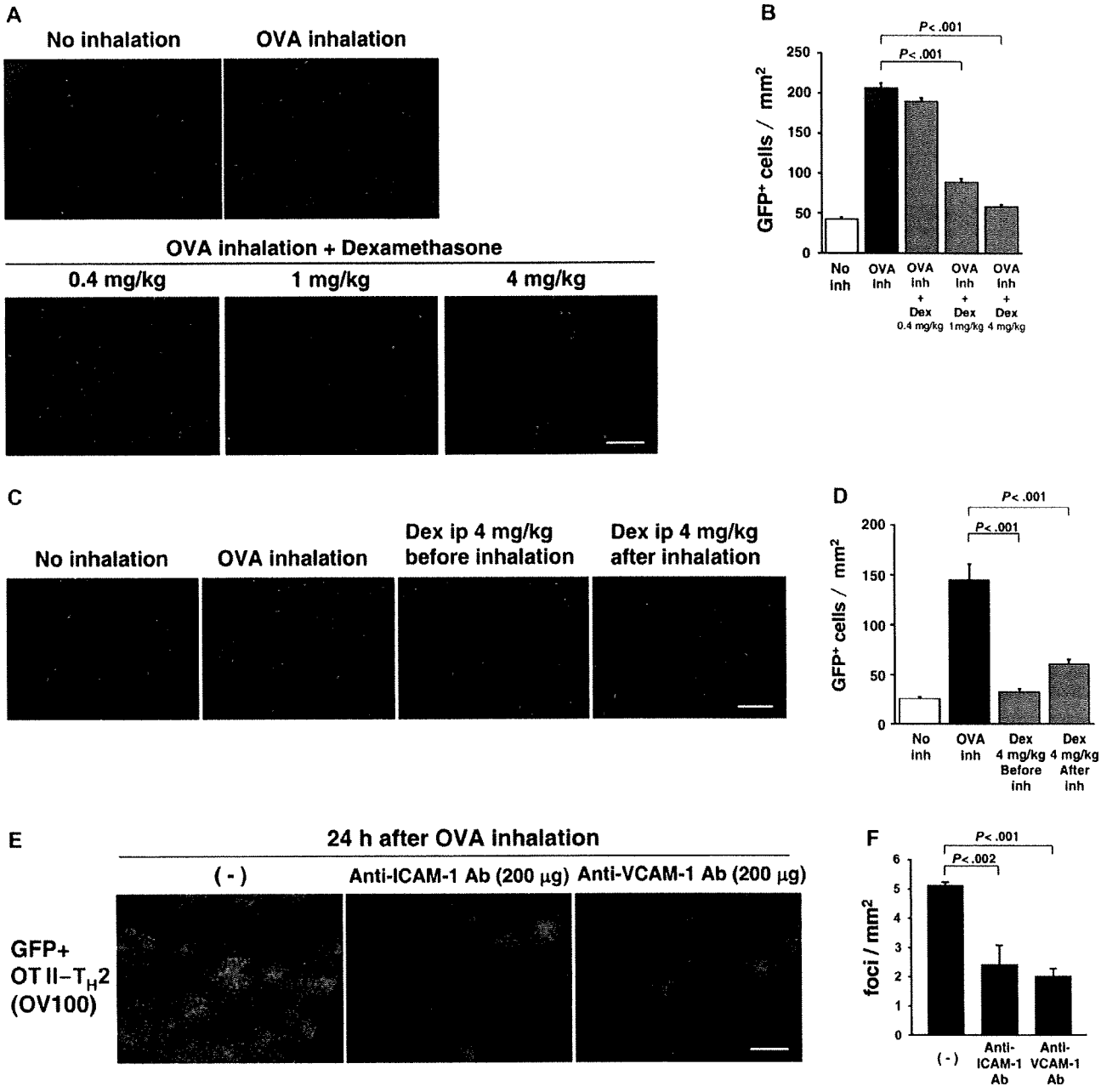

FIG 3. Effects of dexamethasone, anti–ICAM-1, and anti–VCAM-1 treatment on CD4 T-cell accumulation in the lung after ovalbumin (OVA) inhalation. **A,** Allergic airway inflammation was induced as in Fig 1, *A*. Three different doses of dexamethasone (DEX) were injected intraperitoneally 1 hour before OVA inhalation. Twenty-four hours after inhalation (day 16), GFP^+ CD4 T cells were monitored by using the OV100 Small Animal Imaging System. *Bar,* 100 μm. **B,** Summary of the accumulation of fluorescent cells of *A*. Data are from 15 fields from 3 mice with SD. $P < .001$ by the Student's *t* test. **C,** Dexamethasone was injected intraperitoneally 1 hour before or 1 day after OVA inhalation. Two days after inhalation, GFP^+ CD4 T cells were monitored in the excised lung by using the OV100 Small Animal Imaging System. *Bar,* 100 μm. **D,** Summary of the accumulation of fluorescent cells of *C*. The data are from 15 fields from 3 mice with SD. $P < .001$ by the Student's *t* test. **E,** Allergic airway inflammation was induced as in Fig 2, *A*. Anti–ICAM-1 or anti–VCAM-1 mAb was injected intraperitoneally 24 hours before OVA inhalation. Twenty-four hours after OVA inhalation, GFP^+ OT II–T_H2 cells were monitored. *Bar,* 100 μm. **F,** Summary of the generation of T_H2-cell foci in *E*. Data are from 12 fields from 3 mice with SD. $P < .002$, $P < .001$ by the Student's *t* test.

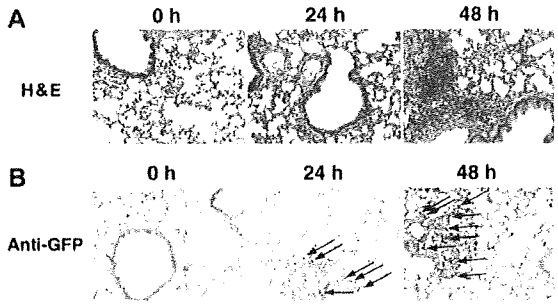

FIG 4. Eosinophilic infiltration and GFP$^+$ T_H2-cell infiltration into the lung in a mouse model of asthma. **A,** The lung specimens were fixed at indicated time point after ovalbumin inhalation and stained with H&E. A representative H&E staining pattern in each group is shown. Magnification ×200. **B,** Representative immunohistochemical staining for GFP is shown. Magnification ×200. *Arrows* indicate some of the representative GFP-positive cells. The results are representative of 3 experiments.

that antigen-specific T_H2 cells accumulated and formed foci in the lung after allergen inhalation. GFP$^+$ OT I–T_H2 cells were intravenously transferred into C57BL/6 mice, and 2 days later, the recipient mice were exposed to ovalbumin. Typical peribronchiolar and perivascular infiltration and focus formation by eosinophils were observed 48 hours after ovalbumin inhalation (Fig 4, *A*). The infiltrated OT II–T_H2 cells were monitored by immunohistochemistry analysis with an anti-GFP antibody. We observed GFP$^+$ transferred T_H2 cells in the region of subsequent eosinophilic infiltration (Fig 4, *B*). These results, in conjunction with the results of our time course experiments (Fig 2), indicate that antigen-specific T_H2 cells accumulate and form foci in the lung before the marked infiltration of eosinophils and thus regulate the initiation of inflammatory processes in this animal model.

DISCUSSION

The behavior of T cells during airway inflammation in mouse models of asthma has been investigated by using flow cytometry and immunohistochemistry.[24-28] These studies showed that T cells migrate into the lung after allergen challenge, but the studies did not address how and when they migrate into the lung or whether only antigen-specific effector T cells migrate into the lung. Several investigators performed lung imaging in a serial but static manner. Hutchison et al[29] have used serial tissue sectioning to describe the time course of proliferating CD4 T cells in the lung and its draining lymph nodes. Bhattacharya's group[30,31,32] imaged whole excised lungs to study signaling by lung resident cells. Until now, the *in vivo* dynamics of cell invasion of the lung during inflammation in living animals has been poorly understood because it has been difficult to arrest motion in the lung as a result of the beating heart or movement during respiration. Our novel imaging model has overcome these problems and has demonstrated, for the first time, the dynamics of migration of allergen-specific T_H2 cells into the lung after allergen inhalation in living animals at the cellular level using the adoptive transfer of GFP$^+$ T cells.

With this novel imaging model, several important findings are demonstrated. We have shown for the first time T_H2-cell focus formation in the lung, a cellular immunologic event occurring during the initiation of airway inflammation (Fig 2). In addition, we demonstrate that unprimed CD4 T cells and naive antigen-specific CD4 T cells did not accumulate around the foci (Fig 1; Figs E1-E3), indicating that the molecules specifically expressed on activated effector T_H2 cells play an important role in migration and focus formation. Moreover, T_H2-cell focus formation occurred before eosinophilic infiltration and thus may determine the eosinophilic inflammatory site (Figs 2 and 4). Focus formation was inhibited by the administration of anti–ICAM-1 and anti–VCAM-1 antibodies (Fig 3, *E and F*), both of which are able to block the induction of eosinophilic airway inflammation, indicating that focus formation is a critical process during the induction of the asthma phenotype.

Several groups have shown time-lapse microscopy of GFP-labeled and/or RFP-labeled cancer cells in live mice. Hoffman's group[22,23] has demonstrated *in vivo* imaging of intracapillary and intralymphatic cancer cell trafficking behavior. Condeelis' group[33] has used *in vivo* imaging to determine molecular mechanisms of cancer metastasis.

Many groups have reported *in vivo* imaging of lymphoid tissues such as lymph nodes, spleen, and bone marrow to visualize antigen presentation and T-cell migration.[34-41] To visualize the cells *in vivo*, they must be labeled by appropriate dyes or express fluorescent proteins. In most of the reports of *in vivo* imaging of lymphoid tissues, T cells were labeled by using appropriate dyes and transferred into recipient mice, although a possible difference in the migration behavior of the cells that were labeled by dyes has been suggested.[42] In our study, we prepared ovalbumin-specific T_H2 cells expressing GFP from GFP Tg mice[16] and transferred the cells intravenously into normal recipient mice. Another technical issue could be the difference in the migration between antigen-specific cells that were injected into the mice before imaging and antigen-specific T cells that were resident in the imaged mice. In the near future, the behavior of antigen-specific T_H2 cells that were resident in the imaged mice will be investigated.

This model allows investigators to monitor the migration of inflammatory lymphocytes into the lung in a real-time manner in live animals and thus provides a new strategy to study the *in vivo* cell biology of inflammatory lung diseases such as asthma. This method can be also applied to various bacteria-induced and virus-induced inflammatory lung diseases, including tuberculosis and influenza virus–induced pneumonia, and to screen for more effective drugs for these respiratory diseases.

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Key messages

- We established a novel *in vivo* real-time color-coded cellular imaging model to visualize the dynamics of migration of T cells in the lung in a mouse model of asthma.
- Accumulating T_H2 cells formed foci in the lungs 6 to 20 hours after allergen inhalation.
- The focus formation was dependent on ICAM-1 and VCAM-1 and appeared to determine the site of eosinophilic infiltration.
- T_H2-cell focus formation appears to be a critical process in the induction of allergic airway inflammation in this animal model.

REFERENCES

1. Eder W, Ege MJ, von Mutius E. The asthma epidemic. N Engl J Med 2006;355: 2226-35.
2. McFadden ER Jr, Gilbert IA. Asthma. N Engl J Med 1992;327:1928-37.
3. Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001;344:350-62.
4. Hamelmann E, Gelfand EW. IL-5-induced airway eosinophilia—the key to asthma? Immunol Rev 2001;179:182-91.
5. Lloyd CM, Gonzalo JA, Coyle AJ, Gutierrez-Ramos JC. Mouse models of allergic airway disease. Adv Immunol 2001;77:263-95.
6. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. J Clin Invest 2003;111:291-7.
7. Kamata T, Yamashita M, Kimura M, Murata K, Inami M, Shimizu C, et al. src homology 2 domain-containing tyrosine phosphatase SHP-1 controls the development of allergic airway inflammation. J Clin Invest 2003;111:109-19.
8. Wills-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev 2004;202: 175-90.
9. Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. Annu Rev Immunol 2004;22:789-815.
10. Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. Immunol Rev 2006;212:238-55.
11. Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. J Exp Med 1997;186:1737-47.
12. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. J Clin Invest 1999;103:175-83.
13. Kaminuma O, Fujimura H, Fushimi K, Nakata A, Sakai A, Chishima S, et al. Dynamics of antigen-specific helper T cells at the initiation of airway eosinophilic inflammation. Eur J Immunol 2001;31:2669-79.
14. Nakajima H, Sano H, Nishimura T, Yoshida S, Iwamoto I. Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue. J Exp Med 1994;179:1145-54.
15. Hoffman RM. The multiple uses of fluorescent proteins to visualize cancer in vivo. Nat Rev Cancer 2005;5:796-806.
16. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407:313-9.
17. Vintersten K, Monetti C, Gertsenstein M, Zhang P, Laszlo L, Biechele S, et al. Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. Genesis 2004;40:241-6.
18. Yang M, Reynoso J, Bouvet M, Hoffman RM. A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. J Cell Biochem 2009;106:279-84.
19. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol 1998;76:34-40.
20. Hasegawa A, Miki T, Hosokawa H, Hossain MB, Shimizu C, Hashimoto K, et al. Impaired GATA3-dependent chromatin remodeling and Th2 cell differentiation leading to attenuated allergic airway inflammation in aging mice. J Immunol 2006;176:2546-54.
21. Hirahara K, Yamashita M, Iwamura C, Shinoda K, Hasegawa A, Yoshizawa H, et al. Repressor of GATA regulates TH2-driven allergic airway inflammation and airway hyperresponsiveness. J Allergy Clin Immunol 2008;122:512-20.
22. Yamauchi K, Yang M, Jiang P, Xu M, Yamamoto N, Tsuchiya H, et al. Development of real-time subcellular dynamic multicolor imaging of cancer-cell trafficking in live mice with a variable-magnification whole-mouse imaging system. Cancer Res 2006;66:4208-14.
23. Hayashi K, Jiang P, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Moossa AR, Bouvet M, Hoffman RM. Real-time imaging of tumor-cell shedding and trafficking in lymphatic channels. Cancer Res 2007;67:8223-8.
24. Catron DM, Rusch LK, Hataye J, Itano AA, Jenkins MK. CD4 + T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J Exp Med 2006;203:1045-54.
25. Kohlmeier JE, Woodland DL. Memory T cell recruitment to the lung airways. Curr Opin Immunol 2006;18:357-62.
26. Mathew A, Medoff BD, Carafone AD, Luster AD. Cutting edge: Th2 cell trafficking into the allergic lung is dependent on chemoattractant receptor signaling. J Immunol 2002;169:651-5.
27. Wells JW, Cowled CJ, Giorgini A, Kemeny DM, Noble A. Regulation of allergic airway inflammation by class I-restricted allergen presentation and CD8 T-cell infiltration. J Allergy Clin Immunol 2007;119:226-34.
28. Xu B, Wagner N, Pham LN, Magno V, Shan Z, Butcher EC, et al. Lymphocyte homing to bronchus-associated lymphoid tissue (BALT) is mediated by L-selectin/PNAd, alpha4beta1 integrin/VCAM-1, and LFA-1 adhesion pathways. J Exp Med 2003;197:1255-67.
29. Hutchison S, Choo-Kang BS, Gibson VB, Bundick RV, Leishman AJ, Brewer JM, et al. An investigation of the impact of the location and timing of antigen-specific T cell division on airways inflammation. Clin Exp Immunol 2009;155:107-16.
30. Ichimura H, Parthasarathi K, Lindert J, Bhattacharya J. Lung surfactant secretion by interalveolar Ca2+ signaling. Am J Physiol Lung Cell Mol Physiol 2006; 291:L596-601.
31. Kuebler WM, Parthasarathi K, Lindert J, Bhattacharya J. Real-time lung microscopy. J Appl Physiol 2007;102:1255-64.
32. Hayashi K, Jiang P, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, et al. Real-time imaging of tumor-cell shedding and trafficking in lymphatic channels. Cancer Res 2007;67:8223-8.
33. Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, et al. Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. Cancer Res 2007;67:3505-11.
34. Miller MJ, Safrina O, Parker I, Cahalan MD. Imaging the single cell dynamics of CD4 + T cell activation by dendritic cells in lymph nodes. J Exp Med 2004;200:847-56.
35. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science 2002;296:1869-73.
36. Bajenoff M, Egen JG, Koo LY, Laugier JP, Brau F, Glaichenhaus N, et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity 2006;25:989-1001.
37. Bousso P, Robey E. Dynamics of CD8 + T cell priming by dendritic cells in intact lymph nodes. Nat Immunol 2003;4:579-85.
38. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature 2004;427:154-9.
39. Hugues S, Scholer A, Boissonnas A, Nussbaum A, Combadiere C, Amigorena S, et al. Dynamic imaging of chemokine-dependent CD8 + T cell help for CD8 + T cell responses. Nat Immunol 2007;8:921-30.
40. Schwickert TA, Lindquist RL, Shakhar G, Livshits G, Skokos D, Kosco-Vilbois MH, et al. In vivo imaging of germinal centres reveals a dynamic open structure. Nature 2007;446:83-7.
41. Cavanagh LL, Bonasio R, Mazo IB, Halin C, Cheng G, van der Velden AW, et al. Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. Nat Immunol 2005;6:1029-37.
42. Germain RN, Miller MJ, Dustin ML, Nussenzweig MC. Dynamic imaging of the immune system: progress, pitfalls and promise. Nat Rev Immunol 2006;6:497-507.

Protective Roles of B and T Lymphocyte Attenuator in NKT Cell-Mediated Experimental Hepatitis

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Although B and T lymphocyte attenuator (BTLA) was originally identified as an inhibitory coreceptor selectively expressed on Th1 cells and B cells, recent studies have revealed that BTLA is expressed on a variety of cells, including macrophages, dendritic cells, and NK cells, and modulates their functions. However, the role of BTLA in the regulation of NKT cell function remains unknown. In this study, we found that BTLA was expressed on NKT cells at the levels similar to those on T cells and that BTLA-deficient ($BTLA^{-/-}$) NKT cells produced larger amounts of IL-4 and IFN-γ upon α-glactosylceramide stimulation as compared with wild-type (WT) NKT cells. In vivo, $BTLA^{-/-}$ mice produced larger amounts of IL-4 and IFN-γ upon Con A injection and were more susceptible to Con A-induced hepatitis than WT mice. In addition, the augmentation of Con A-induced hepatitis in $BTLA^{-/-}$ mice was not observed in BTLA/NKT-double deficient mice. Moreover, $NKT^{-/-}$ mice reconstituted with $BTLA^{-/-}$ NKT cells were significantly more susceptible to Con A-induced hepatitis as compared with $NKT^{-/-}$ mice reconstituted with WT NKT cells. These results suggest that BTLA functions as the inhibitory coreceptor of NKT cells and plays a critical role in the prevention of NKT cell-mediated liver injury. ***The Journal of Immunology*****, 2010, 184: 127–133.**

Signals delivered through stimulatory and inhibitory coreceptors regulate lymphocyte activation in collaboration with primary AgR signals. Stimulatory coreceptors include CD28 and inducible T cell costimulator (ICOS), whereas inhibitory coreceptors include CTLA-4, programmed cell death 1 (PD-1), and B and T lymphocyte attenuator (BTLA) (1, 2). Accumulating evidence indicates that the balance between stimulatory and inhibitory cosignals is crucial for the effective immune responses to pathogens and the maintenance of self-tolerance (1, 2).

BTLA has originally been identified as an inhibitory coreceptor selectively expressed on Th1 cells and B cells (3). Thereafter, flow cytometric analyses using monoclonal Abs against BTLA have revealed that BTLA is expressed on certain lymphocyte subsets including γδ T cells and regulatory T cells as well as on some APCs such as macrophages and dendritic cells (DCs) (4, 5). BTLA has also been reported to be expressed at low levels on NK cells (4, 6). More recently, it has been shown that a TNFR family member herpesvirus entry mediator (HVEM) is a ligand for BTLA (5, 7, 8) and that the ligation of BTLA with HVEM transduces inhibitory cosignals (5).

In vivo function of BTLA has recently been addressed using BTLA-deficient ($BTLA^{-/-}$) mice. Initially, we have shown that the sensitivity to experimental autoimmune encephalomyelitis as well as T cell-dependent Ab responses is increased in $BTLA^{-/-}$ mice (3). It has also been reported that $BTLA^{-/-}$ mice exhibit a rapid rejection of partially MHC-mismatched cardiac allograft (9), persistent allergic airway inflammation following Ag challenge (10, 11), and an acceleration of experimental colitis (12). These findings indicate that BTLA is crucial for dampening immune responses mediated by T cells. Moreover, we have recently shown that aged $BTLA^{-/-}$ mice spontaneously develop autoimmune hepatitis-like disease with an increase of NKT cells in the liver (13), suggesting that BTLA may prevent autoimmune hepatitis through the inhibition of NKT cell function.

NKT cells are characterized by coexpression of T cell markers such as TCR and NK cell markers such as NK1.1 (14). In mice, the majority of NKT cells express an invariant Vα14 TCR, which is essential for their development (14), and recognizes a specific ligand, α-galactosylceramide (α-GalCer), presented on CD1d molecules (14, 15). NKT cells rapidly produce both IL-4 and IFN-γ on activation (15, 16) and play a crucial role in various immune responses, including antitumor immunity, allergic reaction, and autoimmune diseases (14). Although the roles of stimulatory coreceptors in NKT cell function have been addressed (17, 18), the role of inhibitory coreceptors including BTLA in NKT cell function remains largely unknown.

In this study, we examined the role of BTLA in the regulation of NKT cell function. We found that BTLA was expressed on NKT cells at the levels similar to those on T cells. $BTLA^{-/-}$ NKT cells produced larger amounts of IL-4 and IFN-γ on α-GalCer stimulation as compared with wild-type (WT) NKT cells. Importantly, $BTLA^{-/-}$

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Abbreviations used in this paper: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BTLA, B and T lymphocyte attenuator; $BTLA^{-/-}$, BTLA-deficient; DC, dendritic cells; α-GalCer, α-galactosylceramide; HVEM, herpesvirus entry mediator; ICOS, inducible T cell costimulator; PD-1, programmed cell death 1; NKT cell-deficient ($NKT^{-/-}$); WT, wild-type.

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mice were highly susceptible to Con A-induced hepatitis, in which NKT cells have been reported to play pathogenic roles (19, 20). Indeed, the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice was not observed in BTLA/NKT-double deficient mice. In addition, we found that NKT cell-deficient (NKT$^{-/-}$) mice reconstituted with BTLA$^{-/-}$ NKT cells were significantly more susceptible to Con A-induced hepatitis as compared with NKT $^{-/-}$ mice reconstituted with WT NKT cells. Our results indicate that BTLA functions as the inhibitory coreceptor in NKT cells and thus prevents NKT cell-mediated tissue damage.

Materials and Methods

Mice

BTLA$^{-/-}$ mice (3) were backcrossed over eight generations onto C57BL/6 mice (Charles River Laboratories, Kanagawa, Japan). NKT$^{-/-}$ mice (Jα281$^{-/-}$ mice) on a C57/BL6 background were described previously (21). NKT $^{-/-}$ mice were crossed with BTLA$^{-/-}$ mice, and the offspring were intercrossed to obtain BTLA$^{-/-}$ NKT $^{-/-}$ mice. All mice were housed in microisolator cages under specific pathogen-free conditions, and the mice were used for the experiments at 6–12 wk of age. Animal procedures in this study were approved by the Chiba University Animal Care and Use Committee.

Flow cytometry

The following Abs were purchased from BD Biosciences (San Diego, CA): anti-CD3ε FITC, PE (145-2C11), anti-NK1.1 PE (PK136), anti-CD45R/B220 FITC, PE (RA3-6B2), anti-TCR β-chain FITC, PE (H57-597), anti-CD8α FITC (53-6.7), anti-CD11b FITC (M1/70), anti-CD11c FITC (HL3), anti-CD25 FITC (7D4), anti-CD69 FITC ([^{1}H].2F3), anti-CD122 FITC (TM-β1), anti-Fas biotin (Jo2), anti-Fas ligand biotin (MFL3), streptavidin-PE, and streptavidin-allophycocyanin. Anti-BTLA PE (6F7) and anti-BTLA Alexa Fluor 647 (8F4) were purchased from eBioscience (San Diego, CA). After FcRs were blocked with anti-CD16/32 mAb (BD Biosciences), cells were stained with indicated Abs and analyzed on a FACSCalibur (BD Biosciences) using CellQuestPro software (BD Biosciences).

Preparation of allophycocyanin-conjugated α-GalCer/CD1d–dimer

Allophycocyanin-conjugated α-GalCer/CD1d–dimer was prepared as described previously (22). In brief, 2.75 ml α-GalCer (200 mg/ml; Kirin Pharma, Tokyo, Japan) and 6 ml mouse CD1d-Ig fusion protein (0.5 mg/ml; BD Biosciences) was conjugated at 37°C overnight. The α-GalCer/CD1d–Ig conjugates were then incubated with allophycocyanin-conjugated anti-mouse IgG1 (X56; BD Biosciences) for 60 min. Free allophycocyanin-conjugated anti-mouse IgG1 in the mixture was blocked by the addition of excess amounts of control mouse IgG1 mAb (A111-3; BD Biosciences) for 30 min at room temperature.

Preparation of mononuclear cells from the liver

The livers were removed from the mice after perfusion through the portal vein and inferior vena cava with PBS. Each liver was cut into small pieces, passed through a stainless steel mesh, and suspended in RPMI 1640 medium for 3 min. Mononuclear cells were then harvested from the supernatants and enriched by Percoll (GE Healthcare UK, Little Chalfont, United Kingdom) gradient centrifugation according to the manufacturer's instruction.

Preparation of intrahepatic NKT cells by magnetic-activated cell sorting

Liver mononuclear cells were incubated with anti-CD16/32 to block nonspecific binding and then stained with a mixture of FITC-conjugated Abs against B220, CD8α, CD11b, and CD11c, and allophycocyanin-conjugated α-GalCer/CD1d dimer. FITC-positive cells were depleted using anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The remaining cells were then incubated with anti-allophycocyanin MicroBeads (Miltenyi Biotec), and allophycocyanin-positive cells were positively collected twice by magnet cell sorting. The purity of collected cells was determined by flow cytometry and was routinely >95% of TCR-β$^+$ α-GalCer$^+$ cells.

Coculture of NKT cells and α-GalCer–loaded cells

α-GalCer–loaded cells were prepared as described elsewhere (22) with a minor modification. Single cell suspension of splenocytes was irradiated (30 gray) and incubated with α-GalCer (10 or 100 ng/ml) in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 5.5 μM β-mercaptoethanol, 2 mM L-glutamine, non-essential amino acids, and antibiotics) for 12 h. Hepatic NKT cells (5×10^4) were enriched by MACS as described above and were cocultured with α-GalCer loaded cells (5×10^4) in complete RPMI1640 medium in 96-well round bottom plates for 36 h.

Proliferation assay

The proliferation of NKT cells was measured using CellTiter-Glo reagent according to the manufacturer's instruction (Promega, Madison, WI).

Con A-induced hepatitis

Con A (Sigma-Aldrich, St. Louis, MO) was dissolved in pyrogen-free PBS and injected into the mice (10 or 20 mg/kg i.v.). Sera were collected from the individual mice at the indicated time after Con A injection. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were measured by standard protocols (SRL, Tokyo, Japan).

Measurement of cytokine levels by enzyme-linked immunosorbent assay

The levels of TNF-α, IL-4, IFN-γ, and IL-10 in the sera and the supernatants were measured by ELISA kits according to the manufacturer's protocols (TNF-α, IL-4, and IL-10 kits from BD Biosciences; and IFN-γ kit from R&D Systems, Minneapolis, MN). The minimum significant value of the assay was 15 pg/ml TNF-α and IL-4, 30 pg/ml IFN-γ and IL-10.

Adoptive transfer of NKT cells

Purified intrahepatic NKT cells from WT mice or BTLA$^{-/-}$ mice were injected into the liver of NKT $^{-/-}$ mice (1×10^6 cells/mouse) as described previously (19). One hour later, the mice were injected i.v. with Con A (10 mg/kg). Sera were obtained at 12 and 24 h after Con A injection.

Statistical analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t test. p values < 0.05 were considered significant.

Results

BTLA is expressed on NKT cells but is dispensable for the development and maintenance of NKT cells

To determine whether BTLA is involved in the development and function of NKT cells, we first examined the expression of BTLA on NKT cells. Mononuclear cells from thymus, spleen, and liver in C57BL/6 mice were stained with anti-BTLA mAb (6F7), and the expression levels of BTLA on each cell type were evaluated by flow cytometry. As shown in Fig. 1, the expression of BTLA was detected on NKT cells, which were defined as CD3$^+$ α-GalCer/CD1d-dimer$^+$ cells, at the levels similar to those on CD3$^+$ T cells. Consistent with previous reports (3, 6), BTLA was expressed at higher levels on B220$^+$ B cells and at lower levels on NK cells (Fig. 1).

We next examined the development and activation state of NKT cells in BTLA$^{-/-}$ mice. As previously demonstrated (3), the numbers of thymocytes and splenocytes in BTLA$^{-/-}$ mice at 8 wk old were comparable to those in WT mice (data not shown). Flow cytometric analysis revealed that the frequencies of NKT cells (TCRβ$^+$ α-GarCer/CD1d$^+$cells) in the thymus and spleen of BTLA$^{-/-}$ mice were comparable to those in WT mice (Fig. 2*A*). The number of NKT cells in the livers of BTLA$^{-/-}$ mice was also similar to that in WT mice (Fig. 2*A*). In addition, the expression levels of activation markers such as CD25, CD69, and CD122 on NKT cells were similar between BTLA$^{-/-}$ mice and WT mice (Fig. 2*B*). Together, these results indicate that BTLA is dispensable for the development and the maintenance of steady state of NKT cells.

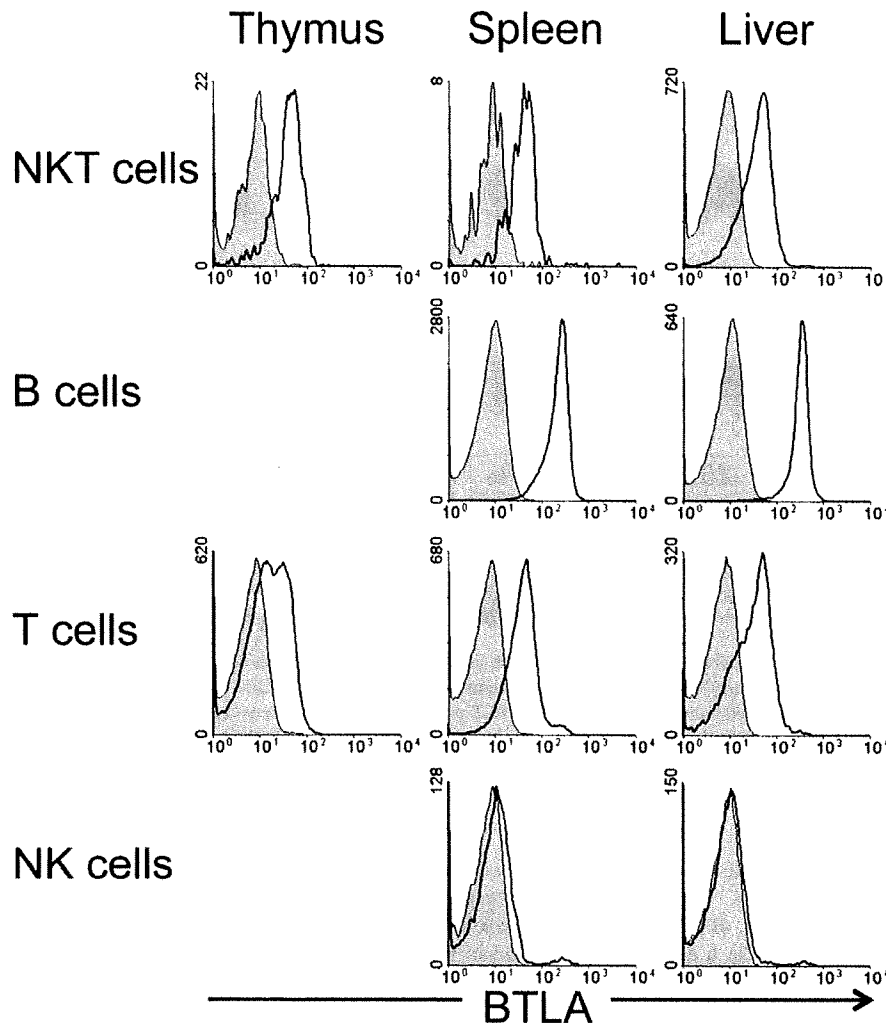

FIGURE 1. BTLA is expressed on NKT cells at the levels similar to those on $CD3^+$ T cells. Mononuclear cells from thymus, spleen, and liver in C57BL/6 mice were stained with anti-BTLA mAb. Representative histograms of BTLA expression (solid line) on NKT cells ($CD3^+$ α-GalCer/CD1d–dimer$^+$), T cells ($CD3^{high}$ α-GalCer/CD1d–dimer$^-$), B cells ($CD3^-$ $B220^+$), and NK cells ($NK1.1^+$ $CD3^-$) are shown (n = 4 mice each). Shaded areas indicate control staining with isotype-matched Ab.

BTLA-deficient NKT cells are hyperreactive to Ag stimulation in vitro

We next examined whether BTLA regulated NKT cell function. Purified NKT cells from the livers of WT mice and $BTLA^{-/-}$ mice were stimulated with α-GalCer–loaded APCs for 36 h, and the levels of IFN-γ, IL-4, and TNF-α in the culture supernatants were measured by ELISA. In response to α-GalCer stimulation (100 ng/ml), $BTLA^{-/-}$ NKT cells secreted significantly larger amounts of IFN-γ and IL-4, compared with WT NKT cells (IFN-γ: $BTLA^{-/-}$ 14.9 ± 7.9 versus WT 3.7 ± 2.5 ng/ml; n = 6; $p < 0.05$; IL-4: $BTLA^{-/-}$ 1.8 ± 0.5 versus WT 1.0 ± 0.4 ng/ml; n = 6; $p < 0.05$; Fig. 3*A*). TNF-α was not detected in the culture supernatants of WT NKT cells and $BTLA^{-/-}$ NKT cells in the experimental condition (data not shown). In addition, $BTLA^{-/-}$ NKT cells showed enhanced proliferation in response to α-GalCer stimulation, compared with WT NKT cells (Fig. 3*B*). These results indicate that $BTLA^{-/-}$ NKT cells are hyperreactive to antigenic stimulation, suggesting that BTLA functions as an inhibitory coreceptor in NKT cell activation.

$BTLA^{-/-}$ mice are highly susceptible to Con A-induced hepatitis

Con A-induced hepatitis is a widely used mouse model that resembles autoimmune hepatitis in humans in many aspects (23). The development of hepatitis after Con A injection has been shown to be attenuated in mice lacking NKT cells (19, 20), indicating that NKT cells are involved in causing Con A-induced hepatitis. We therefore chose this model to test the function of BTLA expressed on NKT cells in vivo. When WT mice and $BTLA^{-/-}$ mice were injected i.v. with Con A (20 mg/kg), all $BTLA^{-/-}$ mice died by 24 h after injection, whereas all WT mice survived over 48 h (Fig. 4*A*). These results indicate that $BTLA^{-/-}$ mice are highly susceptible to Con A-induced hepatitis.

To examine the immune responses to Con A in $BTLA^{-/-}$ mice in detail, we used a sublethal dose of Con A (10 mg/kg) for $BTLA^{-/-}$ mice in the following experiments. First, we performed histologic examination of the liver of Con A-injected $BTLA^{-/-}$ mice and WT mice. As shown in Fig. 4*B*, liver sections from Con A-injected $BTLA^{-/-}$ mice showed a massive necrosis and mononuclear cell infiltration in the portal area. In addition, the levels of ALT and AST in sera of $BTLA^{-/-}$ mice were significantly elevated, compared with those of WT mice at 12 and 24 h after Con A injection (n = 6; $*p < 0.05$; $**p < 0.01$; Fig. 4*C*), confirming the increased susceptibility to Con A-induced hepatitis in $BTLA^{-/-}$ mice.

A number of studies have suggested a proinflammatory role of TNF-α, IFN-γ, and IL-4 and a protective role of IL-10 in the Con A-induced hepatitis (24–26). It has also been demonstrated that IL-4 produced by NKT cells is implicated in liver damage in Con A-induced hepatitis (19, 27). Therefore, we measured cytokine levels in sera of Con A-injected $BTLA^{-/-}$ mice and WT mice.

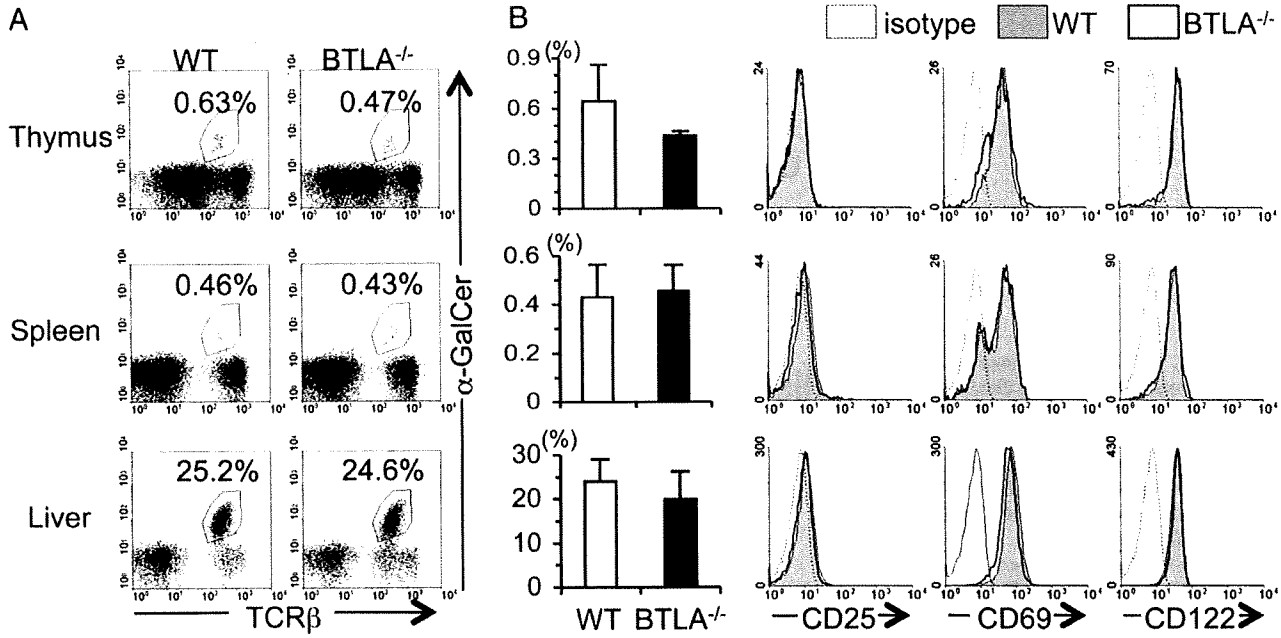

FIGURE 2. Normal development of NKT cells in BTLA$^{-/-}$ mice. Mononuclear cells from thymus, spleen, and liver in WT mice and BTLA$^{-/-}$ mice were stained with anti-TCR-β PE, allophycocyanin-conjugated α-GalCer/CD1d–dimer, and FITC-conjugated Abs against CD25, CD69, or CD122 and analyzed by flow cytometry. *A*, Representative FACS profiles of TCR-β versus α-GalCer/CD1d–dimer staining (*left panels*), and the percentages of TCR-β$^+$ α-GalCer/CD1d–dimer$^+$ cells (*right panels*) are shown. Data are means ± SD for 4 mice in each genotype. *B*, Representative histograms for CD25, CD69, and CD122 expression on TCR-β$^+$ α-GalCer/CD1d–dimer$^+$ cells are shown (n = 4 mice in each genotype). Dotted lines indicate control staining with isotype-matched Ab.

The levels of TNF-α and IFN-γ showed a sharp increase in Con A-injected BTLA$^{-/-}$ mice, and the peaks were higher than those in WT mice (Fig. 4*D*). The levels of TNF-α and IFN-γ reverted to the baseline at 24 h after Con A injection in WT mice, but remained elevated in BTLA$^{-/-}$ mice (Fig. 4*D*). Con A-injected BTLA$^{-/-}$ mice also exhibited prolonged IL-4 production, compared with WT mice (Fig. 4*D*). Alternatively, the levels of IL-10 were comparable between Con A-injected BTLA$^{-/-}$ mice and WT mice (Fig. 4*D*).

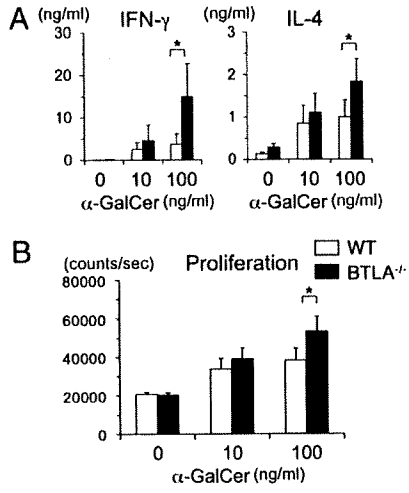

FIGURE 3. Increased activation of BTLA$^{-/-}$ NKT cells on AgR stimulation in vitro. NKT cells (5 × 10^4) were purified from the liver of WT and BTLA$^{-/-}$ mice and cocultured with α-GalCer–loaded irradiated splenocytes (5 × 10^4) as APCs for 36 h. *A*, The levels of IFN-γ and IL-4 in the culture supernatants were measured by ELISA. Data are means ± SD for 6 mice in each group. *$p < 0.05$. *B*, Proliferation of NKT cells was determined using CellTiter-Glo reagent. Data are means ± SD for 6 mice in each group. *$p < 0.05$.

It has been reported that hepatic NKT cells rapidly upregulate FasL expression on the surface and induces apoptosis of hepatocytes upon Con A stimulation (19, 20). We therefore examined FasL expression on hepatic NKT cells in Con A-injected BTLA$^{-/-}$ mice and WT mice. As shown in Fig. 4*E*, FasL was upregulated on NKT cells but not on T cells or NK cells at 1 h after Con A injection in both BTLA$^{-/-}$ mice and WT mice. The levels of FasL on NKT cells were similar between BTLA$^{-/-}$ mice and WT mice, suggesting that FasL expressed on NKT cells could not account for the enhanced susceptibility to Con A-induced hepatitis in BTLA$^{-/-}$ mice.

NKT cells are required for the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice

To determine whether the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice depends on NKT cells, we examined the susceptibility to Con A-induced hepatitis in the mice lacking both BTLA and NKT cells (BTLA$^{-/-}$ NKT$^{-/-}$ mice). We first examined lymphocyte development in BTLA$^{-/-}$ NKT $^{-/-}$ mice and found that BTLA$^{-/-}$ NKT $^{-/-}$ mice lacked NKT cells, but had normal numbers of other lymphoid populations (data not shown). We then compared the levels of ALT and AST in BTLA$^{-/-}$ NKT $^{-/-}$ mice, BTLA$^{-/-}$ mice, NKT $^{-/-}$ mice, and WT mice upon Con A injection. Consistent with a previous study (19), Con A-induced hepatitis was significantly attenuated in NKT $^{-/-}$ mice compared with WT mice (Fig. 5*A*), indicating that NKT cells play an important role in causing Con A-induced hepatitis. Importantly, the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice was not observed in BTLA$^{-/-}$ NKT $^{-/-}$ mice (Fig. 5*A*). Histologic analysis confirmed the reduced liver damage in Con A-injected BTLA$^{-/-}$ NKT $^{-/-}$ mice compared with BTLA$^{-/-}$ mice (Fig. 5*B*). Moreover, serum levels of TNF-α, IFN-γ, and IL-4 were significantly

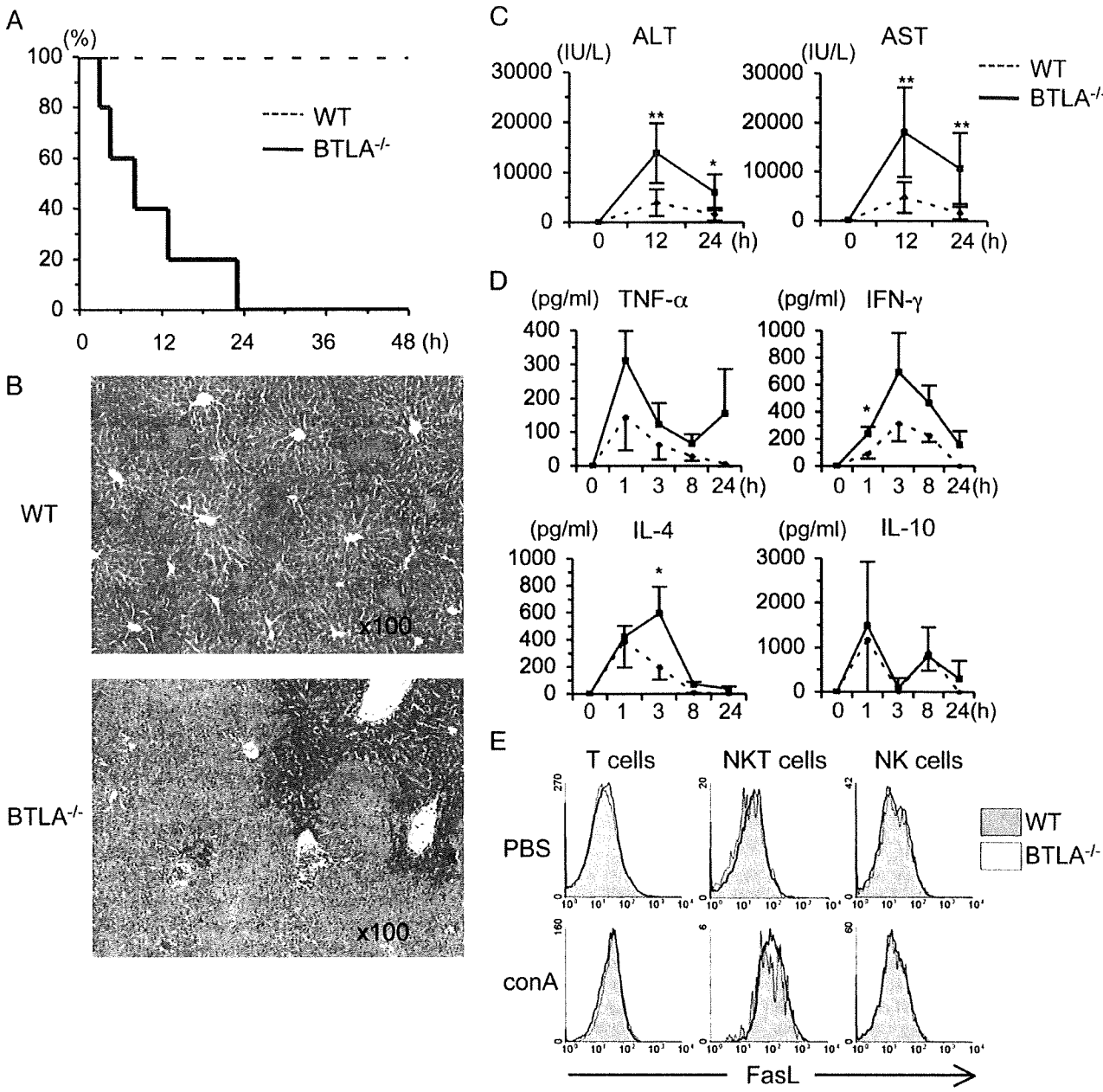

FIGURE 4. Increased susceptibility to Con A-induced hepatitis in BTLA$^{-/-}$ mice. *A*, BTLA$^{-/-}$ mice (solid line) and WT mice (dotted line) were injected i.v. with Con A (20 mg/kg), and the survival of the mice was evaluated for 48 h ($n = 5$ mice in each group). BTLA$^{-/-}$ mice and WT mice were injected i.v. with a sublethal dose of Con A (10mg/kg). *B*, The liver was excised at 24 h after Con A injection. Representative photomicrographs (H&E staining) of the liver of BTLA$^{-/-}$ mice and WT mice are shown ($n = 6$ mice in each group). *C*, Sera were collected at 12 and 24 h after Con A injection, and the levels of ALT and AST were determined. Data are means ± SD ($n = 6$ mice in each group; $*p < 0.05$; $**p < 0.01$). *D*, Sera were collected at 1, 3, 8, and 24 h after Con A injection, and the levels of TNF-α, IFN-γ, IL-4, and IL-10 were determined. Data are means ± SD ($n = 4$ mice in each group; $*p < 0.05$). *E*, One hour after Con A injection, liver mononuclear cells were isolated and the expression levels of Fas ligand (FasL) were analyzed by flow cytometry. Shown are representative histograms for FasL expression on T cells (CD3ε$^+$ α-GalCer/CD1d–dimer$^-$), NKT cells (CD3ε$^+$ α-GalCer/CD1d–dimer$^+$), and NK cells (CD3ε$^-$ NK1.1$^+$) in the liver ($n = 4$ mice in each group).

decreased in BTLA$^{-/-}$ NKT$^{-/-}$ mice compared with BTLA$^{-/-}$ mice (Fig. 5*C*). These results indicate that NKT cells are critical for the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice.

BTLA expressed on NKT cells is involved in the inhibition of Con A-induced hepatitis

To determine whether BTLA expressed on NKT cells is crucial for the inhibition of Con A-induced hepatitis, we performed adoptive transfer experiments in which purified NKT cells from either WT or BTLA$^{-/-}$ mice were transferred to NKT $^{-/-}$ mice. As shown in Fig. 6, NKT$^{-/-}$ mice reconstituted with BTLA$^{-/-}$ NKT cells were significantly more susceptible to Con A-induced hepatitis, compared with NKT $^{-/-}$ mice reconstituted with WT NKT cells ($n = 12$ mice in each group; $p < 0.05$). Together, these results suggest that BTLA expressed on NKT cells is involved in the inhibition of Con A-induced hepatitis.

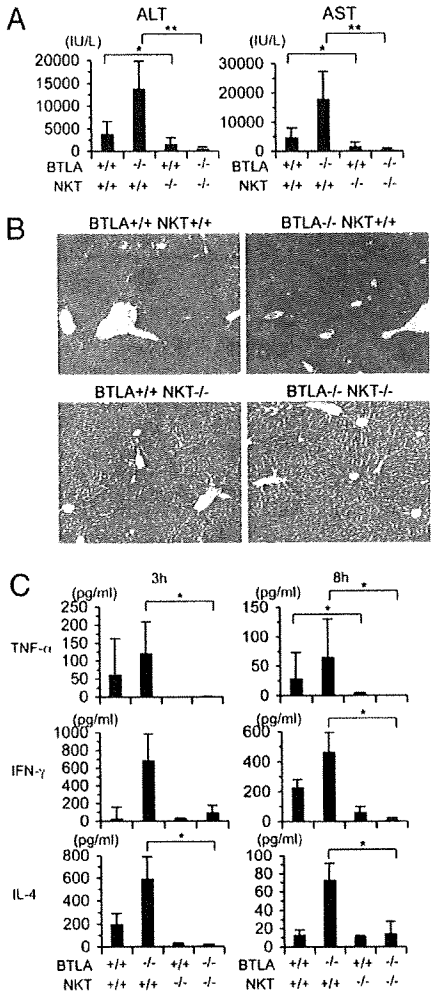

FIGURE 5. NKT cells are required for the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice. WT mice, BTLA$^{-/-}$ mice, NKT$^{-/-}$ mice (Jα281$^{-/-}$ mice), and BTLA$^{-/-}$ NKT$^{-/-}$ mice were injected i.v. with a sublethal dose of Con A (10 mg/kg). *A*, Sera were collected at 12 h after Con A injection, and the levels of ALT and AST were determined. Data are means ± SD (n = 6; $*p < 0.05$; $**p < 0.01$). *B*, The liver was excised from WT mice, BTLA$^{-/-}$ mice, NKT$^{-/-}$ mice, and BTLA$^{-/-}$ NKT$^{-/-}$ mice at 24 h after Con A injection. Shown are representative photomicrographs (H&E staining) of the liver (n = 4 mice in each group). *C*, Sera were collected from WT mice, BTLA$^{-/-}$ mice, NKT$^{-/-}$ mice, and BTLA$^{-/-}$ NKT$^{-/-}$ mice at 3 and 8 h after Con A injection, and the levels of TNF-α, IFN-γ, and IL-4 were determined by ELISA. Data are means ± SD (n = 4; $*p < 0.05$).

Discussion

In this study, we show that BTLA is expressed on NKT cells (Fig. 1) and that BTLA$^{-/-}$ NKT cells produce larger amounts of cytokines on Ag stimulation than WT NKT cells do (Fig. 3), suggesting that BTLA exerts inhibitory effects on NKT cells. In addition, it has recently been demonstrated that PD-1/PD-1 ligand 1 interaction is essential for the induction and maintenance of anergic state of NKT cells (28). It has also been demonstrated that stimulatory coreceptors such as CD28 (17) and ICOS (18) play a significant role in the induction of cytokine production from NKT cells. Together, these findings suggest that, analogous to T cells, the activation of NKT cells is regulated by the balance between stimulatory cosignals and inhibitory cosignals including BTLA.

We also show that BTLA$^{-/-}$ mice are highly susceptible to Con A-induced hepatitis (Fig. 4), in which NKT cells have been shown

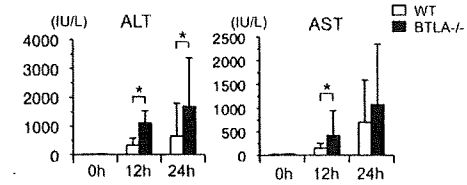

FIGURE 6. NKT$^{-/-}$ mice reconstituted with BTLA$^{-/-}$ NKT cells tended to be susceptible to Con A-induced hepatitis. Purified intrahepatic NKT cells from WT mice or BTLA$^{-/-}$ mice were injected into the liver of NKT$^{-/-}$ mice (1×10^6 cells/mouse), and 1 h later the mice were injected with Con A i.v. Sera were obtained from the mice at 12 and 24 h after Con A injection, and serum ALT and AST levels were determined. Data are means ± SD (n = 12 mice in each group; $*p < 0.05$).

to play a significant role (19, 20). Indeed, although Con A-induced hepatitis was significantly attenuated in NKT$^{-/-}$ mice, compared with WT mice, the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice was not observed in BTLA$^{-/-}$ NKT$^{-/-}$ mice (Fig. 5*A–C*). Recently, Miller et al. (29) also reported that BTLA$^{-/-}$ mice were susceptible to Con A-induced hepatitis and that the increased susceptibility of BTLA$^{-/-}$ mice to Con A-induced hepatitis was diminished in BTLA$^{-/-}$ CD1d$^{-/-}$ mice, which lack most NKT cells (14). Importantly, we also show that NKT$^{-/-}$ mice reconstituted with BTLA$^{-/-}$ NKT cells are significantly more susceptible to Con A-induced hepatitis, compared with NKT$^{-/-}$ mice reconstituted with WT NKT cells (Fig. 6), indicating that BTLA expressed on NKT cells is involved in the regulation of the susceptibility to Con A-induced hepatitis.

Recently, it has been demonstrated that the mice lacking HVEM, a ligand for BTLA (5, 7, 8), are also highly susceptible to Con A injection to induce increased CD4$^+$ T cell activation, but exhibit no significant liver damage (30). Because HVEM is a ligand for BTLA and a costimulatory receptor for lymphotoxins (inducible expression) that compete with HSV glycoprotein D for HVEM (a receptor expressed on T lymphocytes) (5), NKT cell activation might not be sufficient for inducing hepatitis in HVEM-deficient mice.

The effector mechanisms by which NKT cells cause Con A-induced hepatitis remain to be elucidated. Previous studies have shown that NKT cell-derived cytokines including IL-4 are crucial for NKT cell-mediated liver injury (19). Consistent with these findings, we found that IL-4 production from α-GalCer–activated NKT cells, and serum levels of IL-4 in Con A-injected mice were enhanced in BTLA$^{-/-}$ mice (Figs. 3*A* and 4*D*). Alternatively, FasL expression was similarly upregulated on BTLA$^{-/-}$ NKT cells and WT NKT cells on Con A injection (Fig. 4*E*). Together, it is suggested that the elevated levels of cytokines rather than FasL expression might be involved in the augmentation of Con A-induced hepatitis in BTLA$^{-/-}$ mice. In addition, it has recently been suggested that soluble lymphotoxins (inducible expression) that compete with HSV glycoprotein D for HVEM (a receptor expressed on T lymphocytes) released from NKT cells may also be an effector molecule for Con A-induced hepatitis (31).

We found that BTLA$^{-/-}$ NKT cells produced larger amounts of cytokines on α-GalCer stimulation in vitro (Fig. 3*A*). Because the expression levels of activation markers such as CD25, CD69, and CD122 on NKT cells were similar between BTLA$^{-/-}$ mice and WT mice (Fig. 2*B*), it is suggested that the enhanced cytokine production of BTLA$^{-/-}$ NKT cells is caused by a lack of BTLA-HVEM interaction during the in vitro Ag stimulation. However, in preliminary experiments, we found that the addition of anti-BTLA Ab (clone 6F7), which is reported to function as a neutralizing Ab (32), did not significantly enhance cytokine production from α-GalCer–stimulated WT NKT cells in vitro. Although this experiment is

inconclusive, because the binding of 6F7 to BTLA may transduce some signals in NKT cells, this finding raises another possibility that the enhanced cytokine production of BTLA$^{-/-}$ NKT cells may be caused by developmental abnormality of BTLA$^{-/-}$ NKT cells rather than a lack of BTLA-HVEM interaction upon Ag stimulation. Further studies using inducible deletion of the *BTLA* gene in NKT cells are required to address the precise mechanism underlying the enhanced cytokine production from α-GalCer–stimulated BTLA$^{-/-}$ NKT cells.

More recently, it has been demonstrated that PD-1, another inhibitory coreceptor, is expressed on NKT cells (28) and that the engagement of PD-1 on NKT cells by PD-1 ligand 1 inhibits cytokine secretion by α-GalCer–activated NKT cells (33). CD94/NKG2A, an inhibitory receptor on NK cells, is also expressed on NKT cells, and the blockade of NKG2A-mediated inhibitory signal with an antagonistic Ab augments Con A- and α-GalCer–induced liver injury (34). Furthermore, CD4$^+$ CD25$^+$ regulatory T cells have been reported to be important for the suppression of Con A-induced liver injury via a TGF-β–dependent mechanism (35). Therefore, it is suggested that in addition to BTLA, multiple inhibitory pathways are involved in the regulation of NKT cell function in the liver.

In conclusion, we have shown that BTLA functions as an inhibitory coreceptor in NKT cells and prevents NKT cell-mediated experimental hepatitis. Although further studies are required to address the underlying mechanisms, our data suggest that the enhancement of BTLA signaling in NKT cells by agonistic ligands or by a stimulatory Ab might be applicable for the treatment of a number of diseases in which NKT cells play a pathogenic role.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* 23: 515–548.
2. Watts, T. H. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol.* 23: 23–68.
3. Watanabe, N., M. Gavrieli, J. R. Sedy, J. Yang, F. Fallarino, S. K. Loftin, M. A. Hurchla, N. Zimmerman, J. Sim, X. Zang, et al. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat. Immunol.* 4: 670–679.
4. Han, P., O. D. Goularte, K. Rufner, B. Wilkinson, and J. Kaye. 2004. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J. Immunol.* 172: 5931–5939.
5. Murphy, K. M., C. A. Nelson, and J. R. Sedý. 2006. Balancing co-stimulation and inhibition with BTLA and HVEM. *Nat. Rev. Immunol.* 6: 671–681.
6. Hurchla, M. A., J. R. Sedy, M. Gavrieli, C. G. Drake, T. L. Murphy, and K. M. Murphy. 2005. B and T lymphocyte attenuator exhibits structural and expression polymorphisms and is highly Induced in anergic CD4$^+$ T cells. *J. Immunol.* 174: 3377–3385.
7. Gonzalez, L. C., K. M. Loyet, J. Calemine-Fenaux, V. Chauhan, B. Wranik, W. Ouyang, and D. L. Eaton. 2005. A coreceptor interaction between the CD28 and TNF receptor family members B and T lymphocyte attenuator and herpesvirus entry mediator. *Proc. Natl. Acad. Sci. USA* 102: 1116–1121.
8. Sedy, J. R., M. Gavrieli, K. G. Potter, M. A. Hurchla, R. C. Lindsley, K. Hildner, S. Scheu, K. Pfeffer, C. F. Ware, T. L. Murphy, et al. 2005. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat. Immunol.* 6: 90–98.
9. Tao, R., L. Wang, R. Han, T. Wang, Q. Ye, T. Honjo, T. L. Murphy, K. M. Murphy, and W. W. Hancock. 2005. Differential effects of B and T lymphocyte attenuator and programmed death-1 on acceptance of partially versus fully MHC-mismatched cardiac allografts. *J. Immunol.* 175: 5774–5782.
10. Deppong, C., T. I. Juehne, M. Hurchla, L. D. Friend, D. D. Shah, C. M. Rose, T. L. Bricker, L. P. Shornick, E. C. Crouch, T. L. Murphy, et al. 2006. Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. *J. Immunol.* 176: 3909–3913.
11. Tamachi, T., N. Watanabe, Y. Oya, S. Kagami, K. Hirose, Y. Saito, I. Iwamoto, and H. Nakajima. 2007. B and T lymphocyte attenuator inhibits antigen-induced eosinophil recruitment into the airways. *Int. Arch. Allergy Immunol.* 143(Suppl 1): 50–55.
12. Steinberg, M. W., O. Turovskaya, R. B. Shaikh, G. Kim, D. F. McCole, K. Pfeffer, K. M. Murphy, C. F. Ware, and M. Kronenberg. 2008. A crucial role for HVEM and BTLA in preventing intestinal inflammation. *J. Exp. Med.* 205: 1463–1476.
13. Oya, Y., N. Watanabe, T. Owada, M. Oki, K. Hirose, A. Suto, S. Kagami, H. Nakajima, T. Kishimoto, I. Iwamoto, et al. 2008. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. *Arthritis Rheum.* 58: 2498–2510.
14. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol.* 21: 483–513.
15. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278: 1626–1629.
16. Chen, H., and W. E. Paul. 1997. Cultured NK1.1$^+$ CD4$^+$ T cells produce large amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. *J. Immunol.* 159: 2240–2249.
17. Ikarashi, Y., R. Mikami, A. Bendelac, M. Terme, N. Chaput, M. Terada, T. Tursz, E. Angevin, F. A. Lemonnier, H. Wakasugi, et al. 2001. Dendritic cell maturation overrules H-2D-mediated natural killer T (NKT) cell inhibition: critical role for B7 in CD1d-dependent NKT cell interferon γ production. *J. Exp. Med.* 194: 1179–1186.
18. Kaneda, H., K. Takeda, T. Ota, Y. Kaduka, H. Akiba, Y. Ikarashi, H. Wakasugi, M. Kronenberg, K. Kinoshita, H. Yagita, et al. 2005. ICOS costimulates invariant NKT cell activation. *Biochem. Biophys. Res. Commun.* 327: 201–207.
19. Kaneko, Y., M. Harada, T. Kawano, M. Yamashita, Y. Shibata, F. Gejyo, T. Nakayama, and M. Taniguchi. 2000. Augmentation of Valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. *J. Exp. Med.* 191: 105–114.
20. Takeda, K., Y. Hayakawa, L. Van Kaer, H. Matsuda, H. Yagita, and K. Okumura. 2000. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc. Natl. Acad. Sci. U.S.A.* 97: 5498–5503.
21. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623–1626.
22. Watarai, H., R. Nakagawa, M. Omori-Miyake, N. Dashtsoodol, and M. Taniguchi. 2008. Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat. Protoc.* 3: 70–78.
23. Tiegs, G., J. Hentschel, and A. Wendel. 1992. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J. Clin. Invest.* 90: 196–203.
24. Mizuhara, H., E. O'Neill, N. Seki, T. Ogawa, C. Kusunoki, K. Otsuka, S. Satoh, M. Niwa, H. Senoh, and H. Fujiwara. 1994. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. *J. Exp. Med.* 179: 1529–1537.
25. Mizuhara, H., M. Uno, N. Seki, M. Yamashita, M. Yamaoka, T. Ogawa, K. Kaneda, T. Fujii, H. Senoh, and H. Fujiwara. 1996. Critical involvement of interferon γ in the pathogenesis of T-cell activation-associated hepatitis and regulatory mechanisms of interleukin-6 for the manifestations of hepatitis. *Hepatology* 23: 1608–1615.
26. Louis, H., O. Le Moine, M. O. Peny, E. Quertinmont, D. Fokan, M. Goldman, and J. Devière. 1997. Production and role of interleukin-10 in concanavalin A-induced hepatitis in mice. *Hepatology* 25: 1382–1389.
27. Ajuebor, M. N., C. M. Hogaboam, T. Le, and M. G. Swain. 2003. C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. *J. Immunol.* 170: 5252–5259.
28. Chang, W. S., J. Y. Kim, Y. J. Kim, Y. S. Kim, J. M. Lee, M. Azuma, H. Yagita, and C. Y. Kang. 2008. Cutting edge: Programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J. Immunol.* 181: 6707–6710.
29. Miller, M. L., Y. Sun, and Y. X. Fu. 2009. Cutting edge: B and T lymphocyte attenuator signaling on NKT cells inhibits cytokine release and tissue injury in early immune responses. *J. Immunol.* 183: 32–36.
30. Wang, Y., S. K. Subudhi, R. A. Anders, J. Lo, Y. Sun, S. Blink, Y. Wang, J. Wang, X. Liu, K. Mink, et al. 2005. The role of herpesvirus entry mediator as a negative regulator of T cell-mediated responses. *J. Clin. Invest.* 115: 711–717.
31. Anand, S., P. Wang, K. Yoshimura, I. H. Choi, A. Hilliard, Y. H. Chen, C. R. Wang, R. Schulick, A. S. Flies, D. B. Flies, et al. 2006. Essential role of TNF family molecule LIGHT as a cytokine in the pathogenesis of hepatitis. *J. Clin. Invest.* 116: 1045–1051.
32. Crawford, A., and E. J. Wherry. 2009. Editorial: Therapeutic potential of targeting BTLA. *J. Leukoc. Biol.* 86: 5–8.
33. Wang, J., L. Cheng, Z. Wondimu, M. Swain, P. Santamaria, and Y. Yang. 2009. Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *J. Immunol.* 182: 6644–6647.
34. Kawamura, T., K. Takeda, H. Kaneda, H. Matsumoto, Y. Hayakawa, D. H. Raulet, Y. Ikarashi, M. Kronenberg, H. Yagita, K. Kinoshita, et al. 2009. NKG2A inhibits invariant NKT cell activation in hepatic injury. *J. Immunol.* 182: 250–258.
35. Wei, H. X., Y. H. Chuang, B. Li, H. Wei, R. Sun, Y. Moritoki, M. E. Gershwin, Z. X. Lian, and Z. Tian. 2008. CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cells protect against T cell-mediated fulminant hepatitis in a TGF-β-dependent manner in mice. *J. Immunol.* 181: 7221–7229.

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REVIEW ARTICLE

Present Situation of Cedar Pollinosis in Japan and its Immune Responses

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ABSTRACT

Recent observations have suggested significant worldwide increase in the prevalence of allergic rhinitis and cedar pollinosis. In Japan, Japanese cedar (*Cryptometria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) pollens are considered to be the major unique allergens and their extent of dispersal is quite large, travelling more than 100 km and thus causing serious pollinosis. Cedar pollinosis is a typical type 1 allergic disease by an adaptive immune response that occurs through the induction of allergen-specific effector T cells from naïve T cells. We examined the number of Japanese cedar pollen specific memory Th cells in the peripheral blood of the patients and found that the cedar pollen specific IL-4-producing Th2 memory cells increased during the pollen season and decreased during the off-season. However, more than 60% of the cedar-specific memory Th2 cells survived up to 8 months after the pollen season. Natural killer T(NKT) cells represent a unique lymphocyte subpopulation and their activity is not restricted to MHC antigens. NKT cells play an important role in innate immunity, however, the participation in development of allergic rhinitis could not be clarified.

KEY WORDS

cedar pollinosis, cedar specific Th memory cell, epidemiology, natural killer T cell

CEDAR POLLEN

In recent years, many countries have experienced an increase in the prevalence of allergic rhinitis.[1,2] Dust mite allergen is responsible for at least 90% of cases of perennial allergic rhinitis, while arboreal pollen, including that of cedar and Japanese cypress, is important in Japan.[3,4] Cedar forest covers nearly 18% of the total land area of Japan, while Japanese cypress is concentrated in the Kanto region and the western part of the country. Both cedar and Japanese cypress produce enormous amounts of pollen. In Japan, pollen counts are typically measured using the gravimetric method with a Durham sampler, in contrast to Western countries in which a Burkard sampler is typically used. In a study in Chiba Prefecture in 2005, the amount of air-borne pollen counted with a Burkard sampler was about 12 times greater than that counted with a Durham sampler.[5] In addition, distinct from grass pollen, which only spreads less than 100 meters, cedar and cypress pollen travel a long distance and reach major cities, including Tokyo and Osaka, causing wide-spread pollinosis, although no actual data describing the distance traveled was available. A detailed simulation study considering the results of real-time pollen distributing information was conducted using large computers and Figure 1 shows the source and areas from which the cedar pollen detected at Chiba University Hospital had spread. These dark spots indicate the areas where the cedar pollen originated. Pollens blow to Chiba city from the cedar planting areas of Boso Peninsula, as well as from the north Kanto area, Nikko, Izu Peninsula and Shizuoka Prefecture. This study suggests that cedar pollen actually can travel more than 100 km and cause pollinosis in a large area.

Cedar pollen dispersal precedes Japanese cypress pollen dispersal, and approximately 70% of patients with cedar pollinosis are also allergic to Japanese cypress pollen because of a common antigen.[6] Dispersal of cedar and Japanese cypress pollen generally exhibits an arch-shaped pattern with time: cedar pollen dispersal starts in early February and reaches a peak between late February and early March, and is fol-

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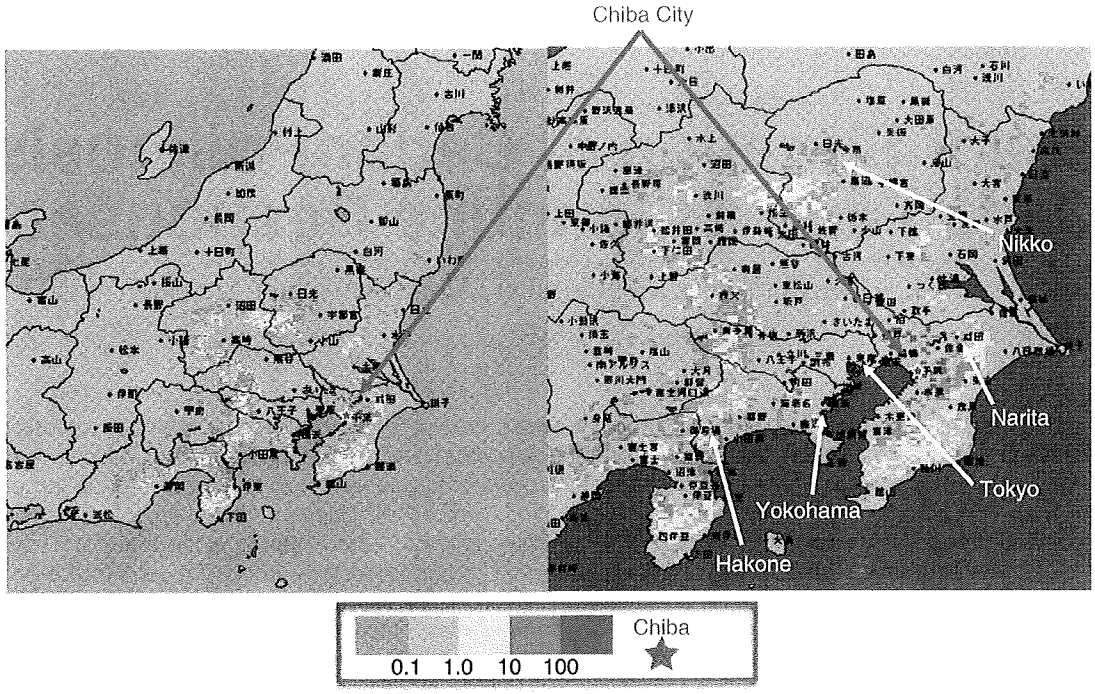

Fig. 1 The source areas from which the cedar pollen detected at Chiba University Hospital spread. This is the computer simulation study done by Mr. Kunihiko Yokota *et al.*, at Weather Service Co.,Ltd..

lowed by dispersal of Japanese cypress pollen, which reaches a peak from late March to early April, with some variation due to changes in the climate each year.[7,8] The pollen dispersal season lasts for more than 10 weeks in and around the Tokyo area.

PREVALENCE OF CEDAR POLLINOSIS IN JAPAN

A survey based only on a questionnaire has the risk of inclusion of a high rate of false-positive cases, because allergic rhinitis is sometimes difficult to distinguish from acute upper respiratory infection and even normal healthy individuals may exhibit mild, nonspecific nasal symptoms, such as sneezing and nasal secretion. In particular, cedar pollen dispersal season is also high flu season. An allergen-specific IgE test is necessary to avoid a high incidence of false positives, but it has been difficult to conduct an epidemiological study in Japan because of laws preventing use of personal information. In 2008, a questionnaire was posed to the Otorhinolaryngologists nationwide to determine whether their families suffered from allergic rhinitis. Although the rate of return of the questionnaire was low, i.e., 40% and the bias of the population could not be ignored, an accurate diagnosis was expected.

According to the analysis of this questionnaire,[9] the prevalence of perennial allergic rhinitis and of cedar pollinosis was 23.4% and 26.5%, respectively. In particular, the prevalence of cedar pollinosis increased more than 10% compared with that observed in a similar questionnaire conducted in 1998. Although the peak of cedar pollinosis is in those in their thirties to forties, the age onset of pollinosis has been decreasing (Fig. 2).

Figure 3 shows the annual amount of cedar pollen dispersal in Japan, which we examined in 2005. The darker brown parts indicate areas where cedar pollen counts were high. We studied the influence of various amounts of pollen exposure on the development of pollinosis and mite allergic rhinitis in elementary school students from schools in rural areas where the movement of students out of or into the school was uncommon. The annual amount of cedar and cypress pollen differed among these five regions. The pollen level was very high in southern Yamanashi: about 7,000/cm^2 on average for the last five years, as determined using Durham pollen samplers. In contrast, the pollen level was low in northern Yamanashi and inland Akita, at about 2,000/cm^2, and very low in coastal Akita, at about 500/cm^2. The pollen level in Chiba was about 4,000/cm^2.

Figure 4 shows the detection rate of cedar- and mite-specific IgE in students in these regions. The positive rate for Japanese cedar was about 60%, except for students in coastal Akita, who had a rate of only 23%. The positive rate for mite IgE was about 50% in each region. These results suggest that the sensitization rate for mite allergen is almost the same nationwide, whereas that for cedar pollen is depend-

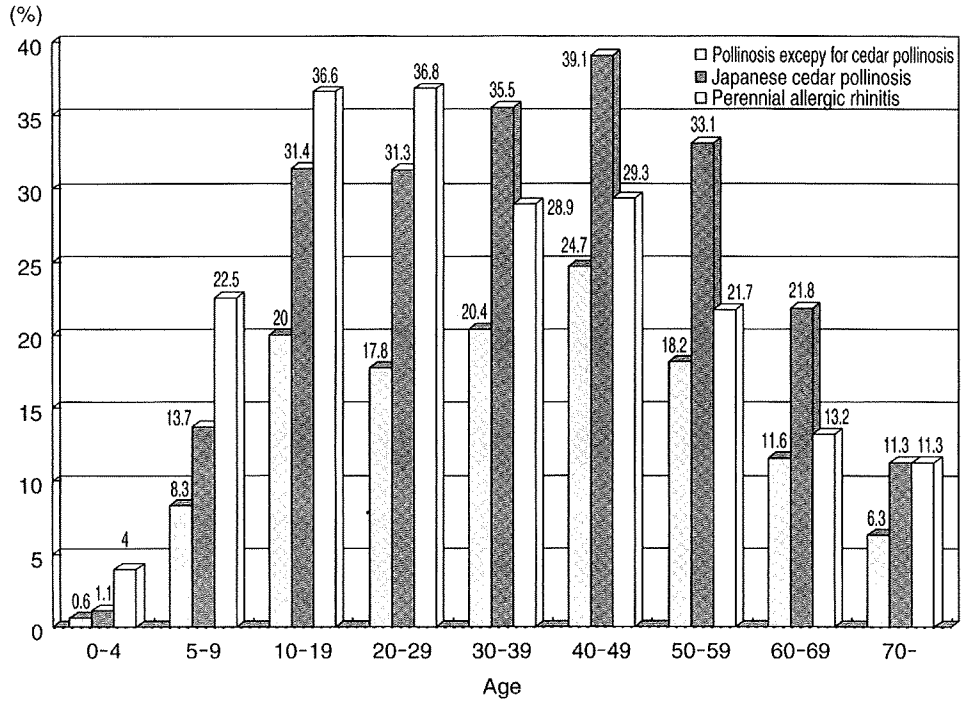

Fig. 2 The prevalence rate of allergic rhinitis in Japan in 2008 (from reference 9).

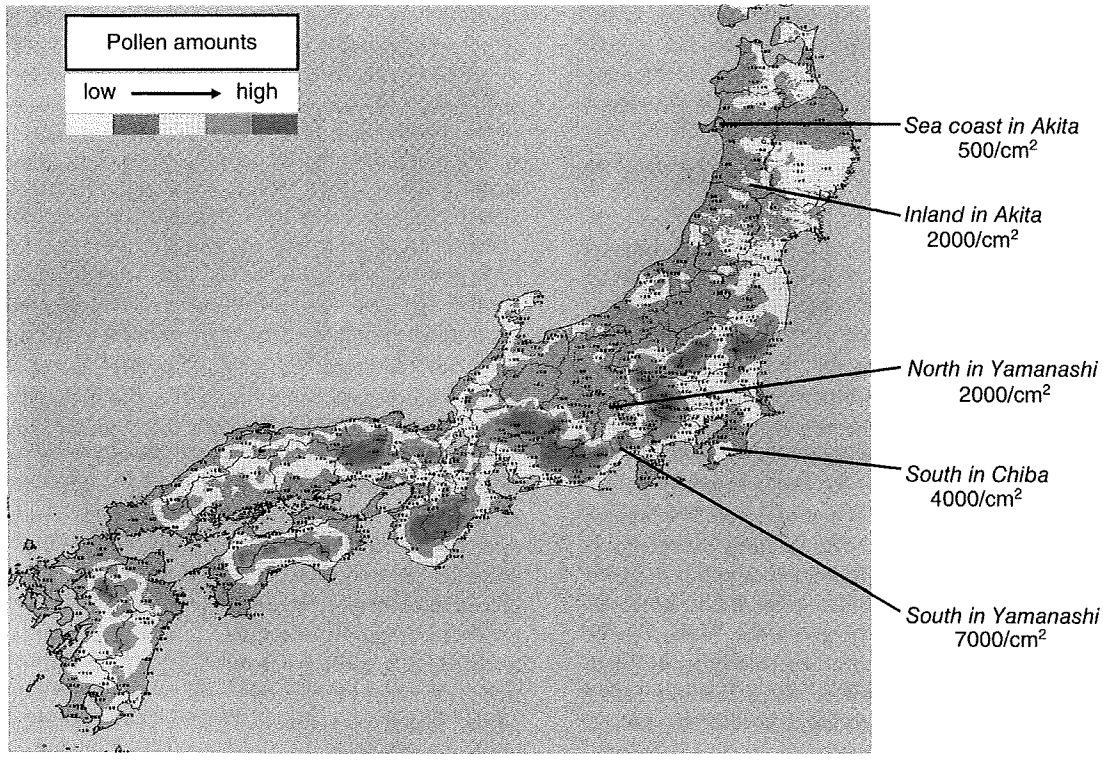

Fig. 3 Annual amount of cedar and cypress pollen dispersal in Japan in 2005.

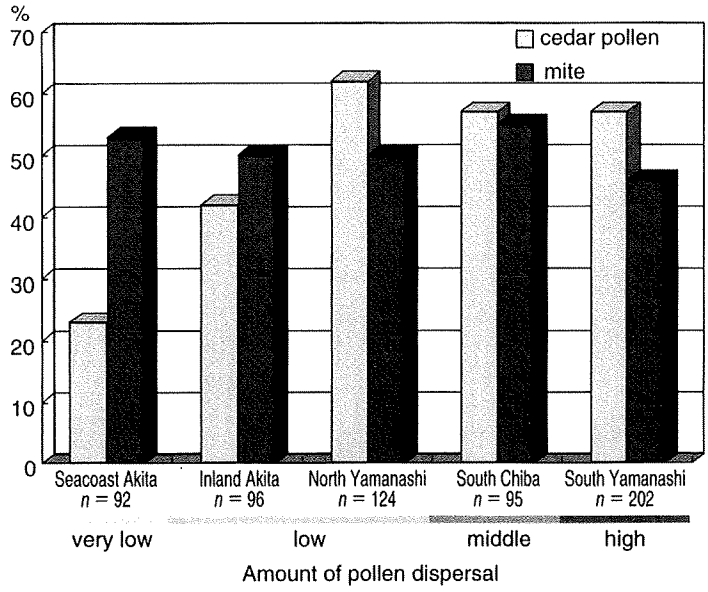

Fig. 4 The detection rate of cedar and cypress pollen-specific IgE in all 4th and 5th grade students in the elementary schools.

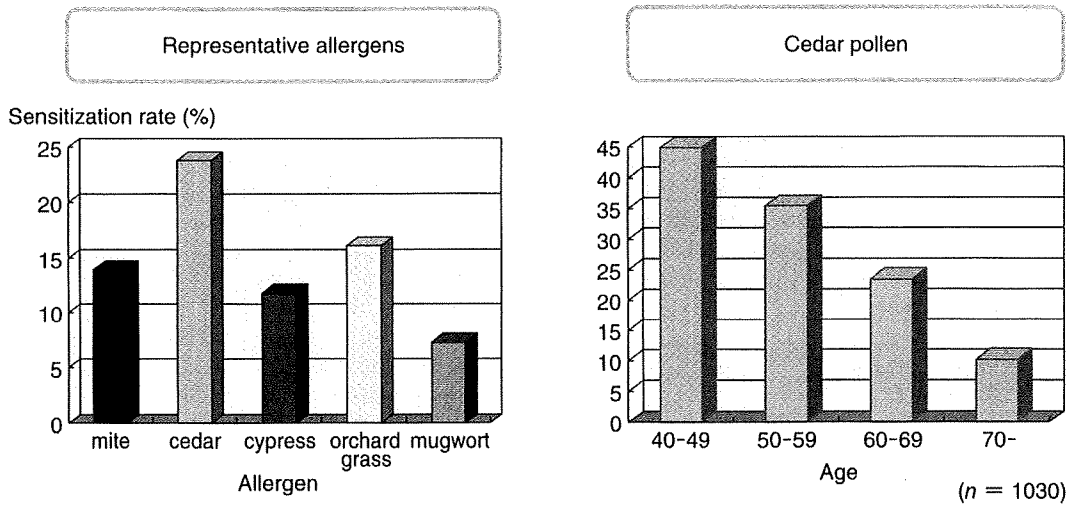

Fig. 5 The sensitization rate to the representative allergen and age distribution of cedar pollen-specific IgE in the adult residents in the forties to seventies in the rural small town in South Chiba.

ent on pollen counts. A very low level of pollen results in a low rate of detection and allergen avoidance is undoubtedly important for prevention. However, a high rate of allergic sensitization can be induced by a relatively small amount of pollen, and it is likely to be very difficult to reduce the amount of pollen exposure to a level that will prevent sensitization. Furthermore, tolerance was not easily induced in students in southern Yamanashi who had been receiving high pollen exposure every year since birth. Interestingly, the incidence of mite allergic rhinitis and pollinosis in these sensitized students was almost the same; about 30 to 35% in each region, respectively.

We have also undertaken medical examination of middle-aged adult residents in their forties to seventies in a rural small town (Maruyama-cho) in South Chiba every year since 1995.[10] The examination includes responses to a questionnaire and testing for specific IgE in serum using a CAP-RAST system. Figure 5 shows the sensitization rate to the representative allergens and the age distribution of cedar pollen-specific IgE. Deterioration of cedar-specific IgE is ob-

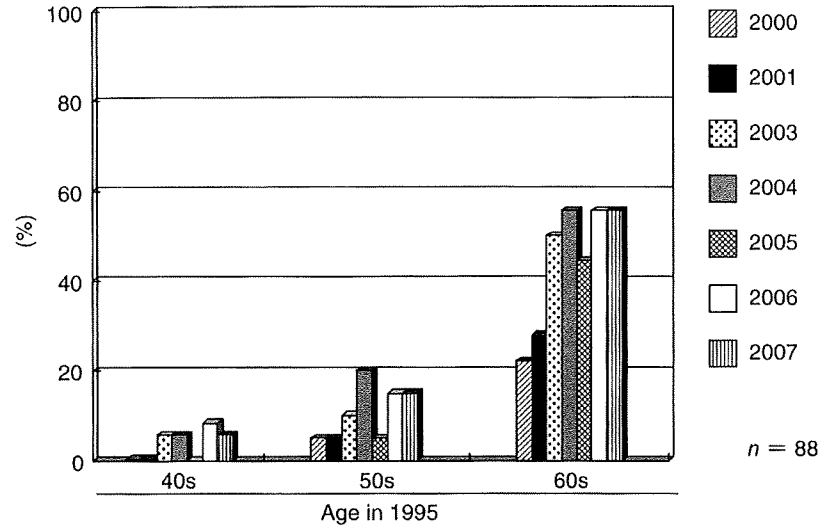

Fig. 6 The rate of change to negative over the last 13 years in cedar pollen-specific IgE in the residents who had tested positive for anti-cedar pollen specific IgE in 1995 and then had received examination every year.

served in elderly subjects. Figure 6 shows the rate of change to negative over the last 13 years in cedar pollen IgE in residents who had tested positive for anti-cedar pollen IgE in 1995. The IgE assays were performed at the end of each cedar pollen season. It appears that the IgE titer is affected by the spread of pollen each year. Interestingly, however, the negative change for 13 years is not commonly observed even in their forties to fifties. The rate of the cedar pollinosis determined by clinical symptoms in combination with positive cedar pollen IgE has also not decreased among these aged subjects.

THE LONG-TERM COURSE OF PATIENTS WITH ALLERGIC RHINITIS

One hundred and seventy-seven patients who were treated in our department from 1970 to 1995 consented to undergo a detailed re-examination. A comparison between the recent symptoms and those observed 10 to 30 years ago showed that 30% of adult patients exhibited some improvements and 10% had resolution. However, only 20% of the pediatric patients exhibited mild improvement of symptoms, whereas the remaining had the same or even worse symptoms as those in childhood (data not shown: in preparation for submitting). Regarding the allergen-specific IgE, a change to negative was not observed in any patients with cedar pollinosis and was seen in only a few of the mite-allergic patients. Thus, natural resolution is not commonly observed in allergic rhinitis and most pediatric patients grow to adulthood without natural improvement of symptoms.

CEDAR POLLEN SPECIFIC MEMORY T CELLS

It has been suggested that dysregulation of cytokine synthesis from Th1 and Th2 cells is fundamental to the pathogenesis of allergic diseases. However, no significant difference was observed between the two groups in the Th1/Th2 cell profile in peripheral blood $CD4^+$ T cells from patients with perennial allergic rhinitis and non-allergic rhinitis by FACS analysis.[11]

Pollinosis is thought to be an adaptive immune response that manifests as a type 1 allergic reaction, and it occurs as a consequence of fundamental allergenic mechanisms involving the induction of pollen-specific T helper type 2 (Th2) effector cells from naïve Th0 cells. Most effector T cells are short-lived, but few effector T cell become long-lived memory T cells. We directly examined the number of allergen-specific Th1/Th2 memory T cells in the peripheral blood of patients of allergic rhinitis by an ELISPOT assay using specific peptides.[12] The Japanese cedar-specific IL-4 producing Th2 cells were detected in all patients examined and increased during the pollen season and decreased during the off-season. However, more than 60% of the cedar-specific memory Th2 cells survived up to 8 months after the pollen season (Fig. 7) .

Allergen-specific immunotherapy is the only current treatment that can change the natural course of allergic rhinitis with long-term effects. However, the conventional immunotherapy with subcutaneous administration is inconvenient because it requires frequent visits to the doctor and also carries the risk of anaphylactic shock.[13] A recent review of randomized

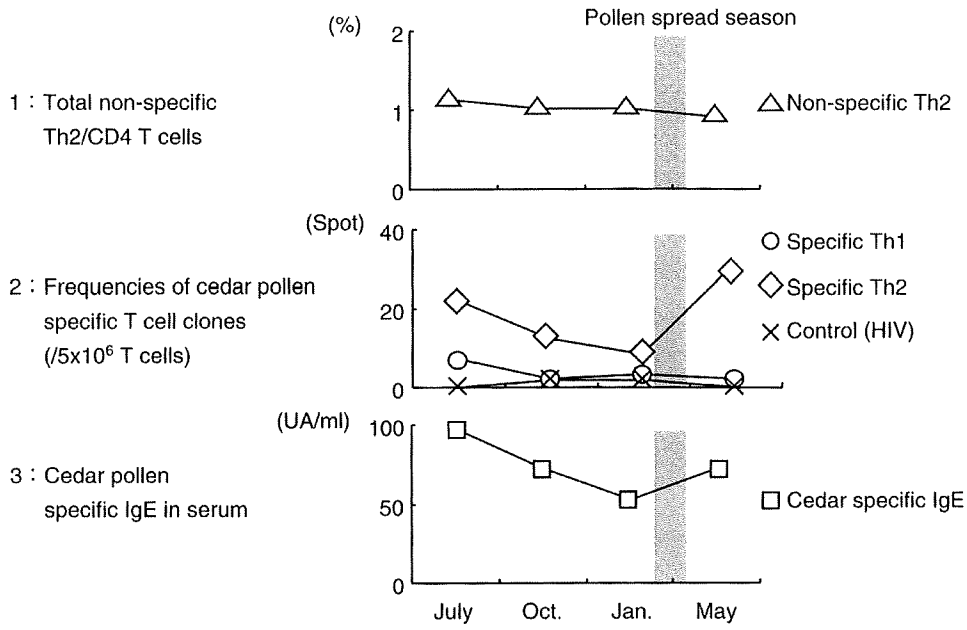

Fig. 7 The seasonal changes of total Th2 cells, frequency of cedar pollen specific T cell clones (spots number) and cedar pollen specific IgE.

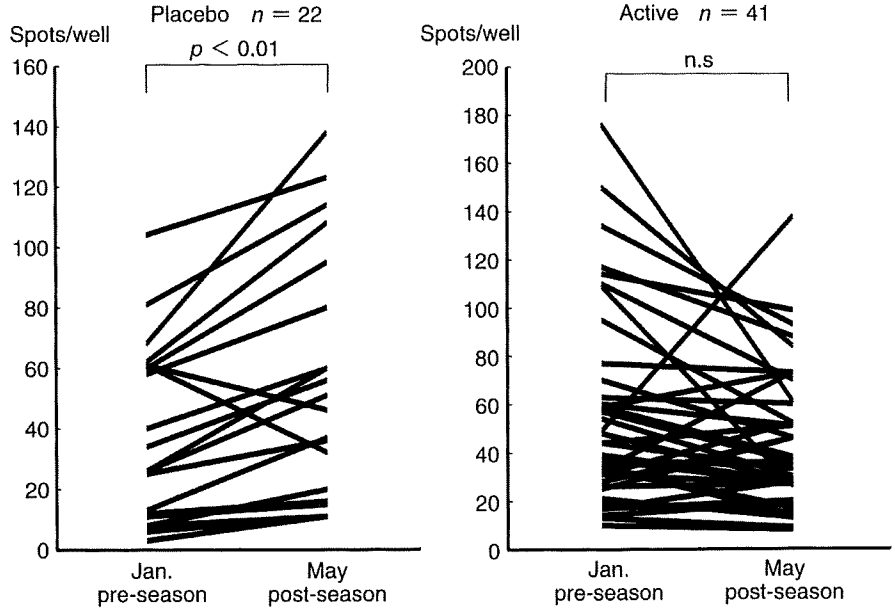

Fig. 8 The number of cedar-specific Th2 cells before and after sublingual immunotherapy.

controlled studies of sublingual immunotherapy suggested that this might be effective as an alternative method of administration.[14-16] To determine the efficacy of sublingual immunotherapy for Japanese cedar pollinosis, we conducted a blinded, randomized, placebo-controlled trial over a period of 6 months (from October 2005 to May 2006).[17] Sixty-seven subjects were enrolled and the nasal symptom scores during the cedar pollen season were evaluated using a symptom diary.

The patients in the active treatment group exhibited significantly lower symptom scores compared to the placebo group. This result suggests that sublingual immunotherapy may offer a safe approach to the management of allergic rhinitis, although the *in vivo* mechanisms of allergen-specific immunotherapy are

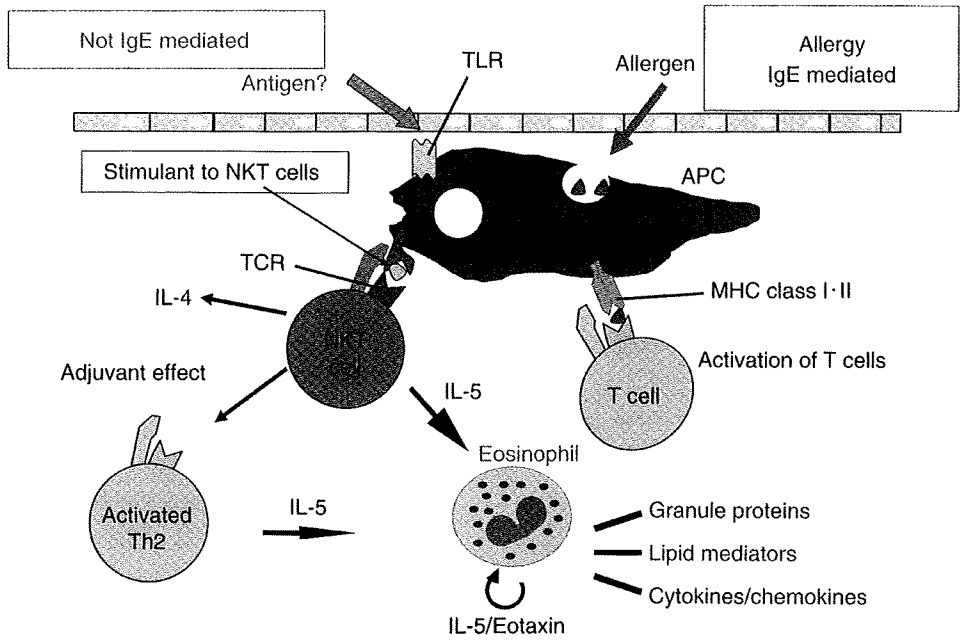

Fig. 9 Mechanism of eosinophil accumulation in respiratory mucosa. Eosinophil accumulation could be observed in MHC class-2 independent.

unknown.

Figure 8 shows the numbers of cedar-specific Th2 cells before and after immunotherapy: the number of Th2 memory cells increased in the placebo group after pollen exposure, but did not increase in the treatment group. Therefore, allergen-specific immunotherapy inhibits an increase in the antigen-specific Th2 memory cell count induced by allergen exposure. Immune-therapeutic intervention might direct at diminishing the size of the clone memory Th2 cells and shifting the cytokine type of memory Th clones.

Natural killer T (NKT) cells represent a unique lymphocyte subpopulation that is characterized by the co-expression of T cells and natural killer receptors.[18,19] Their activity is not restricted to MHC antigens. The relative frequency of NKT cells in the peripheral blood is generally quite low, usually less than 0.1% of PBMCs, and they are not detected in normal peripheral lymph nodes. However, NKT cells play a very important role in innate immunity. Recently, the involvement of NKT cells in the development of airway hypersensitivity in mice and the detection NKT cells in bronchoalveolar-lavage fluid samples from patients with moderate to severe asthma were reported. However, we could not detect the NKT cells in the nasal mucosa of the patients with allergic rhinitis by a polymerase chain reaction. However, NKT cells were detected to varying degrees in the sinus mucosa from asthmatic choronic sinusitis (CS) patients.

These results suggest that NKT cells are not directly related to the development of allergy, but that they may play important roles in the development of sinus disease combined with asthma and in the enhanced Th2 cytokine expression and increased infiltration of Th2 cells and eosinophils observed in the sinus mucosa from asthmatic CS patients via MHC-independent mechanisms (Fig. 9).

SUMMARY

1. The prevalence of allergic rhinitis, in particularly cedar pollinosis, is increasing.

2. Cedar pollen-specific Th1/Th2 dysregulation is observed in patients with pollinosis.

3. Cedar pollen specific memory Th cells increased during the pollen season and decreased during off season, however, morethan 60% of the memory cells survived up to 8 months after the pollen season.

4. NKT cells are not directly related to the development of allergic rhinitis, including pollinosis.

5. Different mechanisms in the accumulation of eosinophilia in the respiratory tract mucosa may exist.

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REFERENCES

1. Bousquet J, Van Cauwenberge P, Khaltaev N; Aria Workshop Group; World Health Organization. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; **108**:S147-334.
2. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjuctivitis, and atopic eczema: ISAAC. *Lancet* 1998;**351**: 1225-32.
3. Okuda M. Epidemiology of Japanese cedar pollinosis throughout Japan. *Ann Allergy Asthma Immunol* 2003;**91**: 288-96.
4. Kaneko Y, Motohashi Y, Nakamura H, Endo T, Eboshida A. Increasing prevalence of Japanese cedar pollinosis: a meta-regression analysis. *Int Arch Allergy Immunol* 2005; **136**:365-71.
5. Delaunay JJ, Sasajima H, Okamoto Y, Yokota M. Side-by-side comparison of automatic pollen counters for use in pollen information systems. *Ann Allergy Asthma Immunol* 2007;**98**:553-8.
6. Ito Y, Takahashi Y, Fujita T, Fukuyama S. Clinical effects of immunotherapy on Japanese cedar pollinosis in the season of cedar and cypress pollination. *Auris Nasus Larynx* 1997;**24**:163-70.
7. Ito H, Nishimura J, Suzuki M *et al.* Specific IgE to Japanese cypress (*Chamaecyparis obtusa*) in patients with nasal allergy. *Ann Allergy Asthma Immunol* 1995;**74**:299-303.
8. Sasaki K, Okamoto Y, Yonekura S *et al.* Cedar and cypress pollinosis and allergic rhinitis: Quality of life effects of early intervention with Leukotriene receptor antagonists. *Int Arch Allergy Immunol* . In press.
9. [*Practical Guideline for the Management of Allergic Rhinitis in Japan—Perennial Rhinitis and Pollinosis—2009 Edition*], 6th edn. Tokyo: Life Science, 2008(in Japanese).
10. Okawa T, Konno A, Yamakoshi T, Numata T, Terada N, Shima M. Analysis of natural history of Japanese cedar pollinosis. *Int Arch Allergy Immunol* 2003;**131**:39-45.
11. Horiguchi S, Okamoto Y, Chazono H, Sakurai D, Kobayashi K. Expression of membrane-bound CD23 in nasal B cells from patients with perennial allergic rhinitis. *Ann Allergy Asthma Immunol* 2005;**94**:286-91.
12. Horiguchi S, Tanaka Y, Uchida T *et al.* Seasonal changes in antigen-specific Th clone sizes in patients with Japanese cedar pollinosis: A 2-year study. *Clin Exp Allergy* 2008;**38**:405-12.
13. Lockey RF, Nicoara-Kasti GL, Theodoropoulos DS, Bukantz SC. Systemic reactions and fatalities associated with allergen immunotherapy. *Ann Allergy Asthma Immunol* 2001;**87**(Suppl 1):47-55.
14. Cox LS, Linnemann DL, Nolte H, Weldon D, Finegold I, Nelson HS. Sublingual immunotherapy: a comprehensive review. *J Allergy Clin Immunol* 2006;**117**:1021-35.
15. Burastero SE. Sublingual immunotherapy for allergic rhinitis: an update. *Curr Opin Otolaryngol Head Neck Surg* 2006;**14**:197-201.
16. Passalacqua G, Lombardi C, Guerra L, Compalati E, Fumagalli F, Canonica GW. Sublingual immunotherapy: no more doubts. *Eur Ann Allergy Clin Immunol* 2005;**37**: 314-20.
17. Horiguchi S, Okamoto Y, Yonekura S *et al.* A randomized controlled trial of sublingual immunotherapy for Japanese cedar pollinosis. *Int Arch Allergy Immunol* 2008;**146**:76-84.
18. Yamamoto H, Okamoto Y, Horiguchi S, Kunii N, Yonekura S, Nakayama T. Detection of natural killer T cells in the sinus mucosa from asthmatics with chronic sinusitis. *Allergy* 2007;**62**:1451-5.
19. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 2003;**21**: 483-513.
20. Kawano T, Cui J, Koezuka Y *et al.* CDld-restricted and TCR-mediated activation of Valpha14 NKT cells by glycosylceramides. *Science* 1997;**278**:1626-9.
21. Akbari O, Stock P, Meyer E *et al.* Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003;**9**: 582-8.
22. Akbari O, Faul JL, Hoyte EG *et al.* CD4+ invariant T-cell-receptor+ naturel killer T cells in bronchial asthma. *N Engl J Med* 2006;**354**:1117-29.

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REVIEW ARTICLE

Antigen-Specific Immunotherapy against Allergic Rhinitis: The State of the Art

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ABSTRACT

Allergic rhinitis is the most prevalent type I allergy in industrialized countries. Pollen scattering from trees or grasses often induces seasonal allergic rhinitis, which is known as pollinosis or hay fever. The causative pollen differs across different areas and times of the year. Impaired performance due to pollinosis and/or medication used for treating pollinosis is considered to be an important reason for the loss of concentration and productivity in the workplace. Antigen-specific immunotherapy is an only available curative treatment against allergic rhinitis. Subcutaneous injection of allergens with or without adjuvant has been commonly used as an immunotherapy; however, recently, sublingual administration has come to be considered a safer and convenient alternative administration route of allergens. In this review, we focus on the safety and protocol of subcutaneous and sublingual immunotherapy against seasonal allergic rhinitis. We also describe an approach to selecting allergens for the vaccine so as to avoid secondary sensitization and adverse events. The biomarkers and therapeutic mechanisms for immunotherapy are not fully understood. We discuss the therapeutic biomarkers that are correlated with the improvement of clinical symptoms brought about by immunotherapy as well as the involvement of Tr1 and regulatory T cells in the therapeutic mechanisms. Finally, we focus on the current immunotherapeutic approach to treating Japanese cedar pollinosis, the most prevalent pollinosis in Japan, including sublingual immunotherapy with standardized extract, a transgenic rice-based edible vaccine, and an immunoregulatory liposome encapsulating recombinant fusion protein.

KEY WORDS

allergic rhinitis, biomarker, immunotherapy, pollinosis, regulatory T cell

INTRODUCTION

Allergic rhinitis is the most prevalent type I allergy, and pollen grains are one of the most common causes of respiratory allergies. In western Europe, the prevalence of clinically confirmable allergic rhinitis was estimated to be 23%, with more than 50% of the allergic subjects possessing specific IgE against grass pollen.[1] In Japan, the prevalence of allergic rhinitis was estimated to be 39.4% and that of pollinosis was 29.8%.[2]

Pollinosis is induced by the invasion of pollen grains onto the ocular and nasal mucosa. Pollen grains easily access internal binding sites on contact with the aqueous phases of nasal and ocular mucosal membranes. After pollens are hydrated on aqueous membranes, they swell, rupture, and release their cytoplasmic components. It has been reported that grass pollen grains rupture in water and release large amounts of respirable particles, such as starch granules containing allergens.[3] Although pollinosis patients have a low rate of asthma attacks during pollen season, the attacks that do occur may be attributable to these respirable particles bearing allergens from pollen grains.[4] Pollen grains release not only allergen-bearing particles but also immunomodulatory mediators such as pollen-associated lipid mediators (PALMs) and NADPH oxidases. Proinflammatory PALMs such as leukotriene B4-like substances attract and activate human peripheral blood eosino-

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phils and polymorphonuclear granulocytes from both allergic and non-allergic donors.[5,6] Immunomodulatory PALMs, such as phytoprastanes, inhibit IL12 production in dendritic cells and Th1-type cytokine production in antigen-specific T cells, while inducing antigen-specific Th2 responses.[7] NADPH oxidase rapidly increases the level of reactive oxygen species (ROS) in lung epithelium and induces neutrophil recruitment to the airway independent of the adaptive immune responses.[8,9] These reports strongly suggest that pollen grains themselves act primarily as adjuvants to induce pollen-antigen-specific Th2 responses and to enhance inflammatory processes during the elicitation phase of allergic responses.

The most common treatments against pollinosis are medications like antihistamines, leukotriene inhibitors, and corticosteroids. However, these treatments are not curative and sometimes induce impaired performance as a results of their side effects.[10,11] Antigen-specific immunotherapy can change the natural course of allergic rhinitis and is recognized as a curative treatment against type I allergy without impaired performance. In this century, since the first report on subcutaneous immunotherapy (SCIT), SCIT has been developed and improved and has become safer and more effective.[12,13] Recently, sublingual immunotherapy (SLIT) has been developed and has become a safer and more beneficial immunotherapy for patients.

This review focuses on the recent approach of using antigen-specific immunotherapy to treat allergic rhinitis, and focuses especially on the use of SLIT against pollinosis using standardized extract or recombinant allergens. We also discuss the therapeutic mechanisms and therapeutic biomarkers for SLIT. Finally, we discuss the recent immunotherapeutic approach to treat Japanese cedar (*Cryptomeria japonica*) pollinosis, which is the most common pollinosis in Japan.

ANTIGENS FOR IMMUNOTHERAPY

For immunotherapy, extracts from an allergen source, i.e. pollen extract, are widely used after the concentration of their major allergen is adjusted so as to be standardized. To standardize such extracts, it is important to analyze their component allergens and establish a quantification system for major allergens.[14] The World Allergy Organization (WAO) recommends that standardized vaccines be used for immunotherapy if they are available.[15] However, the protocols and methods for the standardization of allergen extract are different among different suppliers, which use their own in-house reference materials and their own unique allergen units. This made it difficult to compare the therapeutic effects and safety among clinical trials involving different products. It has been proposed that vaccines be standardized using a protocol based on mass units of major allergens and that the active ingredients of the treatment be quantified. The CREATE project has been working to select major allergens for use in the standardization of vaccines and to establish a quantification system and recombinant allergens for the standardization.[16]

To improve the safety and clinical therapeutic effects of a vaccine, the selection of allergens for vaccination is an important issue. Extract from pollen may contain many allergens that cross-react with those from fruit, vegetables, and latex. These allergens may cause minor local side effects, especially in SLIT, among patients who suffer from oral allergies and/or latex-fruit syndrome. Latex-fruit syndrome sometimes induces severe systematic reactions such as anaphylactic shock in response to natural rubber and some latex fruits.[17] The cross-reactive allergens may have to be removed from vaccines in order to avoid severe systematic adverse reactions caused by cross-reactivity with latex allergens for safer SLIT. For the elucidation of reactive allergens, protein microarray techniques have recently been applied to allergy diagnosis. Microarray-chip technology using a glass slide with the immobilization of large numbers of proteins on the surface enable us to simultaneously test IgE-binding reactivity against large numbers of allergens from various sources.[18,19] This diagnostic technique is applicable to the diagnosis of allergens from a single allergen source. This component-resolved diagnosis is a powerful tool for selecting components of allergens for immunotherapy vaccines and may improve the safety and clinical therapeutic efficacy of the vaccines in comparison to traditional immunotherapy using crude extract.[20] Such an allergen diagnosis enables us to choose only IgE-binding allergens that are individually sensitized for antigen-specific immunotherapy. This approach, in which only sensitized allergens are used for immunotherapy, avoids secondary additional sensitization against nonreactive proteins that can occur with the use of crude extracts or a mixture of allergens (Fig. 1).

Recombinant technology has been used to construct vaccines for immunotherapy.[21] Immunotherapy clinical trials were performed using a mixture of five recombinant grass allergens (rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, and rPhl p 6), and the results suggested that a recombinant allergen vaccine can be an effective and safe treatment to ameliorate the symptoms of allergic rhinitis.[22] Immunotherapy using recombinant Bet v 1 was also recently reported to show clinical efficacy, and its therapeutic effects were comparable with those obtained using native Bet v 1 against birch pollen allergy.[23]

Vaccines using allergoids and modified allergens, such as T cell-epitopes, pathogen-related molecular pattern molecule-conjugated allergens, and others, are under development, and some of them are considered to be promising for use as therapeutic vaccines.[13,24]