through mammalian evolution with a lack of allelic polymorphism (5, 6). After activation, human Vα24 NKT cells show a strong antitumor activity against various malignant tumors both *in vitro* and *in vivo* (2, 7, 8) and produce high levels of cytokines, such as IFN-γ and interleukin-4 (IL-4), thereby activating other antitumor effector cells (9–12). Decreased numbers of Vα24 NKT cells in human peripheral blood mononuclear cells (PBMC) have been shown in patients with malignant diseases (13–15). At the same time, functional alterations of Vα24 NKT cells have also been reported after *in vitro* stimulation with αGalCer in patients with some malignant diseases (13, 16–18). For the patients possessing severely decreased or functionally deficient Vα24 NKT cells, the expansion and activation of these cells *in vitro* and the subsequent adoptive transfer may be therapeutically beneficial. The *in vitro* expansion of Vα24 NKT cells has been reported to be successful in the presence of αGalCer and IL-2 in both healthy volunteers and cancer-bearing patients (19–21).

Based on theses findings, we carried out a phase I study using *in vitro* expanded Vα24 NKT cells in patients with recurrent or advanced non–small cell lung cancer. The goal of this study was to confirm the safety profile of activated Vα24 NKT cell immunotherapy, and no severe adverse events were observed.

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We detected an increased number of Vα24 NKT cells and also an increased number of IFN-γ-producing cells in the PBMCs of the patients receiving $5 \times 10^7/m^2$ *in vitro* expanded Vα24 NKT cells.

Patients and Methods

Patient eligibility criteria. Patients between 20 and 80 years of age, with a histologic or cytologic diagnosis of non-small cell lung cancer for which no standard treatment was available, were eligible for the study. Further inclusion criteria were a performance status of 0, 1, or 2; an expected survival of ≥6 months; normal or near normal renal, hepatic, and hematopoietic function; and no chemotherapy or radiotherapy received for at least 4 weeks before enrollment. In enrolled patients, $V\alpha24^+V\beta11^+$ NKT cells were detected at a level of >100 cells in 1 mL peripheral blood by flow cytometry. The exclusion criteria were a positive response to HIV, hepatitis C virus, or human T-cell lymphotrophic virus antibodies; positive for hepatitis B antigen; the presence of active inflammatory disease or active autoimmune disease; a history of hepatitis; pregnancy or lactation; concurrent corticosteroid therapy; and evidence for another active malignant neoplasm. The histologic type, tumor-node-metastasis classification, and the antitumor effect of treatment were classified according to the general rules for clinical and pathologic recording of lung cancer as described by the Japan Lung Cancer Society.

Clinical protocol and study design. The study was carried out in the Department of Thoracic Surgery, Chiba University Hospital, Japan, according to the standards of Good Clinical Practice for Trials on Medicinal Products in Japan. The protocol was approved by the Institutional Ethics Committee (no. 1972). In addition, this trial underwent *ad hoc* reviews by the Chiba University Quality Assurance Committee on Cell Therapy.

The study design is illustrated in Fig. 1. Written informed consent was obtained from all of the patients before undergoing a screening evaluation to determine eligibility. Clinical and laboratory assessments were conducted once a week, consisting of a complete physical examination and standard laboratory values. Any adverse events and changes in laboratory values were graded according to the National Cancer Institute Common Toxicity Criteria version 2.0. All patients underwent an assessment of the tumor status at baseline and 4 weeks after the second NKT cell administration (7 weeks after study entry). Disease progression was defined as >25% increase in target lesions and/or the appearance of new lesions.

Preparation of activated Vα24NKT cells from peripheral blood. All procedures were done according to the Good Manufacturing Practice standards. Eligible patients underwent peripheral blood leukapheresis (Spectra, COBE). PBMCs were collected and then further separated by density gradient centrifugation (OptiPrep, Axis-Shield, Oslo, Norway). After washing three times, the cells were resuspended in AIM-V (Invitrogen Corp., Carlsbad, CA) with 100 JRU/mL of recombinant human IL-2 (Imunace, Shionogi, Japan) and 100 ng/mL of αGalCer (KRN7000; Kirin Brewery Co., Gunma, Japan). Restimulation with αGalCer-pulsed autologous PBMCs was done on days 7 and 14. After 14 or 21 days of cultivation, the cells were harvested, washed thrice, and then resuspended in 100 mL of 2.5% albumin in saline. The patients received the cultured cells i.v. The criteria for activated Vα24 NKT cell administration included a negative bacterial culture 48 hours before Vα24NKT cell injection, cell viability >70%, and an endotoxin test 48 hours before cell injection with a result <0.7 EU/mL. The activated Vα24 NKT cells were given in a dose escalation design at a dose level per cohort of 1×10^7 and 5×10^7 cells/m^2 per injection. The cell dose represents the $V\alpha24^+V\beta11^+$ NKT cell number but not the bulk population of the cells.

Activated Vα24NKT cell phenotype evaluation. The phenotypes of peripheral blood lymphocytes and activated Vα24NKT cells were determined by a flow cytometry analysis. The monoclonal antibodies (mAb) used were FITC-labeled anti-TCR Vα24 mAb (C15; Immunotech, Marseilles, France), anti-CD8 mAb (HIT8a; BD Biosciences PharMingen, San Diego, CA), phycoerythrin-labeled anti-TCR Vβ11 mAb (C21; Immunotech), anti-CD56 mAb (B159; BD Biosciences PharMingen), and cychrome-labeled anti-CD3ε mAb (UCTH1; BD Biosciences PharMingen), anti-CD4mAb (RPA-T4; BD Biosciences PharMingen). Phycoerythrin-labeled αGalCer-loaded CD1d tetramer was prepared in our laboratory using a baculovirus expression system kindly provided by Dr. M. Kronenberg (La Jolla Institute, La Jolla, CA; ref. 22). Isotype-matched control mAbs were used as negative controls. To determine the percentages of Vα24NKT cells in the *in vitro* αGalCer/IL-2-cultured PBMCs, we presented the values of $V\alpha24^+V\beta11^+CD3^+$ cells because the percentages of αGalCer/CD1d tetramer-positive cells and $V\alpha24^+V\beta11^+CD3^+$ cells are basically identical (Supplementary Fig. S1).

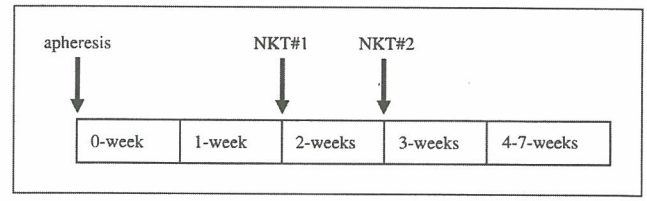

Fig. 1. Experimental design of activated Vα24 NKT cell administration. The patients received *in vitro* expanded activated Vα24 NKT cells (*NKT#1* and *NKT#2*). Timing for both apheresis and activated Vα24 NKT cell administration.

^{51}Cr release cytotoxic assay. Target Daudi cells (B lymphoma) and PC-13 cells (lung large cell carcinoma; 5×10^6) were labeled with 100 μCi sodium chromate (Amersham LIFE SCIENCE, Little Chalfont, Buckinghamshire, England) for 1 hour. Activated NKT cells were seeded into 96-well round-bottomed plates at the indicated effector/target ratios on ^{51}Cr-labeled Daudi cells or PC-13 cells (1×10^4). Radioactivity released from lysed target cells was counted on a γ-counter after 4 hours of incubation at 37°C in 5% CO_2 incubator. The percentage of ^{51}Cr release was calculated by the following formula: % specific lysis = (sample cpm − spontaneous cpm) × 100 / (maximum cpm − spontaneous cpm) as described (19). Spontaneous cpm was calculated from the supernatant of target cells alone, and the maximum release was obtained by adding 1 N HCl to the target cells. The data are expressed as the mean value of triplicate cultures with SDs.

Cytokine measurement. The amount of cytokines in the culture supernatant was measured by ELISA. Cultured Vα24 NKT cells were plated at 2×10^5 per well with 1×10^6 irradiated PBMCs that had been previously pulsed for 2 to 3 hours with 100 ng/mL αGalCer. The supernatants were collected after 24 to 48 hours of activation and stored −80°C. IFN-γ and IL-4 were measured using ELISA kits (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Flow cytometric analysis of the peripheral blood NKT cells and NK cells. PBMC samples were obtained from the patients twice before Vα24 NKT cell administration and weekly until 4 weeks after the final injection. The frequencies of Vα24 NKT cells ($V\alpha24^+V\beta11^+CD3^+$), NK cells ($CD3^-CD56^+$), T cells ($CD3^+$ cell), and other cells, including B cells ($CD3^-CD56^-$ cells) in PBMCs, were assessed by flow cytometry using automated full blood counts. The frequency of monocyte was calculated from the cytogram findings. The absolute numbers of these cells were also calculated.

Single-cell enzyme-linked immunospot assay. The PBMCs were washed thrice with PBS and then were stored in liquid nitrogen until use. For detecting IFN-γ-secreting cells, 96-well filtration plates (Millipore, Bedford, MA) were coated with mouse anti-human IFN-γ (10 μg/mL; Mabtech, Nacka Strand, Sweden). PBMCs (5×10^5 per well) were incubated for 16 hours with or without αGalCer (100 ng/mL) in 10%FCS containing RPMI. Phorbol 12-myristate 13-acetate (10 μg/mL) plus ionomycin (10 nmol/L) was used as a positive control. After culture, the plates were washed and incubated with biotinylated

anti-IFN-γ (1 μg/mL; Mabtech). Spot-forming cells were quantified by a microscopy. In our enzyme-linked immunospot assay protocol, the majority of the IFN-γ-producing cells detected after αGalCer stimulation for 16 hours was $CD56^+$ NK and NKT cells (Supplementary Table S1).

Results

Patient characteristics. In accordance with the protocol, a total of six patients were enrolled in the study from July 2003 to March 2004. The patient characteristics are summarized in Table 1. All patients showed recurrent lung cancer after surgical treatment. The study included four patients with adenocarcinoma and two patients with squamous cell carcinoma. All patients received previous treatments, including two cases who had undergone an incomplete surgical resection of the recurrent legions, one who had received radiation therapy, and four who had received chemotherapy >4 weeks before enrollment into this study.

Phenotypes of activated Vα24NKT cells cultured with αGalCer and IL-2. The surface phenotypes of activated Vα24 NKT cells were analyzed by flow cytometry for each administration. Representative profiles (Vα24/Vβ11 and CD3/CD56) of a patient in the level 2 (case 005) are shown in Fig. 2A. Freshly isolated PBMCs contained very small percentages of $V\alpha24^+V\beta11^+$ NKT cells (0.06%; Fig. 2A, *top left*). After cultivation in the presence of αGalCer and IL-2, this population efficiently expanded (25%, 2 weeks and 21.5%, 3 weeks; Fig. 2A, *top center* and *right*). The CD3-negative population decreased after cultivation (Fig. 2A, *bottom*). The percentages of NKT cells, $CD3^+$ cells, and $CD3^-CD56^+$ cells in the cultured cells of six patients (before, 2 weeks, and 3 weeks after cultivation) are summarized in Table 2. The CD4/CD8 expression of αGalCer/CD1d tetramer-positive cells was also investigated. $CD8^+$ and $CD4^-CD8^-$ phenotype were dominant in one patient (case 005), and $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ phenotypes were all observed in other two patients in the levels 2 (Fig. 2B). It is interesting to note that we detected a substantial number of $CD8^+V\alpha24^+V\beta11^+$ NKT cells, which correlated with the findings of a previous report (23). Next, functional evaluations of the levels of cytotoxic activities and cytokine production were done. Activated Vα24 NKT cells simulated with αGalCer and IL-2 showed efficient cytotoxic activities against human tumor cell lines, including Daudi cells and PC-13 cells (Fig. 2C). In addition, they also produced a large amount of IFN-γ and a small amount of IL-4 in response to a rechallenge with αGalCer-pulsed PBMCs (Fig. 2D). The number of $V\alpha24^+V\beta11^+$ NKT cells in the PBMC cultures for 1 to 3 weeks increased efficiently with some variations (Fig. 2E). The expansion rate of Vα24 NKT cells in six donors averaged 1,290-fold (range: 50- to 3,460-fold) for the 14-day culture period and 2,380-fold (range: 280- to 5,250-fold) for the 21-day culture period, and these results are consistent with those shown in our previous report (19).

Adverse events. No major (grade >2) toxicity or severe side effects were observed in any patients (Table 1). One patient in the level 1 group (case 001) experienced a transient flush and headache soon after the Vα24 NKT cell injection, but no additional treatment was required. One patient in the level 1 group (case 003) experienced transient arrhythmia a few hours after the Vα24 NKT cell injection, and the symptom disappeared in several minutes without any medication. One patient in the level 2 group (case 004) experienced a mild facial paralysis (numbness) on day 42 (21 days after the final injection). A zygomatic bone metastasis was found after precise medical check-up. The numbness was improved by a radiation therapy on the metastatic legion in the zygomatic bone. One patient in the level 2 group (case 006) experienced an increased fever (grade 1) a few hours after the Vα24 NKT cell administration, which abated 6 hours after the use of loxoprofen sodium. Regarding the laboratory data, an elevation in the serum glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase (day 42), and total bilirubin level (day 22) was observed in one patient in the level 1 group (case 001); a lactate dehydrogenase elevation (day 28) was observed in one patient in the level 1 group (case 003); and an elevation in the γ-GTP level (day 22) was observed in one patient in the level 2 group (case 006). The Common Terminology Criteria for Adverse Events grade of these abnormal data was categorized as grade 1, and no cases needed any additional treatment.

Table 1. Profiles of each patient and any adverse events observed

Level	Case	Age/gender	Diagnosis	Cancer lesion	PS	Pre-Tx	Adverse events
I	001	66/F	Recurrence after complete surgical resection, adenocarcinoma	Lung, pleura	0	ST, CT	Hot flash, headache, GOT, GPT, total bilirubin elevation
I	002	78/M	Recurrence after complete surgical resection, squamous cell carcinoma	Lung, SCLN; mediastinal LN	1	RT	None
I	003	78/M	Recurrence after complete surgical resection, squamous cell carcinoma	Lung	1	CT	Arrhythmia, LDH elevation
II	004	66/M	Recurrence after complete surgical resection, adenocarcinoma	Lung	0	CT	Facial paralysis
II	005	55/M	Recurrence after complete surgical resection, adenocarcinoma	Lung, pleura	0	CT	Headache, general fatigue
II	006	61/F	Recurrence after complete surgical resection	Lung, brain	0	ST	Fever, γ-GTP elevation

Abbreviations: SCLN, supraclavicular lymph node; LN, lymph node; PS; performance status; pre-Tx, treatment after recurrence and before clinical trial; ST, surgical treatment of the recurrent lesions; RT, radiation therapy; CT, chemotherapy; GOT, glutamic-oxalacetic transaminase; GPT, glutamic-pyruvic transaminase; LDH, lactate dehydrogenase.

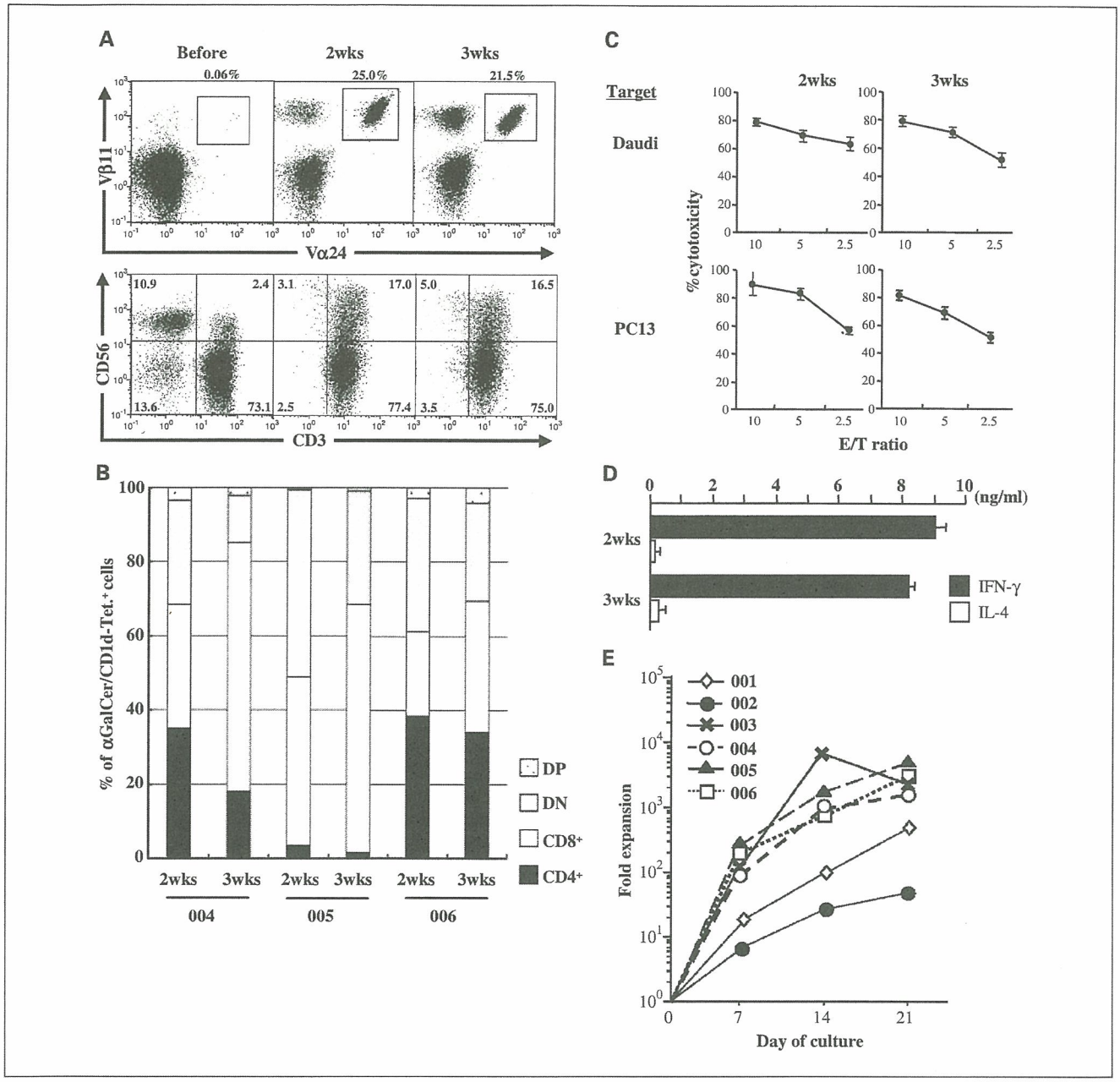

Fig. 2. Phenotypic and functional evaluation of activated Vα24 NKT cells. PBMCs from a patient were cultured with αGalCer and IL-2 for 2 or 3 weeks, and whole cultured cells were used for analysis. *A*, flow cytometric analysis of precultured (*Before*) or cultured cells (*2 wks* or *3 wks*). Top, percentages of $V\alpha24^+V\beta11^+$ NKT cells. Bottom, NK cells ($CD56^+CD3^-$), NKT cells ($CD56^+CD3^+$), and T cells ($CD3^+CD56^-$). Representative results of a patient (case 005) in the level 2 group. *B*, CD4/CD8 expression on αGalCer/CD1d tetramer-positive cells. Cultured cells were stained with phycoerythrin-labeled αGalCer/CD1d tetramer followed by FITC-labeled anti-CD8 mAb and cychrome-labeled anti-CD4 mAb. CD4/CD8 expression was determined by a flow cytometry analysis at the end of the 2- or 3-week culture period. *C*, cytotoxic activity of *in vitro* expanded Vα24 NKT cells. Whole cultured cells for 2 or 3 weeks were incubated with two target cell lines (1×10^4 per well) for 4 hours at various effector/target ratios (*E/T ratio*) in triplicate. Percentages of specific ^{51}Cr release with SD. *D*, cytokine production from activated Vα24 NKT cells. After 2- or 3-week cultivation with αGalCer and IL-2, whole cells were collected and seeded onto a 96-well plate (2×10^5 per well) in triplicate. Restimulation was done with αGalCer-pulsed, irradiated, autologous PBMCs for 24 hours, and the amounts of cytokines (IFN-γ and IL-4) in the culture supernatant were determined by ELISA. *E*, expansion of Vα24 NKT cells after cultivation with αGalCer and IL-2. The PBMCs were cultured with αGalCer (100 ng/mL) and IL-2 (100 JRU/mL). On days 7, 14, and 21, all cultured cells were harvested, and the total cell number and the percentage of $V\alpha24^+V\beta11^+$ NKT cells were evaluated by a flow cytometry analysis. The fold increase in $V\alpha24^+V\beta11^+$ NKT cells was determined as follows: [(total number of live cells recovered on days 7, 14, and 21) × (%$V\alpha24^+V\beta11^+CD3^+$ cells)] / [(total number of live cells before culture) × (%$V\alpha24^+V\beta11^+CD3^+$ cells before culture)]. Number (*001-006*) represent each enrolled patient.

Immunologic monitoring. Immunologic assays were done for the six patients who completed the study. The frequency of peripheral blood $V\alpha24^+V\beta11^+$ NKT cells in all patients was measured by a flow cytometry analysis (Fig. 3A-F). A level 1 patient (case 003) showed increased circulating Vα24 NKT cells on days 35 to 49 (Fig. 3C). Two patients in the level 2 group showed increases in the Vα24 NKT cells after the administration on day 28 (Fig. 3D) or on days 21 and 28

Table 2. Profiles of αGalCer/IL-2–cultured PBMCs

Level	Case	-NKT cell (%)			$CD3^+$ cell (%)			$CD3^-CD56^+$ cell (%)		
		Before	2 wk	3 wk	Before	2 wk	3 wk	Before	2 wk	3 wk
I	001	0.02	3.5	3.2	75.5	76.0	65.4	10.9	21.8	32.8
I	002	0.01	0.1	0.3	44.1	34.9	51.8	ND	ND	ND
I	003	0.01	10.3	2.5	76.4	93.3	ND	10.4	6.6	ND
II	004	0.04	3.3	1.3	51.4	96.0	95.7	28.0	3.6	3.8
II	005	0.06	25.0	21.5	75.5	94.4	91.5	10.9	3.1	5.0
II	006	0.05	2.5	2.0	78.9	64.2	59.2	10.8	34.0	38.3

NOTE: NKT cells, $V\alpha24^+V\beta11^+$ cells.
Abbreviation: ND, not done.

(Fig. 3E) compared with the initial NKT cell numbers detected (before or on day 14: before 1st injection). The absolute numbers of Vα24 NKT cells decreased transiently to a nadir around 1 day after the Vα24 NKT cell injection in these three cases. The number of NK cells slightly increased in one patient on day 28 (Fig. 3D), but no obvious increase was detected in the other four patients. In addition, we monitored the absolute numbers of peripheral blood $CD3^+$ cells, $CD3^-CD56^-$ cells, and monocytes in patients 005 and 006 (Fig. 3G and H). We observed a slight increase in the number of peripheral blood $CD3^+$ cells, but no obvious changes in the number of $CD3^-CD56^-$ cells or monocytes.

Concurrently, we assessed the ability to produce IFN-γ after restimulation with αGalCer. The patient PBMCs were stimulated with αGalCer for 16 hours, and the IFN-γ-producing cells in the culture were assessed by an enzyme-linked immunospot assay. The number of cells with IFN-γ production increased 1 and 2 weeks after the second injection in a patient in level 1 (case 001, Fig. 4A) and a level 2 patient (case 004, Fig. 4C), immediately after the first injection in a patient in level 2 (case 005, Fig. 4D), and 2 weeks after the second injection in case 006 (Fig. 4E). Regarding the production of IL-4, we did not observe a detectable number of αGalCer-dependent IL-4-producing cells in any cases in our assay system (data not shown).

Clinical outcome. All six cases were evaluated at the end of the clinical trial period. From the chest X-ray and computed tomography findings, there were no cases of complete response or partial response, four cases of stable disease (cases 001, 003, 005, and 006), and two cases of progressive disease (cases 002 and 004). Two patients receiving the level 2 dose were followed up for 9 and 12 months after the clinical trial period, and both of them were classified as stable disease.

Discussion

The primary aim of this study was to assess the feasibility and toxicity of adoptive immunotherapy using activated Vα24 NKT cells in patients with advanced or recurrent non–small cell lung cancer. Because this is the first clinical trial for the administration of Vα24 NKT cells activated *in vitro* with αGalCer and IL-2, we did the trial very carefully. Our results indicate that activated Vα24 NKT cell therapy has no major side effects and is well tolerated with minor adverse events, even in patients with advanced stages of lung cancer. There were no clinical symptoms suggesting the development of an autoimmune disease during the observation period. Furthermore, the therapy was safe with minor adverse events, and it can be easily done on an outpatient basis. Although activated iNKT cells in the mouse liver induced severe hepatitis (24, 25), only slight liver dysfunction was detected in two patients in the level 1 group and one patient in the level 2 group. These patients recovered without any additional treatments. This could be due to the limited number of activated Vα24 NKT cells that migrated into the liver of the patients receiving the Vα24 NKT cell administration.

We chose PBMCs as a source of Vα24 NKT cells for administration. An effective method to obtain a large number of purified, functional Vα24 NKT cells from human peripheral blood has been reported (21). In this report, autologous whole PBMCs were used as antigen-presenting cells instead of the additional preparation of monocyte-derived dendritic cells for stimulation. Our culture system was similar to that described in this report. The expansion of Vα24 NKT cells was sufficient (Fig. 2E); thus, this simple procedure seemed to be the most appropriate for the clinical use. The fold increase in the Vα24 NKT cell number varied among the patients (Fig. 2E), and it seems to be partially depended on the initial frequency of Vα24 NKT cells (see Table 2). We previously reported that the number of Vα24 NKT cells in the peripheral blood significantly decreased in patients with lung cancer (14). We, therefore, set relatively tight entry criteria (>100 mL) regarding the number of peripheral blood Vα24 NKT cells. The number of $V\alpha24^+V\beta11^+CD3^+$ NKT cells and αGalCer/CD1d tetramer-positive cells were almost identical in the cultures with αGalCer and IL-2 (Supplementary Fig. S1); therefore, the expansion of Vα24-negative αGalCer/CD1d tetramer-reactive T cells reported by Gadola et al. seemed to be negligible in our culture system (26).

Deficiencies in IFN-γ production or the proliferative potential of Vα24 NKT cells have recently been reported in some patients with advanced malignancies (13, 15, 17). In prostate cancer patients, *ex vivo* expansion of αGalCer-activated Vα24 NKT cells as well as their IFN-γ production was decreased (13). The number of peripheral blood Vα24 NKT cells was decreased in the cancer patients regardless of the tumor type or tumor load, but the ability to produce IFN-γ at a per cell basis did not decrease (15). Vα24 NKT cells from different types of solid cancer patients failed to proliferate even with αGalCer stimulation, thus producing reduced levels of cytokines compared with those from healthy individuals (17). In lung cancer patients, a reduced proliferative response of Vα24 NKT cells to αGalCer was detected, and the reduction was partially

recovered by granulocyte-colony stimulating factor (27). These studies indicate that Vα24 NKT cells in cancer patients have some numerical and functional defects. In contrast, the number and the function of Vα24 NKT cells has been reported not to be suppressed in glioma patients (28). We previously reported the ability to produce IFN-γ in peripheral blood Vα24 NKT cells to be preserved in non–small cell lung cancer patients (14). The reason for the discrepancy between the NKT cell function in the lung cancer patients in our report (14) and that in the report by Konishi et al. (27) is still unclear at this time. However, the levels of expansion and the ability to produce IFN-γ in our prepared activated Vα24 NKT cells were sufficient for the use of clinical trials (Table 2; Fig. 2). Although we still do not know whether there is functional alteration in the Vα24 NKT cells in the lung, we detected an accumulation of Vα24 NKT cells in the cancerous lesion in the lung, thus suggesting the occurrence of antitumor reactions against tumor cells (14).

In patients with either decreased or functionally altered Vα24 NKT cells, the expansion and activation of these cells *in vitro* and subsequent adoptive transfer should be therapeutically beneficial, if these deficiencies can be rectified through *in vitro* cultivation. From the results shown in this report, *in vitro* expanded Vα24 NKT cells seemed to be functional regarding IFN-γ production (Fig. 2). More importantly, we detected an increased number of IFN-γ-producing cells in the peripheral blood after the injection of activated Vα24 NKT cells. The timing of the increase detected was from a day to 2 weeks (Fig. 4). Because the number of injected activated Vα24 NKT

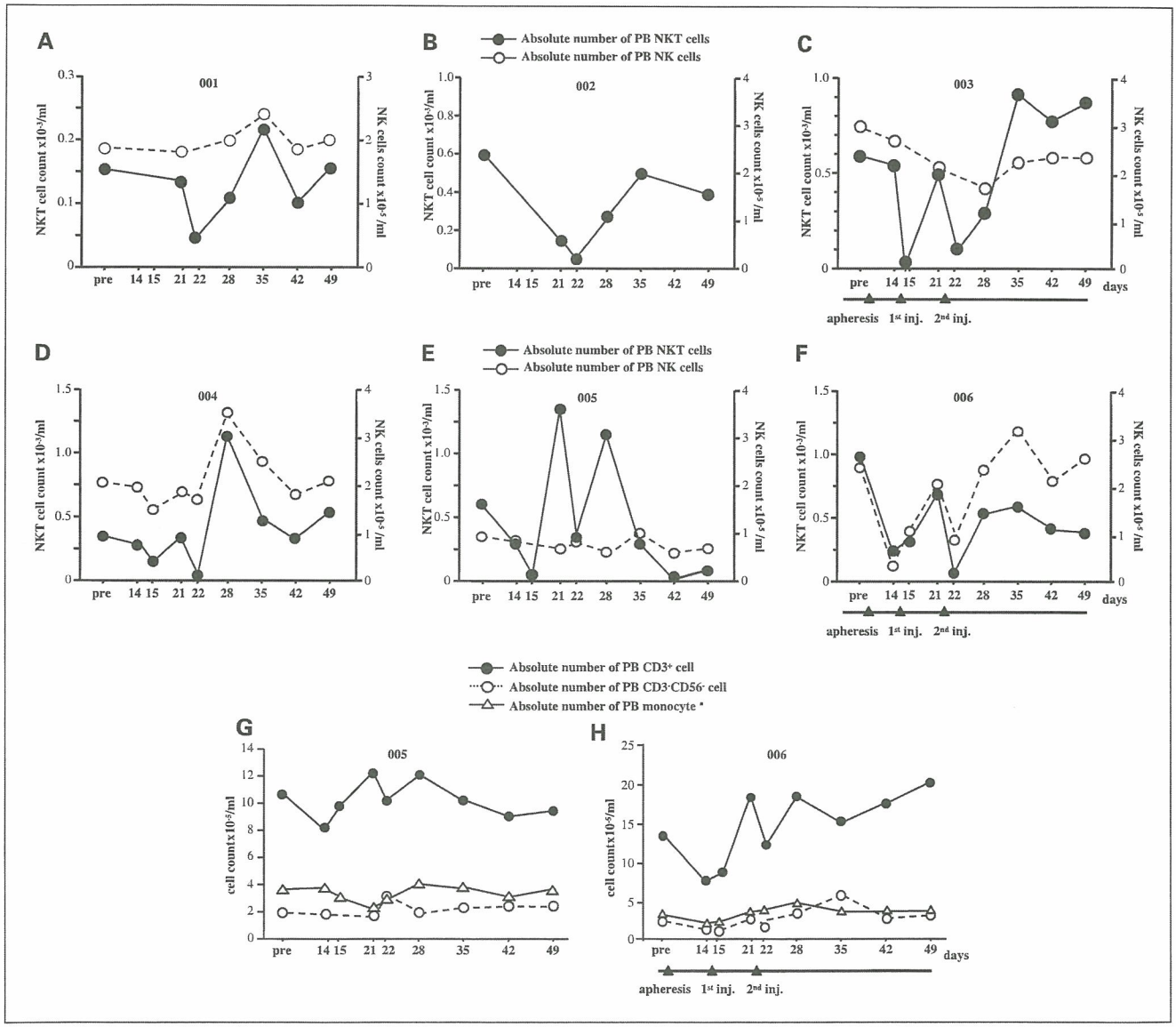

Fig. 3. Immunologic monitoring of PBMCs of patients with Vα24 NKT cell administration. Absolute number of peripheral blood NKT cells ($V\alpha24^+V\beta11^+$ cells) and NK cells ($CD56^+CD3^-$ cells; *A-F*) and that of T cells ($CD3^+$ cell), other cells including B cell ($CD3^-CD56^-$ cells), and monocytes (*G* and *H*) during the course of treatment in each patient. With the use of the results of a flow cytometry analysis and automated full blood counts (Chiba University Hospital), the absolute number of NKT cells, NK cells, T cells, and other cells was calculated. The absolute number of monocytes was calculated from the cytogram. 1st inj., first *in vitro* expanded NKT cell injection; 2nd inj., second *in vitro* expanded NKT cell injection.

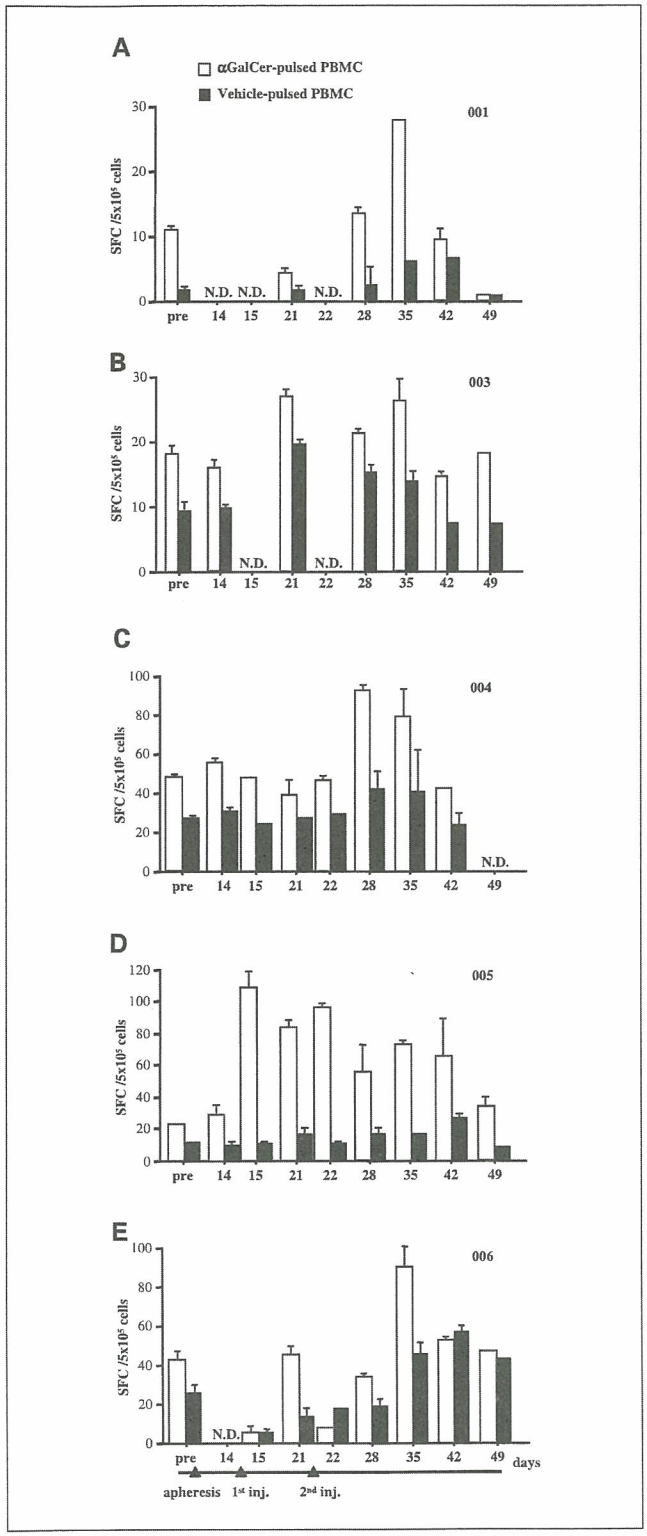

Fig. 4. Monitoring of IFN-γ-producing cells in PBMCs of patients with Vα24 NKT cell administration. Detection of αGalCer-reactive IFN-γ-producing cells by enzyme-linked immunospot assay. Cryopreserved PBMCs were thawed and cultured overnight with either αGalCer or vehicle. The presence of IFN-γ-producing cells was quantified by an enzyme-linked immunospot assay. Spot number of IFN-γ with SD for triplicate cultures. N.D., not done; 1st inj., first *in vitro* expanded NKT cell injection; 2nd inj., second *in vitro* expanded NKT cell injection; SFC, spot-forming cells.

cells was within the order of 1×10^8, it is very likely that some important change occurred in patients after the administration of activated Vα24 NKT cells, such as a robust expansion of endogenous Vα24 NKT cells and the activation of NK cells. In fact, the specific activation of iNKT cells has been reported to lead to a rapid induction of extensive NK cell proliferation and cytotoxicity, partially depending on the IFN-γ production by iNKT cells (9, 10, 29, 30). Regarding the transient decrease in the number of Vα24 NKT cells observed after NKT cell administration (Fig. 3C, D, and E), such a decrease was also observed in our previous clinical trials with αGalCer-pulsed dendritic cells (31). We do not know the reason at this time, but a possibility for the down-modulation of Vα24 NKT cell receptors from the cell surface has been suggested (32–34).

Immunotherapy methods with αGalCer or αGalCer-pulsed dendritic cells in patients with malignant diseases have been recently described (31, 35–37). In these trials, it is expected that the given αGalCer or αGalCer-pulsed dendritic cells induces the activation of Vα24 NKT cells *in situ*. We detected an increase in the number of Vα24 NKT cells in the peripheral blood of patients receiving αGalCer-pulsed dendritic cells (31). The most characteristic difference in the present study from these studies is that the activation process of Vα24 NKT cells with αGalCer presented on dendritic cells is done *in vitro*. We detected notable immune responses *in vivo* after the administration of activated Vα24 NKT cells (Figs. 3 and 4). Although we need to more precisely assess the nature of immune responses induced by the administration of activated Vα24 NKT cells with an increased number of patients, the results shown in this report provide an alternative procedure of cancer immunotherapy aimed at the activation of Vα24 NKT cells and the subsequent activation of NK cells.

No clear antitumor effect was observed in the present clinical trials. However, these trials are still small scale; thus, we need to await the findings of relatively large-scale studies to evaluate the clinical efficacy of this immunotherapy with activated Vα24 NKT cells. A study with a relatively longer monitoring period (at least a few years) may help to answer the question whether this immunotherapy can induce a long stable disease status in advanced cancer patients. In addition, a combined immunotherapy with the administration of αGalCer or αGalCer-pulsed dendritic cells is expected to result in a more effective clinical effect.

Primary lung cancer is hard to cure, although the primary tumor lesions tend to be small enough to be diagnosed at an early stage. Approximately half or more of patients with lung carcinomas who undergo complete resection had clinically undetectable local or distant micrometastases (38–40). These findings point to the importance of preoperative or postoperative immunotherapy to suppress the growth of micrometastasis. For this purpose, among the lymphocytes possessing antitumor activity, cells for tumor surveillance, such as NK and NKT cells, are considered to be the most appropriate. From this point of view, non–small cell lung cancer patients undergoing radical surgery may thus be the optimal candidates for immunotherapy aimed at Vα24 NKT cell activation.

In summary, immunotherapy of activated Vα24 NKT cell administration is well tolerated, and it can be carried out safely with minor adverse events even in patients with advanced disease. *In vivo* immunologic responses, including the elevation of IFN-γ-producing cells in the peripheral blood, were detected

after the administration of activated Vα24 NKT cells. With a greater number of treatments, we should eventually obtain more conclusive evidence regarding evaluation of the antitumor effect of this therapy. Furthermore, a combination of this therapy with a potentially additive or synergistic therapeutic strategy may also result in a more prominent antitumor effect.

Acknowledgments

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Crucial Role of MLL for the Maintenance of Memory T Helper Type 2 Cell Responses

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Summary

The *Mixed-Lineage Leukemia* (*MLL*) gene, a mammalian homolog of the *Drosophila trithorax*, is implicated in regulating the maintenance of *Hox* gene expression and hematopoiesis. The physiological functions of MLL in the immune system remain largely unknown. Although $MLL^{+/-}$ CD4 T cells differentiate normally into antigen-specific effector Th1/Th2 cells in vitro, the ability of memory Th2 cells to produce Th2 cytokines was selectively reduced. Furthermore, histone modifications at the Th2 cytokine gene loci were not properly maintained in $MLL^{+/-}$ memory Th2 cells. The reduced expression of MLL in memory Th2 cells resulted in decreased GATA3 expression accompanied with impaired GATA3 locus histone modifications. The direct association of MLL with the GATA3 locus and the Th2 cytokine gene loci was demonstrated. Memory Th2 cell-dependent allergic airway inflammation was decreased in $MLL^{+/-}$ Th2 cell-transferred mice. Thus, a crucial role for MLL in the maintenance of memory Th2 cell function is indicated.

Introduction

After stimulation with antigens, naive CD4 T cells differentiate into two distinct helper T (Th) cell subsets, Th1 and Th2 cells (Mosmann et al., 1986; Reiner and Locksley, 1995; Seder and Paul, 1994). The IL-12-induced activation of STAT4 is required for Th1 cell differentiation, whereas IL-4-induced STAT6 activation is crucial for Th2 cell differentiation (Constant and Bottomly, 1997; Murphy et al., 2000; Nelms et al., 1999; O'Garra, 1998). In addition to cytokine-induced signals, the activation of TCR-mediated signaling is indispensable for both Th1 and Th2 cell differentiation. In particular, Th2 cell differentiation is largely dependent on the activation of $p56^{lck}$, calcineurin, and the Ras-ERK MAPK cascade (Yamashita et al., 1998, 1999, 2000). The critical transcription factors for Th1/Th2 cell differentiation have been revealed, i.e., GATA3 for Th2 cells and T-bet for Th1 cells (Lee et al., 2000; Szabo et al., 2000; Zhang et al., 1997; Zheng and Flavell, 1997). We recently reported that the Ras-ERK MAPK cascade controls the GATA3 stability through the ubiquitin-proteasome-dependent pathway (Yamashita et al., 2005).

Changes in the chromatin structure of the Th2 cytokine (IL-4/IL-5/IL-13) gene loci occur during Th2 cell differentiation (Ansel et al., 2003; Lohning et al., 2002). Covalent modifications of histones play critical roles in the epigenetic regulation of transcription. Recently, we and others demonstrated that histone hyperacetylation of the Th2 cytokine gene loci occurs in developing Th2 cells in a Th2-specific and STAT6/GATA3-dependent manner (Avni et al., 2002; Fields et al., 2002; Yamashita et al., 2002). In addition, the long-range histone hyperacetylation region within the IL-13/IL-4 gene loci in developing Th2 and Tc2 cells was also revealed (Omori et al., 2003; Yamashita et al., 2002).

Some of the effector Th2 cells are maintained as memory Th2 cells for a long time in vivo (Dutton et al., 1998; Sprent and Surh, 2002). In contrast to CD8 memory T cells, CD4 memory T cells may not require any specific cytokine signals for their homeostatic maintenance (Jameson, 2002; Schluns and Lefrancois, 2003). Recent reports, however, suggest that IL-7 plays a role in the regulation of the generation and survival of CD4 memory T cells (Kondrack et al., 2003; Li et al., 2003; Seddon et al., 2003). GATA3 is required for the maintenance of Th2 cytokine production (Pai et al., 2004; Yamashita et al., 2004b; Zhu et al., 2004) and chromatin remodeling of the Th2 cytokine gene loci (Yamashita et al., 2004b). Memory Th2 cells maintain the Th2 features, such as selective Th2 cytokine production, high-level expression of GATA3 mRNA, and histone modifications of the Th2 cytokine gene loci in an IL-4-independent manner (Yamashita et al., 2004a). However, the molecules that control the maintenance of these Th2 features in memory Th2 cells have not yet been clarified.

The *Mixed-Lineage Leukemia* (*MLL*) gene was isolated as a common target of chromosomal translocations observed in human acute leukemias (Gu et al., 1992; Popovic and Zeleznik-Le, 2005; Thirman et al., 1993; Tkachuk et al., 1992; Ziemin-van der Poel et al., 1991). Sequence analyses and genetic studies have identified *MLL* as a functional ortholog of the *Drosophila trithorax* (*trx*) gene (Tkachuk et al., 1992; Yu et al., 1995). MLL protein belongs to the Trithorax protein family and is involved in the nuclear regulatory mechanism that establishes an epigenetic transcriptional memory system (Francis and Kingston, 2001). MLL forms a multicomponent complex and mediates its epigenetic transcriptional effector functions via the SET domain-dependent histone methyl transferase activity (Milne et al., 2002; Nakamura et al., 2002). MLL specifically methylates lysine 4 present in the N-terminal tail on histone H3, a modification typically associated with the transcriptionally active regions of chromatin. As a result, specific gene expression patterns are maintained throughout subsequent mitotic cell cycles, which in turn allow the cells to cope with their cellular fate, or their specific differentiation pathways. The best-studied downstream target of MLL and trx function is the *homeobox* (*HOX*) genes, which control the segment specificity and cell fate in the developing

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