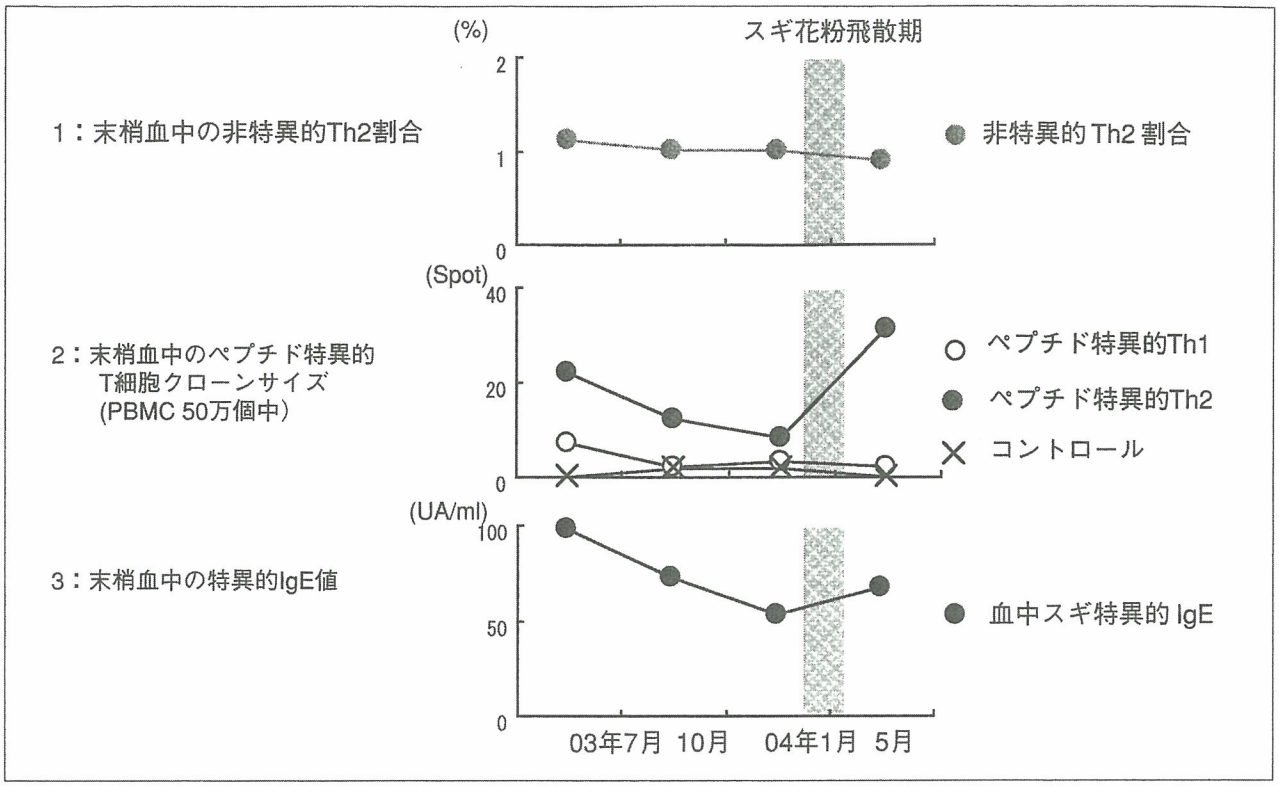

図6　スギ花粉症患者の特異クローンサイズ変動の典型例

明より，いかにメモリーの維持機構に介入するかが重要であるが，一旦成立したアレルゲン特異的Th2細胞がどの様に維持されていくのかはよく分かっていない。Th2メモリー維持機構の解明は動物モデルでも検討が始まったばかりであり，ヒトでの検証が待たれている。

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小児アレルギー性鼻副鼻腔炎の病態，診断と治療

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アレルギー性鼻副鼻腔炎とは

アレルギー性鼻炎には副鼻腔炎を合併することが以前より知られている。しかしながら，この病態については従来より議論がある。だが，少なくとも根底にはアレルギー性鼻炎の存在が必須であることは疑いない。したがって，アレルギー性鼻副鼻腔炎の定義としては，アレルギー性鼻炎症状を有し，IgE 抗体の関与が証明でき，画像的には副鼻腔に陰影を認めるものと理解される[1]。しかしながら，臨床的には様々な病態を包含しており治療においても考慮する必要がある。

アレルギー性鼻炎に伴う副鼻腔陰影

X 線検査における上顎洞の陰影出現率は，ダニおよびスギ花粉症いずれにおいても約40％程度である。陰影の型をびまん型，粘膜肥厚型，ポリープ型に分類すると，アレルギー性鼻副鼻腔炎では粘膜肥厚型（58％）が最も多く，ポリープ型も含めると全体の 3/4 を占めるのに対し，化膿性副鼻腔炎では約 6 割がびまん性陰影である。すなわち，アレルギー性鼻副鼻腔炎では陰影は軽度のものが多く，画像的にも両者は異なる。

アレルギー性鼻副鼻腔炎の病態

アレルギー性副鼻腔炎では IgE 性炎症が鼻腔に起こっていることが前提となる。しかし，併発する副鼻腔炎の発症機序にはいくつかの要因が関与している。すなわち狭義と広義の発症機序である。狭義とはⅠ型アレルギー反応が副鼻腔でダイレクトにあるいはインダイレクトに（血行性）起こるケースである。次に，広義のそれは，鼻腔に起こったアレルギー炎症が副鼻腔に波及する，副鼻腔自然孔の閉塞により二次的な反応が副鼻腔に起こる，神経反射あるいは神経原性炎症が何らかの機序で起こる，副鼻腔に感染が合併しているケースなどが想定される。ところで，Ⅰ型アレルギー反応が副鼻腔で直接起こるかどうかは抗原・粒子の大きさと自然孔の関係などから否定的な意見も多い。しかし，断片化した抗原粒子の存在も報告されており，また血行性あるいは鼻腔のアレルギー反応の波及が起こる可能性は否定できない。また，鼻腔のアレルギー反応やその他の原因による鼻粘膜腫脹による自然孔閉塞そのものも副鼻腔に浮腫や血管の透過性亢進を惹起する引き金になることが示唆されている。さらに，アレルギー性鼻副鼻腔炎では上顎洞貯留液からグラム陰性桿菌に含まれるエンドトキシンやそのリガンドである sCD14が高頻度に検出され，その濃度は化膿性副鼻腔炎と差を認めないという[2]。すなわち，感染もアレルギー性鼻副鼻腔炎の病態形成には重要な因子であることが窺える。

さて，アレルギー性鼻副鼻腔炎症例の鼻汁細胞検査では，好酸球80％，好中球40％の出現率である。年齢別で比較すると，好酸球の出現率は年齢別で差はないが，好中球は10歳未満の症例において出現率が高く，さらに好中球が出現している症例のほうが副鼻腔陰影出現率が高いことがわかった（図）[3]。このことは，特に10歳未満の小児アレルギー性鼻副鼻腔炎症例では，副鼻腔におけるアレルギー反応，鼻腔におけるアレルギー反応の波及や自然孔閉塞などの要因に加え，感染がその病態形成に関わっているものと思われる。

診　　断

症状：くしゃみ，鼻汁，鼻閉，後鼻漏，頭痛などの鼻副鼻腔炎症状を認める。

所見：ダニなどの通年性の典型例では，下鼻甲介粘膜の蒼白，腫脹が認められる。中鼻道視診により，中鼻甲介の肥厚や浮腫，中鼻道の閉塞や中

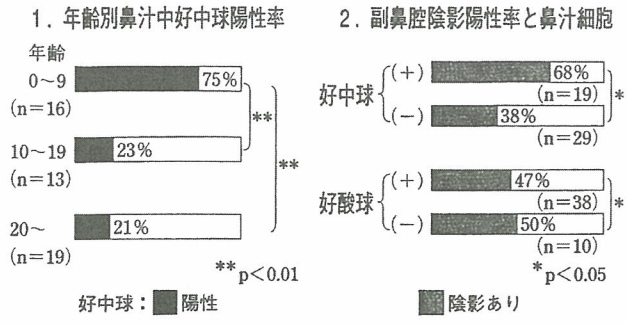

図　副鼻腔陰影と鼻汁中好中球

鼻道に漿液性あるいは膿粘性の鼻汁の貯留を認める。ただし中甲介，中鼻道に明らかな所見を認めない場合もある。

検査：副鼻腔X線検査にて上顎洞に肥厚性，ポリープ様陰影（あるいはびまん性）を認める。必要に応じて副鼻腔CT検査を施行する。

鼻汁好酸球検査（ハンセル染色）にて鼻汁好酸球の証明。

抗原の検査：皮膚テスト（皮内テスト，スクラッチテスト，プリックテスト）陽性。血清IgE抗体検査陽性。鼻誘発テスト陽性。

（アレルギーの診断は鼻汁好酸球，皮膚テストあるいは血清IgE抗体検査，鼻誘発テストのいずれか2つが陽性であれば可である）

上咽頭あるいは中鼻道からの菌検査で陰性あるいは常在菌のみ。

鑑別疾患

化膿性副鼻腔炎

アレルギー検査は陰性。鼻汁検査で好中球多数。中鼻道に膿粘性鼻汁貯留。膿性後鼻漏。副鼻腔X線検査にて典型例はびまん性陰影を示す。特に小児ではアレルギー性鼻炎に化膿性副鼻腔炎を合併している場合があるので注意が必要である。

好酸球性鼻副鼻腔炎

小児ではまれ。嗅覚障害，耳閉感，難聴などを訴えることがある。典型例では乳白色のにかわ状の鼻汁。鼻汁中には多数の好酸球を認める。時に中耳炎を併発しにかわ状の耳漏（好酸球を多数認める）が特徴的である。アスピリン喘息など成人型発症の気管支喘息を合併することが多い。

アレルギー性真菌性副鼻腔炎

小児ではまれ。慢性副鼻腔炎症例の4～7%を占める。Bentらの診断基準では，1）CTにて慢性副鼻腔炎の所見，2）鼻茸を有する，3）アレルギー性ムチンを有する，4）組織学的あるいは培養検査にて真菌の証明，5）既往歴あるいは皮膚テスト，血清IgE抗体検査によるI型アレルギーの診断を満たすものをいう。

治　　療

1．明らかな膿性鼻汁を認め中鼻道よりの分泌があり好中球が多数を占める場合：感染が病態に強く関わっておりまずその治療を行う。急性増悪などを繰り返し，起炎菌が判明すれば感受性のある抗生剤の投与を行う。その後，マクロライドの少量長期投与（2～3カ月をめどに）を行う。並行してアレルギーの治療として抗ヒスタミン薬を使用し，感染の徴候が消えた時点で局所ステロイド薬を併用する。エリスロマイシンと抗ヒスタミン薬（エバスチン，塩酸フェキソフェナジン，ロラタジン）とは併用注意がある。

2．明らかな膿性鼻汁がなく好酸球主体の鼻汁の場合：マクロライドは好酸球性炎症には効果が低いとされている。アレルギー性鼻炎の治療法の選択の中等症/重症の薬物療法に準じ，抗ヒスタミン薬と局所ステロイド薬の併用治療を数カ月行い，症状の軽減とともにステップ・ダウンしていく。

3．中鼻道を越えて鼻腔内に突出する鼻茸を有する場合：中鼻道開放処置により自然孔の閉塞状態を改善することが望ましい。その後は，1あるいは2に準じて薬物治療を行う。

ま と め

以上，アレルギー性鼻副鼻腔炎の病態形成に関わるいくつかの因子を取り上げ，診断と治療について解説を試みた。個々の症例においても病態が変化する可能性があり，その病態に応じた治療法の選択が必要であると思われる。

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IL-21–induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses

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Epidemiological studies have suggested that the recent increase in the incidence and severity of immunoglobulin (Ig)E-mediated allergic disorders is inversely correlated with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) vaccination; however, the underlying mechanisms remain uncertain. Here, we demonstrate that natural killer T (NKT) cells in mice and humans play a crucial role in the BCG-induced suppression of IgE responses. BCG-activated murine Vα14 NKT cells, but not conventional CD4 T cells, selectively express high levels of interleukin (IL)-21, which preferentially induces apoptosis in Bε cells. Signaling from the IL-21 receptor increases the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor, resulting in Bε cell apoptosis. Similarly, BCG vaccination induces IL-21 expression by human peripheral blood mononuclear cells (PBMCs) in a partially NKT cell–dependent fashion. BCG-activated PBMCs significantly reduce IgE production by human B cells. These findings provide new insight into the therapeutic effect of BCG in allergic diseases.

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Abbreviations used: α-GalCer, α-galactosylceramide; BCG, *Mycobacterium bovis* bacillus Calmette Guerin; BM-DC, BM-derived DC; Bmf, Bcl-2–modifying factor; γc, common γ-chain; IRAK, IL-1R–associated kinase; MNC, mononuclear cell; MyD88, myeloid differentiation factor 88; PGN, peptidoglycan; TLR, Toll-like receptor.

The prevalence of IgE-mediated allergic diseases such as asthma, hay fever, and atopic dermatitis has increased dramatically over the past two decades, especially in industrialized countries (1). For example, the incidence of asthma has nearly doubled since 1980 in the United States as well as in Japan (1, 2). However, the precise mechanisms underlying the increased incidence of allergic diseases are not fully understood. One possible explanation has been termed "the hygiene hypothesis," which proposes that improved hygiene combined with the excessive use of antibiotics in industrial countries has markedly reduced the incidence of infections, particularly in children. This lack of early exposure to infectious agents is associated with accelerated IgE production and an increased incidence of allergic disorders (1–3). Epidemiological studies support this hypothesis (4–6), and bacterial and viral products have been proposed as therapeutic strategies to suppress the development of allergic responses. For example, vaccination with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) has been reported to suppress IgE production and inhibit the development of allergic diseases in mouse models (7–9) and in humans (10). Furthermore, injection of CpG oligodeoxynucleotides, bacterial DNA surrogates recognized by Toll-like receptor (TLR)9, reduces serum IgE levels in mice (11).

It has been widely accepted that IgE production is totally dependent on Th2 cells, whose functions are reciprocally inhibited by Th1 cells. Mechanistically, therefore, the hygiene hypothesis is based on an imbalance in

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the Th1/Th2 ratio because bacterial components stimulate Th1 responses that in turn inhibit Th2 responses and IgE production (12). On the other hand, recent findings have indicated that a spectrum of T cells with immunoregulatory properties is involved in the regulation of IgE production and the pathophysiology of allergic diseases (13). For example, $CD4^+CD25^+$ regulatory T cells inhibit Th2 responses by producing immunosuppressive cytokines that can directly inhibit B cell activation (14, 15). Furthermore, NKT cells expressing an invariant antigen receptor (Vα14-Jα281 for mice and Vα24-JαQ for humans; reference 16) suppress Th2 and IgE responses via their production of IFN-γ (17).

In addition to these cellular mechanisms, it has also been reported that IL-21 is involved in the suppression of IgE production in both mice and humans (18, 19). IL-21 is a type I cytokine produced by activated $CD4^+$ T cells and has a broad capacity to regulate lymphoid cell functions (20–22). Among these functions, IL-21 directly inhibits antibody production by IgE-bearing B (Bε) cells induced by CD40L and IL-4 (18). Conversely, IL-21R–deficient mice exhibit enhanced IgE production (23). IL-21 has been shown to specifically inhibit germ line transcription of the IgE constant region (Cε) gene but not of other isotype genes (18). However, there is no direct evidence that this inhibition of germ line transcription is responsible for the suppression of IgE production, as class switch recombination of Ig genes and subsequent antibody secretion are differentially regulated events (24). IL-21 also induces apoptosis in B cells (25, 26), which could partially explain the reduction of IgE production; however, this effect was not shown to be specific for IgE. Hence, the mechanism by which IL-21 specifically inhibits IgE production is not yet fully understood.

Here, we have investigated BCG-mediated IgE suppression and found that NKT cells specifically induced apoptosis in Bε cells through the production of IL-21, resulting in a dramatic decrease in IgE production. IL-21 increased the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor (Bmf), which is selectively expressed in Bε cells and counteracts the antiapoptotic activity of Bcl-2. We have found that similar mechanisms are operative in humans. This is the first report demonstrating that IL-21 produced by Vα14 NKT cells plays an important role in the regulation of IgE responses in both mouse and human immune systems.

RESULTS

Vα14 NKT cell–dependent IgE suppression by BCG treatment

We used an OVA-patched sensitization protocol (27) to determine if BCG activates Vα14 NKT cells. Vα14 NKT cells were detected by α-galactosylceramide (α-GalCer)–loaded CD1d tetramer staining. In control mice treated with PBS or OVA without BCG, ~15% of the liver mononuclear cells (MNCs) were Vα14 NKT cells (Fig. 1 A, left and middle). However, BCG treatment significantly increased the frequency of Vα14 NKT cells to >25% (Fig. 1 A, right). BCG treatment also increased the absolute number of Vα14 NKT

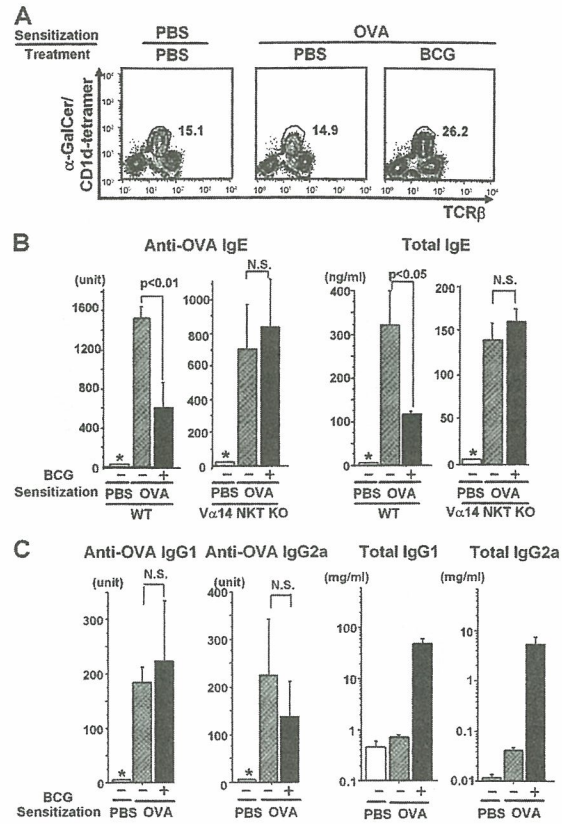

Figure 1. Requirement of Vα14 NKT cells in BCG-mediated IgE suppression. (A) FACS profiles of liver MNCs. The liver MNCs obtained 1 wk after the last immunization were stained with α-GalCer/CD1d tetramer and anti-TCRβ mAb. Three mice per each group were analyzed and representative data are shown. (B and C) Effects of BCG on antibody responses in WT and Vα14 NKT KO mice. Total and OVA-specific serum IgE (B), IgG1, and IgG2a (C) were assayed by ELISA. Five mice were used in each group. Values are expressed as mean ± SD. The asterisks (*) indicate that the amount of IgE was below the detection level for anti-OVA IgE (<31.2 U/ml), anti-OVA IgG1 (<0.002 U/ml), or anti-OVA IgG2a (<1.25 U/ml). N.S., not significant. All experiments were repeated three times with similar results.

cells because the total number of liver MNCs was also increased by 50–80% (not depicted). Sera were collected from these mice 1 wk after the last sensitization, and IgE levels were evaluated. In WT mice, both total and OVA-specific IgE levels were suppressed by BCG treatment (Fig. 1 B). In mice lacking the Jα18 gene (Vα14 NKT KO), there was no significant BCG-induced suppression of IgE responses, suggesting that suppression requires Vα14 NKT cells.

The effect of BCG administration on Th1/Th2 responses

It is well known that the isotype commitment of B cells during Ig class switching is tightly regulated by Th1/Th2 cell cytokines (28) and that Vα14 NKT cells play a regulatory role in T cell differentiation (17, 29, 30). Therefore, we measured serum IgG2a (Th1) and IgG1 (Th2) levels to assess any changes in the Th1/Th2 balance. BCG administration did

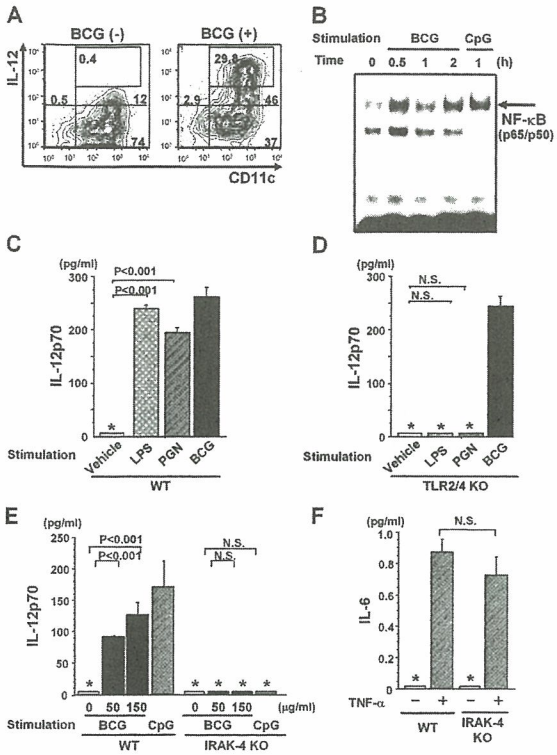

Figure 2. Activation of DCs by BCG. IL-12 production (A) and NF-κB activation (B). (A) Intracellular staining of BM-DCs with anti-IL-12p40/p70 and anti-CD11c mAbs with or without in vitro BCG (50 μg/ml) treatment for 12 h. BCG-treated BM-DCs (10,000 cells) were analyzed by FACS, and the number in each panel indicates the percentage of total cells. (B) NF-κB activation. 2×10^5 BM-DCs were stimulated with or without 50 μg/ml BCG or 1 μM CpG in vitro. NF-κB activity was determined by EMSA. (C and D) No requirement of TLR2 and TLR4 in BCG-mediated IL-12 production. 2×10^5 BM-DCs derived from WT (C) or TLR2/4 double KO (D) mice were stimulated in vitro with or without 10 μg/ml LPS, 10 μg/ml PGN, or 150 μg/ml BCG for 48 h, and IL-12p70 levels were measured by ELISA. (E and F) Requirement of IRAK-4 for IL-12 production. 2×10^5 BM-DCs were assayed for IL-12p70 by ELISA after stimulation with 0, 50, or 150 μg/ml BCG or 1 μM CpG (E), and for IL-6 with 10 ng/ml TNF-α stimulation for 48 h (F). In C–F, values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the levels were below the detection limits for IL-12p70 (<62.5 pg/ml) and IL-6 (<15.6 pg/ml). N.S., not significant. All experiments were repeated twice with similar results.

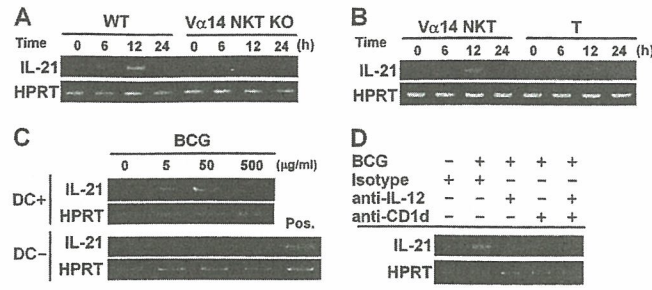

Figure 3. IL-21 expression. (A) Vα14 NKT cell–dependent IL-21 production. Liver MNCs were obtained after BCG injection (500 μg/mouse) and examined for IL-21 mRNA expression. (B) Identification of the source of IL-21. Vα14 NKT and conventional T cells were sorted from liver MNCs and examined for IL-21 mRNA expression. (C) Requirement of DCs for BCG-induced IL-21 expression by Vα14 NKT cells. Liver $TCR\beta^+$ cells were cultivated in the presence of 50 μg/ml BCG with (top) or without (bottom) BM-DCs for 24 h and analyzed for IL-21 mRNA expression. Liver $TCR\beta^+$ cells stimulated with 10 μg/ml anti-CD3 mAb were used as a positive (Pos.) control. (D) Requirement of IL-12– and CD1d-mediated signals for IL-21 mRNA expression upon BCG stimulation. An isotype control, anti-IL-12p40/p70, or anti-CD1d mAb (20 μg/ml) was added to the cultures of liver $TCR\beta^+$ cells and BM-DCs as described in C. All experiments were repeated twice with similar results.

not significantly alter the levels of OVA-specific IgG1 or IgG2a, although total levels of both isotypes were significantly enhanced (Fig. 1 C).

Innate signaling pathway for BCG-mediated IL-12 production

During microbial infection, both CD1d- and IL-12–mediated signals are required for the rapid activation of Vα14 NKT cells (31). Thus, we assessed IL-12 production after BCG treatment. BM-derived DCs (BM-DCs) were stimulated in vitro with 50 μg/ml BCG and examined for IL-12 production by intracellular cytokine staining using an IL-12p40/p70 mAb. Upon BCG stimulation, a large fraction of $CD11c^{high}$ cells produced IL-12 (Fig. 2 A). NF-κB activation is crucial for IL-12 production, and BCG treatment activated NF-κB to the same extent as treatment with the positive control CpG, as demonstrated by electrophoretic mobility shift assay (Fig. 2 B). These results indicate that BCG directly induces IL-12 production in DCs by activating NF-κB.

It has been reported that mycobacterial cell wall antigens such as peptidoglycan (PGN) or lipoarabinomannan induce proinflammatory gene transcription through TLR2 and TLR4 (32). However, when we compared IL-12p70 production by BCG-stimulated WT and TLR2/TLR4 double KO BM-DCs, there was no difference (Fig. 2 C). As expected, however, the TLR2/4-deficient cells failed to respond to LPS or PGN (Fig. 2 D). These results indicate that receptor(s) other than TLR2 and TLR4 are responsible for the recognition of whole BCG organisms.

To analyze intracellular signaling pathways activated by BCG, we measured IL-12p70 production by BM-DCs from WT and IL-1R–associated kinase (IRAK)-4 KO mice. BM-DCs from IRAK-4 KO mice produced less IL-12p70 than those from WT mice in response to both BCG and CpG (Fig. 2 E), whereas they produced comparable levels of IL-6 in response to TNF-α stimulation (Fig. 2 F). Similarly, BM-DCs from myeloid differentiation factor 88 (MyD88) KO mice produced nearly undetectable IL-12p70 upon BCG stimulation, whereas IL-6 production remained unchanged (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). Therefore, the recognition of BCG organisms is mediated by innate receptors other than TLR2 and TLR4 that signal through both IRAK-4 and MyD88.

BCG-induced IL-21 expression in Vα14 NKT cells

The recently identified IL-21 and its receptor (IL-21R), members of the common γ-chain (γc)-dependent cytokine family, have been shown to regulate IgE production without influencing Th2 cell differentiation (18, 20, 23). Thus, we examined the possibility that IL-21 might be induced by BCG stimulation and might suppress IgE responses in a Vα14 NKT cell–dependent manner. We first measured IL-21 mRNA expression in TCRβ$^+$ liver MNCs by a RT-PCR. IL-21 mRNA was detected in liver TCRβ$^+$ liver MNCs of WT mice within 6 h after BCG injection (Fig. 3 A). In contrast, no IL-21 mRNA was detected in the Vα14 NKT KO mice (Fig. 3 A), suggesting that Vα14 NKT cells are the source of IL-21 in response to BCG. To test this hypothesis, we separated conventional T cells and Vα14 NKT cells and found that IL-21 mRNA was more abundant in the Vα14 NKT cells after BCG injection (Fig. 3 B). Similarly, after stimulation with anti-CD3, IL-21 mRNA levels in Vα14 NKT cells were more than seven times higher than in CD4 T cells, confirming that these cells are the major source of IL-21 in this model (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1).

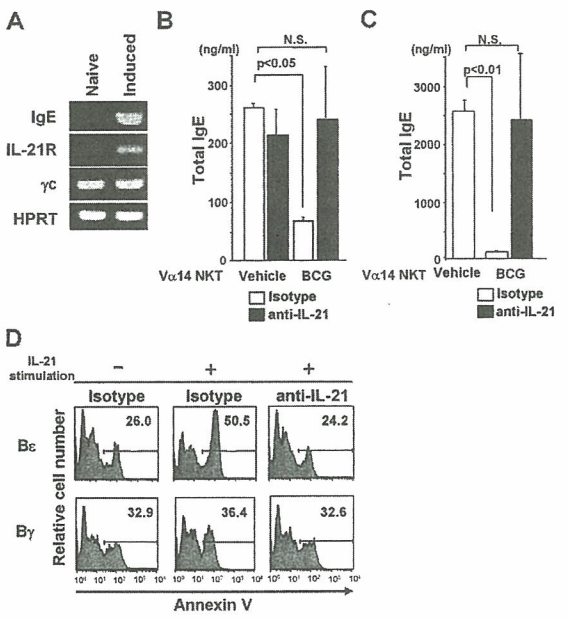

Figure 4. IL-21–mediated Bε cell apoptosis. (A) RT-PCR analysis. Expression of IgE (Cε), IL-21R, and γc was investigated in naive B (left) and Bε (right) cells. (B) Suppression of IgE production in naive B cell cultures. Naive B cells and Vα14 NKT cells (10^5 each) were cocultured in the presence of sCD40L and IL-4. (C) Suppression of IgE production in the Bε cell culture. 10^5 Vα14 NKT cells were added to the Bε cell (10^5) cultures. In B and C, 20 μg/ml anti-IL-21 mAb or isotype control mAb was added at the same time as the Vα14 NKT cells. The concentration of total IgE was measured by ELISA in triplicate. Values are expressed as mean ± SD. N.S., not significant. The experiments were repeated three times with similar results. (D) IL-21–mediated Bε cell apoptosis. 2 × 10^5 Bε and Bγ cells were generated and then further cultured with or without 30 ng/ml IL-21 for 30 h. Annexin V staining was then performed. The numbers represent percentage of the gated cells. Annexin V$^+$ cells among Bε and Bγ cells just before IL-21 treatment was 25.7 and 29.2%, respectively (not depicted). The experiments were repeated three times with similar results.

Requirement for IL-12 and CD1d in IL-21 expression by Vα14 NKT cells

We next analyzed the role of DCs in BCG-induced IL-21 mRNA expression. Co-culture of Vα14 NKT cells with DCs plus IL-12 strongly induced IL-21 mRNA expression, whereas no IL-21 mRNA was induced in the absence of DCs (Fig. 3 C). Furthermore, IL-21 mRNA expression was inhibited by the addition of anti–IL-12, anti-CD1d, or both into the cultures (Fig. 3 D), indicating that both IL-12 and CD1d are required for IL-21 expression by Vα14 NKT cells.

IL-21–mediated IgE suppression

To examine whether BCG-activated Vα14 NKT cells actually suppress IgE production, Bε cells were generated from naive CD19$^+$ splenic B cells using the 3-d culture system described by Snapper et al. (33). The starting population of naive B cells expressed negligible IL-21R and contained no Bε cells as defined by Cε transcripts (Fig. 4 A). However, after 3 d of the culture, the majority of CD19$^+$ B cells became Bε cells and expressed IL-21R (Fig. 4 A). We then investigated the effects of BCG treatment on B cells, before and after IgE class switching. The addition of BCG-treated liver MNCs at the onset of the naive B cell cultures significantly suppressed IgE production (~50%; Fig. 4 B). However, when BCG-activated Vα14 NKT cells were added to the Bε cell culture on day 3 and the cells were further cultivated for 5 d, IgE production was even more strongly inhibited (>90% suppression; Fig. 4 C). These results indicate that, even after B cells have undergone Cε class switching, BCG-activated Vα14 NKT cells can potently suppress IgE production. The inhibition of IgE production was IL-21 dependent, as an anti–IL-21 mAb completely abrogated the inhibitory effects (Fig. 4, B and C). When the B cells in these cultures were assessed for apoptosis by annexin V staining, there was a significant increase

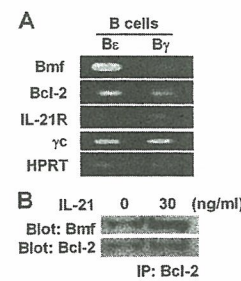

Figure 5. Bmf-mediated Bε cell apoptosis. (A) RT-PCR. RNA from Bε and Bγ cells was analyzed for its expression of the indicated genes by RT-PCR. Note that no significant differences in Bcl-2 and IL-21R expression between Bε and Bγ cells were observed. (B) Western blotting. Bε cells were stimulated with IL-21 at 37°C for 30 min, and their cell lysates (6 × 10^6) were subjected to immunoprecipitation with anti-Bcl-2 mAb and immunoblotting with anti-Bmf antibody (top) or anti-Bcl-2 mAb (bottom). All experiments were repeated three times with similar results.

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).

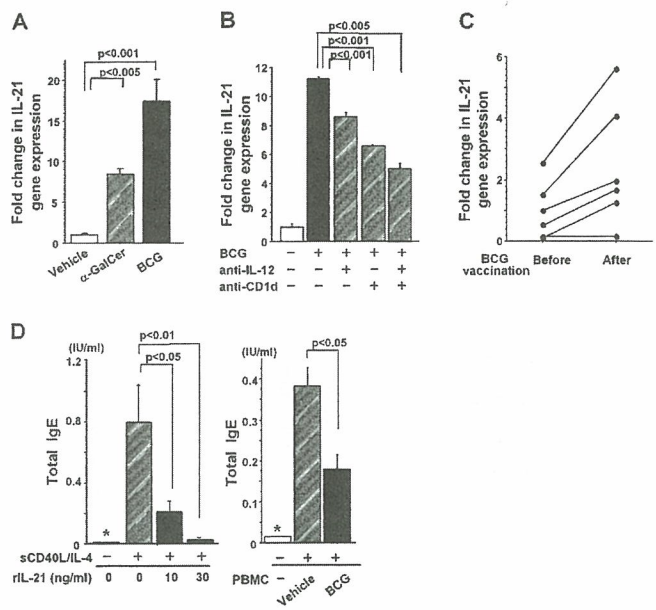

Figure 6. IL-21 mRNA expression and IL-21–induced IgE suppression in humans. (A) IL-21 mRNA expression in PBMCs. 10^6 human PBMCs were stimulated with 100 ng/ml α-GalCer or 50 μg/ml BCG and examined for IL-21 expression by quantitative real-time PCR with Taqman probes. The data are representative of 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
T1 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
T2 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
F1 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.

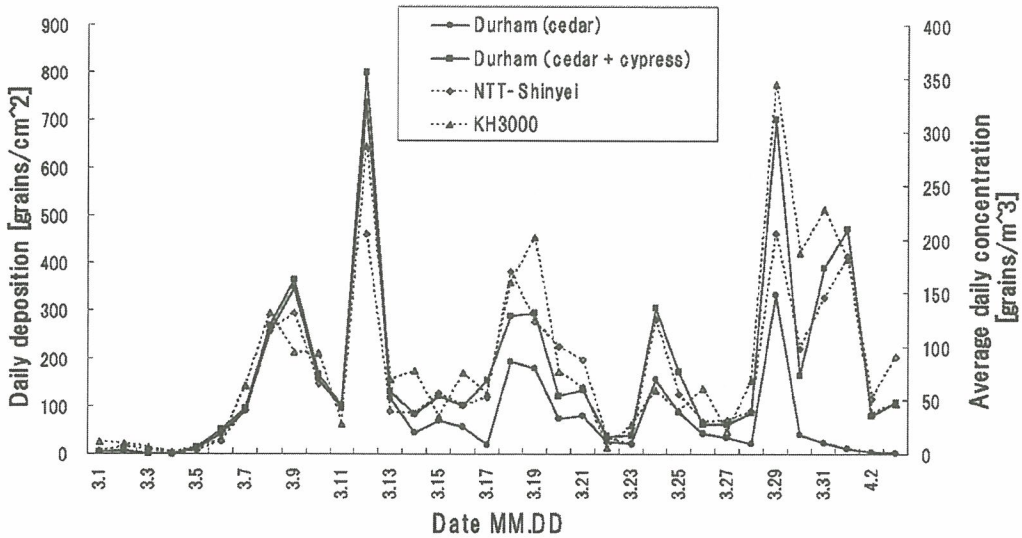


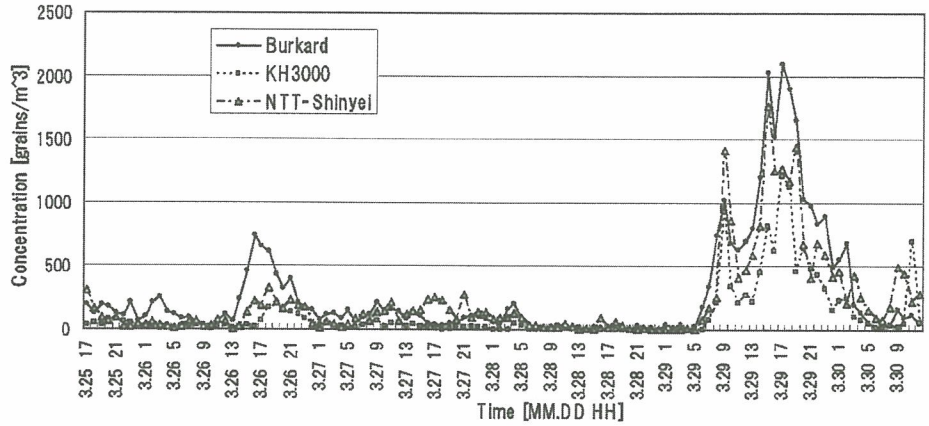
Figure 1. Comparison between daily deposition and daily average concentration of pollen at the Chiba site for the complete pollen season. The daily deposition counts for the cedar and hinoki total and for the cedar only are shown to illustrate the contribution of hinoki to the total count of pollen. 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.

F3,F4 In Figure 3 and Figure 4, we examine in more detailed 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

a)



b)

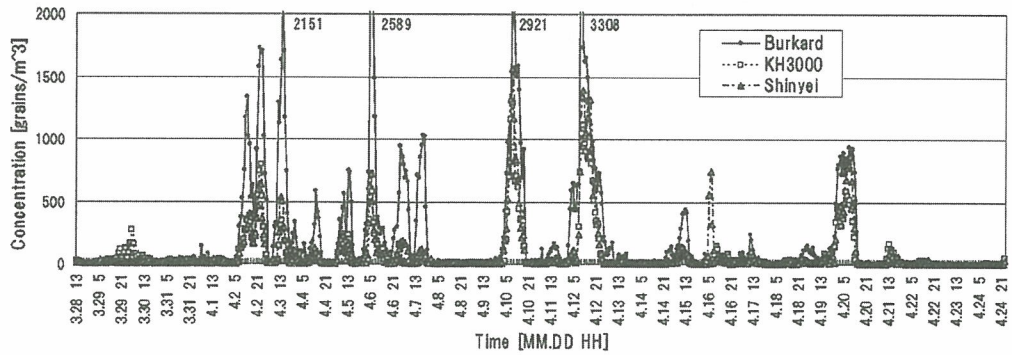


Figure 2. Comparison between pollen concentrations measured with a Burkard, KH3000, and Shinyei counter at the Chiba site (a) and the Akita site (b). In section b, the maximum value of the concentration axis was set to 2,000 grains/m^3 to make visible the variations for all counters (actual values for 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

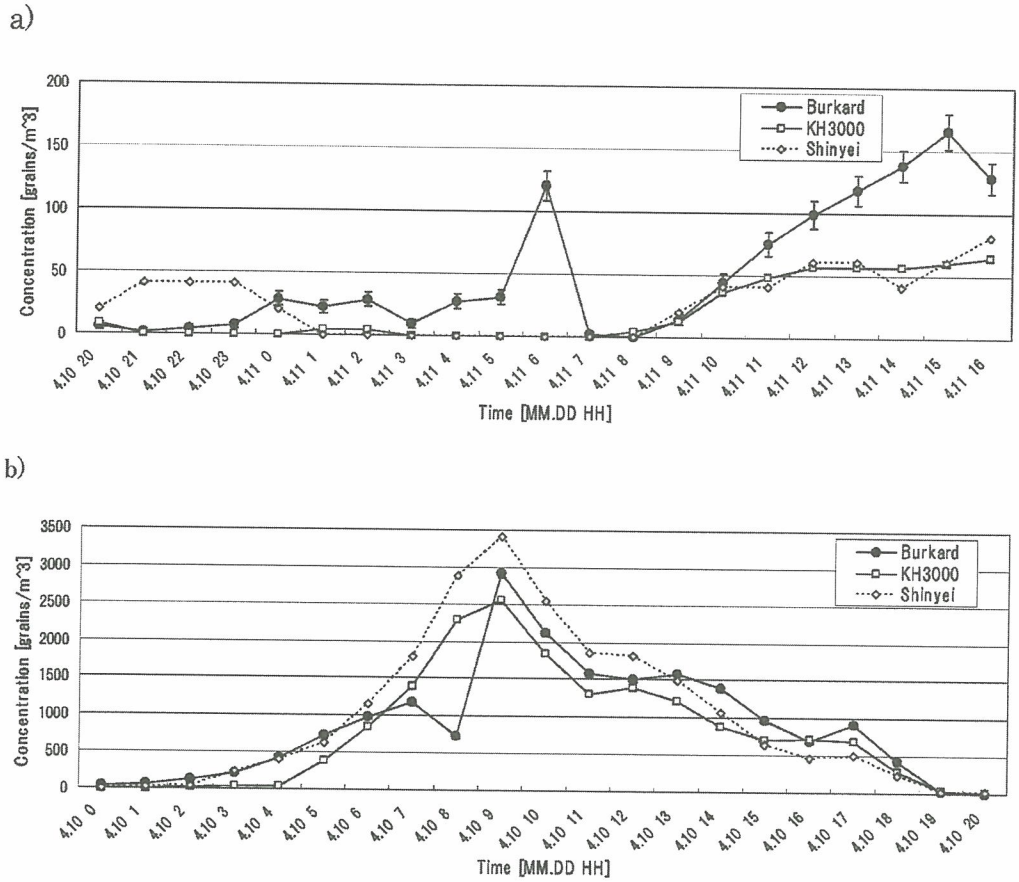


Figure 3. Detailed variations in pollen concentration with time at Akita as measured with a Burkard sampler and a KH3000 and Shinyei counter for a low-concentration region (a) and a high-concentration (b) region. The error bars of the Burkard series represent statistical uncertainty, which arises from 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.

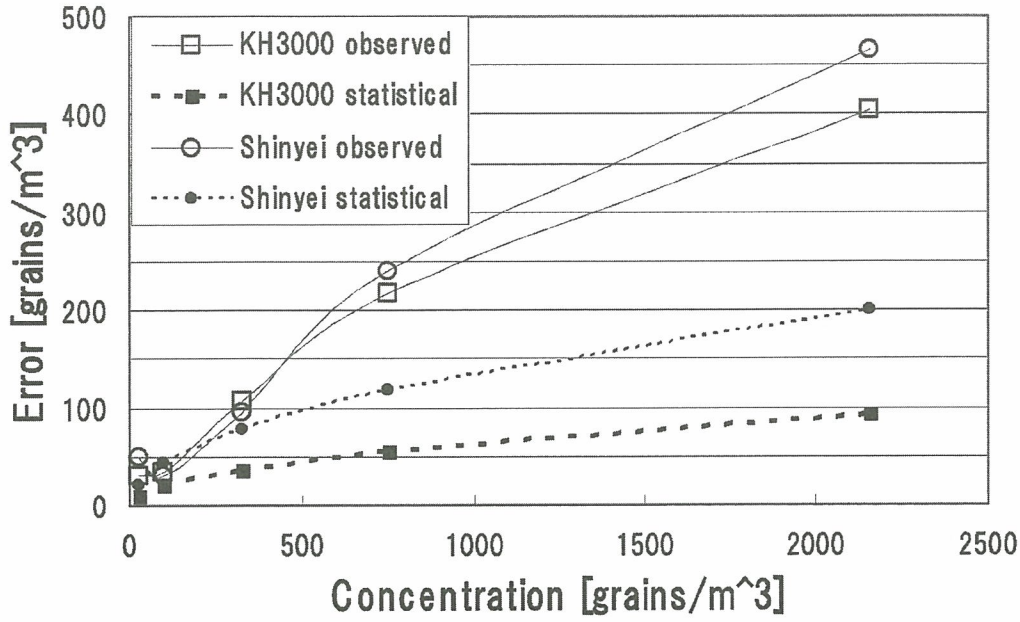


Figure 4. Error analysis of automatic pollen counters performed on the Akita dataset. Absolute errors are computed between the concentrations measured with the Burkard reference sampler and the automatic counters. The 2-hour average concentrations were used and errors computed in the following bins: 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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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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embryo (Krumlauf, 1994). Korsmeyer and colleagues reported that targeted homozygous disruption of *MLL* in mice is embryonic lethal, and similar to *trx* mutants in flies, the *Hox* gene expression is initiated but not maintained in these mice (Yu et al., 1995). Lethality at day E10.5 is associated with numerous defects in the segmental identity. Another line of *MLL*-deficient mice, where exon 12–14 of *MLL* is targeted, showed a delayed lethality for a couple of days (Yagi et al., 1998). Yolk sac and fetal liver hematopoiesis is disrupted in both *MLL* mutant mice (Hess et al., 1997; Yagi et al., 1998). In addition, MLL is also required for definitive hematopoiesis (Ernst et al., 2004). Interestingly, even animals carrying only one normal *MLL* allele show a phenotype that differs from wild-type mice (Yagi et al., 1998; Yu et al., 1995). The significant phenotypes of the $MLL^{+/-}$ mutant mice suggest that two copies of the gene are essential during development. However, no definitive analysis has yet been reported regarding the function of MLL in the immune system.

We herein investigate the role of MLL in the regulation of Th1/Th2 cell differentiation and memory Th1/Th2 cell function by using $MLL^{+/-}$ mice. Our results indicate that MLL plays a crucial role in the maintenance of Th2 function in memory Th2 cells involving the regulation of chromatin status of the Th2 cytokine gene loci and the GATA3 gene locus.

Results

Phenotypic and Functional Characterization of Splenic CD4 T Cells in *MLL* Heterozygous Mice

Homozygous knockouts of the *MLL* gene are embryonic lethal, and animals carrying one normal *MLL* allele are viable but show significant changes during development (Yagi et al., 1998; Yu et al., 1995). As a result, we used $MLL^{+/-}$ mice (Yagi et al., 1998) to investigate the potential role of MLL in the Th1/Th2 cell differentiation and function. In $MLL^{+/-}$ mice, there was a marginal reduction of mature CD4 and CD8 T cells in the thymus with equivalent numbers of CD4 and CD8 T cells in the spleen when compared to wild-type controls (see Figure S1A in the Supplemental Data available with this article online). The cell-surface phenotype (Figure S1B), antigen-induced proliferative responses, and primary IL-2 production (data not shown) were within the normal range. The reduction of *MLL* mRNA in $MLL^{+/-}$ CD4 T cells was confirmed (Figure S1C). The expression levels of *MLL* mRNA were substantial in the freshly prepared wild-type splenic naive CD4 T cells, and they decreased after antigen stimulation in either a Th1 or Th2 cell differentiation culture (Figure 1A). The levels of *MLL* mRNA increased again in memory CD4 T cells, especially in memory Th2 cells.

The Ability to Produce Th2 Cytokines Is Not Maintained in $MLL^{+/-}$ Memory Th2 Cells

First, we assessed the capability of naive $MLL^{+/-}$ CD4 T cells to differentiate into Th1 and Th2 effector cells. Naive CD4 T cells ($CD4^+$ $CD44^{low}$) from $MLL^{+/-}$ × OVA-specific TCR-αβ Tg (DO11.10 Tg) mice were cultured under Th1 or Th2 culture conditions in vitro. Naive CD4 T cells from $MLL^{+/-}$ mice differentiated normally into both IFNγ-producing Th1 (96.1% versus 95.8%) and IL-4-producing Th2 (42.5 versus 43.0%) cells (Figure 1B, left). Similarly, the production of both Th1 and Th2 cytokines was equivalent (Figure 1C, top). Although the number of memory Th2 cells slightly decreased in the $MLL^{+/-}$ CD4 T cells (Figure S2A), the cell-surface expressions of IL-4Rα, γC, IL-2Rβ, IL-7Rα, CD69, CD25, CD44, and CD62L on memory Th2 cells were normal (Figure S2B). Interestingly, however, the number of IL-4-producing cells in $MLL^{+/-}$ memory Th2 cells decreased dramatically (39.8% versus 4.7%), whereas those of IFNγ-producing cells in memory Th1 cells did not decrease but was rather slightly increased (55.1% versus 65.3%) (Figure 1B, right). An assessment of the levels of cytokine production after antigenic restimulation of the memory Th1/Th2 cells revealed, as expected, a dramatic decrease in the production of IL-4, IL-5, and IL-13 from memory Th2 cells, whereas the IFNγ production from memory Th1 cells was slightly increased (Figure 1C, bottom). Taken together, these results indicate that in $MLL^{+/-}$ memory Th2 cells, the maintenance of the ability to produce Th2 cytokines in memory Th2 cells is selectively impaired.

Methylation of Histone H3-K4 and Acetylation of Histone H3-K9 at the Th2 Cytokine Gene Loci Are Not Maintained in $MLL^{+/-}$ Memory Th2 Cells

MLL is known to have intrinsic histone methyl transferase activity for histone H3-K4 (Milne et al., 2002; Nakamura et al., 2002), and the methylation of histone H3-K4 is important for the maintenance of the acetylation of histone H3-K9. We previously reported that the hyperacetylation of histone H3-K9 and methylation of histone H3-K4 at the Th2 cytokine gene loci are preserved in memory Th2 cells, particularly at the IL-4/IL-13 gene loci in an IL-4-independent manner (Yamashita et al., 2004a). Consequently, we examined these histone modifications at the Th2 cytokine gene loci in $MLL^{+/-}$ effector and memory Th2 cells. The induction of both histone modifications at the Th2 cytokine gene loci (CGRE, CNS1, and V_A enhancer, IL-4p, IL-13p, IL-5p) occurred normally in $MLL^{+/-}$ effector Th2 cells (Figure 2A). In $MLL^{+/-}$ memory Th2 cells, however, the levels of both histone modifications at the Th2 cytokine gene loci significantly decreased, while those at RAD50 promoter and IFNγ promoter regions were equivalent (Figure 2B). An assessment of the methylation of histone H3-K4 by a quantitative PCR analysis showed a similar decrease in $MLL^{+/-}$ memory Th2 cells (Figure 2C). Looking at the levels of histone modifications within the IL-13/IL-4 gene loci more precisely, we found that the levels were decreased in $MLL^{+/-}$ memory Th2 cells at almost all regions tested (Figure 2D and Figure S3). These results indicate that histone modifications such as hyperacetylation of histone H3-K9 and methylation of histone H3-K4 at the Th2 cytokine gene loci are not properly maintained in $MLL^{+/-}$ memory Th2 cells.

$MLL^{+/-}$ Effector Th2 Cells Fail to Maintain the Ability to Produce Th2 Cytokines and the Histone Modifications In Vitro

Next, we assessed whether $MLL^{+/-}$ Th2 cells properly maintained the ability to produce Th2 cytokines and the histone modifications at the Th2 cytokine gene loci under resting culture conditions in vitro. IL-4-producing