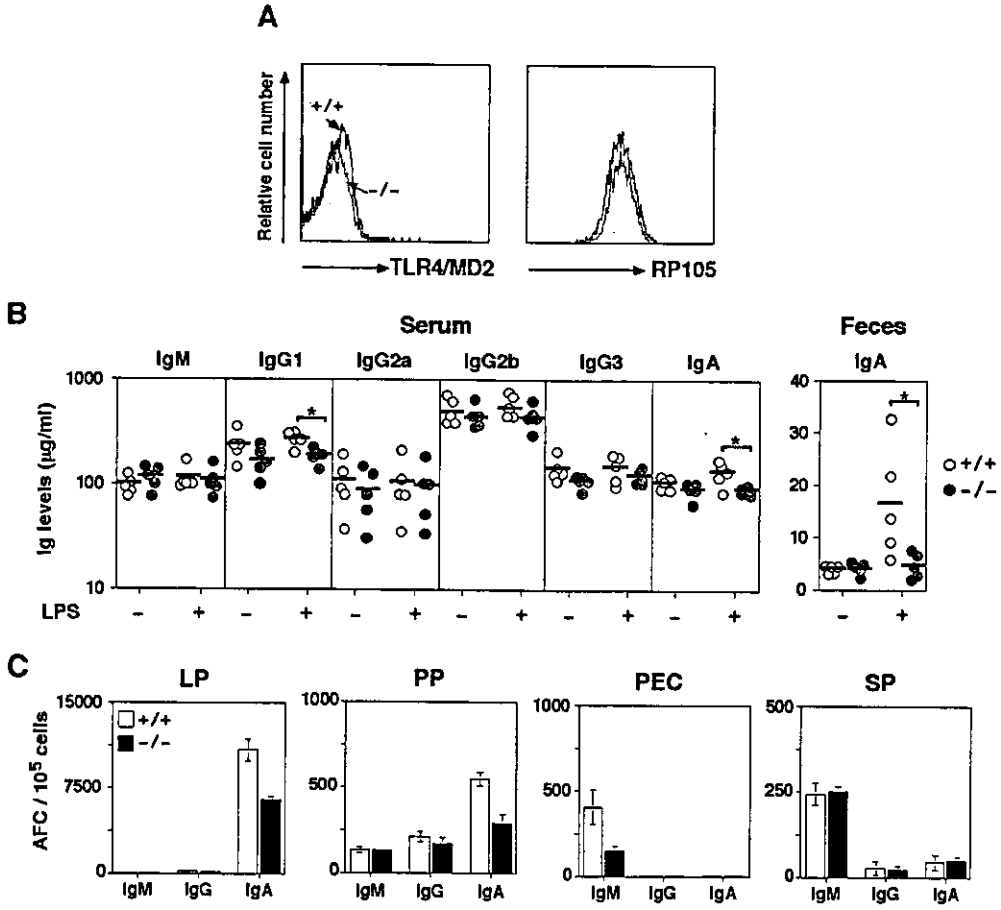

FIGURE 8. Ab production in IL-5R$\alpha^{-/-}$ mice that were administered LPS orally. *A*, IL-5R$\alpha^{-/-}$ B-1 cells express normal levels of TLR4/MD2 and RP105. Representative histograms show TLR/MD2 or RP105 expression on B-1 cells from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice. *B*, IgA and IgG1 levels are not elevated in IL-5R$\alpha^{-/-}$ mice by oral injection of LPS. LPS (0.1 mg/200 μl/week) was injected orally into the gut of IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice for 3 wk. On day 7 after the last injection, the serum and fecal Ig levels of the mice were analyzed by isotype-specific ELISA. The mean Ig levels of the indicated group of mice are represented as a bar. *, $p < 0.05$. *C*, The numbers of Ab-producing cells are reduced in LPS-injected IL-5R$\alpha^{-/-}$ mice. IgM-, IgG-, or IgA-producing cells were examined in the LP, PP, peritoneal cavity, and spleen (SP) from LPS-injected mice by isotype-specific ELISPOT assay. The results represent the mean ± SD of the duplicate wells. Representative results of three independent experiments are shown (*A* and *C*).

on B-1 cells, not on B-2 cells (Fig. 7). Moreover, IL-5R$\alpha^{-/-}$ B-1 cells showed a defective response to anti-CD40 or anti-CD40 plus IL-4 (Fig. 6*A*). Taking these results together, we propose that constitutive stimulation by IL-5 is important for the full activation of B-1 cells in T cell-dependent response as well as LPS-dependent response in mucosal tissues as described below.

IL-5 and B-1 cell-derived IgA

IL-5 is an important cytokine for the mucosal immune system, which distinguishes it from the systemic immune compartment (54). IL-5 is postulated to be a major cytokine that induces sIgA$^+$ B-2 cells to differentiate into IgA-producing plasma cells in PP and to a lesser extent in the spleen (25, 54). Approximately one-half of IgA plasma cells in the LP of the intestine appear to be derived from B-1 cells in the peritoneal cavity, and B-1 cell-derived IgA is specific for commensal bacteria (55). Hiroi et al. (37) have reported the critical role of IL-5 in IgA secretion in mucosal tissues using IL-5R$\alpha^{-/-}$ mice. In IL-5R$\alpha^{-/-}$, the number of sIgA$^+$ B-1 cells from the effector site are significantly reduced, and IgA levels in mucosal secretions are reduced (37). Interestingly, there were significant differences in serum and fecal IgA levels in LPS-treated IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice (Fig. 8*B*). Although the B-1 cells of IL-5R$\alpha^{-/-}$ mice showed defective proliferation and Ig production upon LPS stimulation in vitro (Fig. 6), the expression levels of TLR4/MD2 and RP105 and the sensor of LPS signals on IL-5R$\alpha^{+/+}$ B-1 cells were comparable with those on IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 8*A*). The IL-5-mediated signaling pathway may couple or cross-talk with the LPS-induced signaling pathway. Because LPS, CD40, and BCR triggering of B cells results in the activation of NF-κB factors (56), NF-κB activation induced by LPS or CD40 may be influenced by IL-5 in B-1 cells.

In summary, the present study provides new insight for an understanding of the important role of IL-5 in homeostatic proliferation and survival of mature B-1 cells. Furthermore, constant IL-5 stimulation may be required for optimal B-1 cell activation in response to CD40 or LPS requirements.

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References

1. Kantor, A. B. 1991. The development and repertoire of B-1 cells (CD5 B cells). *Immunol. Today 12:389.*
2. Hardy, R. R., and K. Hayakawa. 1994. CD5 B cells, a fetal B cell lineage. *Adv. Immunol. 55:297.*
3. Kantor, A. B., and L. A. Herzenberg. 1993. Origin of murine B cell lineages. *Annu. Rev. Immunol. 11:501.*

4. Berland, R., and H. H. Wortis. 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol. 20:253.*
5. Takatsu, K., S. Takaki, and Y. Hitoshi. 1994. Interleukin-5 and its receptor system: implications in the immune system and inflammation. *Adv. Immunol. 57:145.*
6. Hayakawa, K., R. R. Hardy, and L. A. Herzenberg. 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med. 161:1554.*
7. Hardy, R. R. 1992. Variable gene usage, physiology and development of Ly-1$^+$ (CD5$^+$) B cells. *Curr. Opin. Immunol. 4:181.*
8. Kroese, F. G., and N. A. Bos. 1999. Peritoneal B-1 cells switch in vivo to IgA and these IgA antibodies can bind to bacteria of the normal intestinal microflora. *Curr. Top. Microbiol. Immunol. 246:343.*
9. Macpherson, A. J., D. Gatto, E. Sainsbury, G. R. Harriman, H. Hengartner, and R. M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science 288:2222.*
10. Taki, S., M. Schmitt, D. Tarlinton, I. Forster, and K. Rajewsky. 1992. T cell-dependent antibody production by Ly-1 B cells. *Ann. NY Acad. Sci. 651:328.*
11. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, L. A. Herzenberg, et al. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity 3:283.*
12. Kerner, J. D., M. W. Appleby, R. N. Mohr, S. Chien, D. J. Rawlings, C. R. Maliszewski, O. N. Witte, and R. M. Perlmutter. 1995. Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity 3:301.*
13. Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky. 1996. Immunodeficiency in protein kinase cβ-deficient mice. *Science 273:788.*
14. Engel, P., L. J. Zhou, D. C. Ord, S. Sato, B. Koller, and T. F. Tedder. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity 3:39.*
15. Fruman, D. A., S. B. Snapper, C. M. Yballe, L. Davidson, J. Y. Yu, F. W. Alt, and L. C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85α. *Science 283:393.*
16. Tarakhovsky, A., M. Turner, S. Schaal, P. J. Mee, L. P. Duddy, K. Rajewsky, and V. L. Tybulewicz. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature 374:467.*
17. Ahearn, J. M., M. B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R. G. Howard, T. L. Rothstein, and M. C. Carroll. 1996. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity 4:251.*
18. Maecker, H. T., and S. Levy. 1997. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med. 185:1505.*
19. Cyster, J. G., and C. C. Goodnow. 1995. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity 2:13.*
20. O'Keefe, T. L., G. T. Williams, S. L. Davies, and M. S. Neuberger. 1996. Hyperresponsive B cells in CD22-deficient mice. *Science 274:798.*
21. Pan, C., N. Baumgarth, and J. R. Parnes. 1999. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity 11:495.*
22. Takatsu, K., A. Tominaga, N. Harada, S. Mita, M. Matsumoto, T. Takahashi, Y. Kikuchi, and N. Yamaguchi. 1988. T cell-replacing factor (TRF)/interleukin 5 (IL-5): molecular and functional properties. *Immunol. Rev. 102:107.*
23. Mita, S., Y. Hosoya, I. Kubota, T. Nishihara, T. Honjo, T. Takahashi, and K. Takatsu. 1989. Rapid methods for purification of human recombinant interleukin-5 (IL-5) using the anti-murine IL-5 antibody-coupled immunoaffinity column. *J. Immunol. Methods 125:233.*
24. Mita, S., A. Tominaga, Y. Hitoshi, K. Sakamoto, T. Honjo, M. Akagi, Y. Kikuchi, N. Yamaguchi, and K. Takatsu. 1989. Characterization of high-affinity receptors for interleukin 5 on interleukin 5-dependent cell lines. *Proc. Natl. Acad. Sci. USA 86:2311.*
25. Sonoda, E., R. Matsumoto, Y. Hitoshi, T. Ishii, M. Sugimoto, S. Araki, A. Tominaga, N. Yamaguchi, and K. Takatsu. 1989. Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. *J. Exp. Med. 170:1415.*
26. Ogata, N., T. Kouro, A. Yamada, M. Koike, N. Hanai, T. Ishikawa, and K. Takatsu. 1998. JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor α and βc subunit, respectively, and are activated upon IL-5 stimulation. *Blood 91:2264.*
27. Sato, S., T. Katagiri, S. Takaki, Y. Kikuchi, Y. Hitoshi, S. Yonehara, S. Tsukada, D. Kitamura, T. Watanabe, O. Witte, et al. 1994. IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. *J. Exp. Med. 180:2101.*
28. Alam, R., K. Pazdrak, S. Stafford, and P. Forsythe. 1995. The interleukin-5/ receptor interaction activates Lyn and Jak2 tyrosine kinases and propagates signals via the Ras-Raf-1-MAP kinase and the Jak-STAT pathways in eosinophils. *Int. Arch. Allergy Immunol. 107:226.*
29. Takaki, S., H. Kanazawa, M. Shiiba, and K. Takatsu. 1994. A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor α chain and its function in IL-5-mediated growth signal transduction. *Mol. Cell. Biol. 14:7404.*
30. Li, T., S. Tsukada, A. Satterthwaite, M. H. Havlik, H. Park, K. Takatsu, and O. N. Witte. 1995. Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. *Immunity 2:451.*
31. Kikuchi, Y., M. Hirano, M. Seto, and K. Takatsu. 2000. Identification and characterization of a molecule, BAM11, that associates with the pleckstrin homology domain of mouse Btk. *Int. Immunol. 12:1397.*
32. Kouro, T., Y. Kikuchi, H. Kanazawa, K. Hirokawa, N. Harada, M. Shiiba, H. Wakao, S. Takaki, and K. Takatsu. 1996. Critical proline residues of the cytoplasmic domain of the IL-5 receptor α chain and its function in IL-5-mediated activation of JAK kinase and STAT5. *Int. Immunol. 8:237.*
33. Tominaga, A., S. Takaki, N. Koyama, S. Katoh, R. Matsumoto, M. Migita, Y. Hitoshi, Y. Hosoya, S. Yamauchi, Y. Kanai, et al. 1991. Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin-5) develop eosinophilia and autoantibody production. *J. Exp. Med. 173:429.*
34. Katoh, S., M. M. Bendig, Y. Kanai, L. D. Shultz, Y. Hitoshi, K. Takatsu, and A. Tominaga. 1993. Maintenance of CD5$^+$ B cells at an early developmental stage by interleukin-5: evidence from immunoglobulin gene usage in interleukin-5 transgenic mice. *DNA Cell Biol. 12:481.*
35. Yoshida, T., K. Ikuta, H. Sugaya, K. Maki, M. Takagi, H. Kanazawa, S. Sunaga, T. Kinashi, K. Yoshimura, J. Miyazaki, S. Takaki, and K. Takatsu. 1996. Defective B-1 cell development and impaired immunity against *Angiostrongylus cantonensis* in IL-5Rα-deficient mice. *Immunity 4:483.*
36. Moon, B. G., T. Yoshida, M. Shiiba, K. Nakao, M. Katsuki, S. Takaki, and K. Takatsu. 2001. Functional dissection of the cytoplasmic subregions of the interleukin-5 receptor α chain in growth and immunoglobulin G_1 switch recombination of B cells. *Immunology 102:289.*
37. Hiroi, T., M. Yanagita, H. Iijima, K. Iwatani, T. Yoshida, K. Takatsu, and H. Kiyono. 1999. Deficiency of IL-5 receptor α-chain selectively influences the development of the common mucosal immune system independent IgA-producing B-1 cell in mucosa-associated tissues. *J. Immunol. 162:821.*
38. Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young, and K. I. Matthaei. 1996. IL-5-deficient mice have a developmental defect in CD5$^+$ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity 4:15.*
39. Bao, S., K. W. Beagley, A. M. Murray, V. Caristo, K. I. Matthaei, I. G. Young, and A. J. Husband. 1998. Intestinal IgA plasma cells of the B1 lineage are IL-5 dependent. *Immunology 94:181.*
40. Harada, N., T. Takahashi, M. Matsumoto, T. Kinashi, J. Ohara, Y. Kikuchi, N. Koyama, E. Severinson, Y. Yaoita, T. Honjo, et al. 1987. Production of a monoclonal antibody useful in the molecular characterization of murine T-cell-replacing factor/B-cell growth factor II. *Proc. Natl. Acad. Sci. USA 84:4581.*
41. Hitoshi, Y., N. Yamaguchi, M. Korenaga, S. Mita, A. Tominaga, and K. Takatsu. 1991. In vivo administration of antibody to murine IL-5 receptor inhibits eosinophilia of IL-5 transgenic mice. *Int. Immunol. 3:135.*
42. Hitoshi, Y., N. Yamaguchi, S. Mita, E. Sonoda, S. Takaki, A. Tominaga, and K. Takatsu. 1990. Distribution of IL-5 receptor-positive B cells: expression of IL-5 receptor on Ly-1(CD5)$^+$ B cells. *J. Immunol. 144:4218.*
43. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol. 3:667.*
44. Miyake, K., Y. Yamashita, Y. Hitoshi, K. Takatsu, and M. Kimoto. 1994. Murine B cell proliferation and protection from apoptosis with an antibody against a 105-kD molecule: unresponsiveness of X-linked immunodeficient B cells. *J. Exp. Med. 180:1217.*
45. Yamamoto, M., K. Fujihashi, K. W. Beagley, J. R. McGhee, and H. Kiyono. 1993. Cytokine synthesis by intestinal intraepithelial lymphocytes: both $\gamma\delta$ T cell receptor-positive and $\alpha\beta$ T cell receptor-positive T cells in the G_1 phase of cell cycle produce IFN-γ and IL-5. *J. Immunol. 150:106.*
46. Xu-Amano, J., H. Kiyono, R. J. Jackson, H. F. Staats, K. Fujihashi, P. D. Burrows, C. O. Elson, S. Pillai, and J. R. McGhee. 1993. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J. Exp. Med. 178:1309.*
47. Corcoran, L. M., and D. Metcalf. 1999. IL-5 and Rp105 signaling defects in B cells from commonly used 129 mouse substrains. *J. Immunol. 163:5836.*
48. Kushnir, N., N. A. Bos, A. W. Zuercher, S. E. Coffin, C. A. Moser, P. A. Offit, and J. J. Cebra. 2001. B2 but not B1 cells can contribute to CD4$^+$ T-cell-mediated clearance of rotavirus in SCID mice. *J. Virol. 75:5482.*
49. Fort, M. M., J. Cheung, D. Yen, J. Li, S. M. Zurawski, S. Lo, S. Menon, T. Clifford, B. Hunte, R. Lesley, et al. 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity 15:985.*
50. Gordon, J. R., P. R. Burd, and S. J. Galli. 1990. Mast cells as a source of multifunctional cytokines. *Immunol. Today 11:458.*
51. Broide, D. H., M. M. Paine, and G. S. Firestein. 1992. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J. Clin. Invest. 90:1414.*
52. Walker, C., J. Checkel, S. Cammisuli, P. J. Leibson, and G. J. Gleich. 1998. IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. *J. Immunol. 161:1962.*
53. Erickson, L. D., T. M. Foy, and T. J. Waldschmidt. 2001. Murine B1 B cells require IL-5 for optimal T cell-dependent activation. *J. Immunol. 166:1531.*
54. McGhee, J. R., J. Mestecky, C. O. Elson, and H. Kiyono. 1989. Regulation of IgA synthesis and immune response by T cells and interleukins. *J. Clin. Immunol. 9:175.*
55. Kroese, F. G., E. C. Butcher, A. M. Stall, P. A. Lalor, S. Adams, and L. A. Herzenberg. 1989. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Int. Immunol. 1:75.*
56. Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol. 12:141.*

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The role of antigenic peptide in CD4$^+$ T helper phenotype development in a T cell receptor transgenic model

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Abstract

CD4$^+$ Th1 cells play a critical role in the induction of cell-mediated immune responses that are important for the eradication of intracellular pathogens. Peptide-25 is the major Th1 epitope for Ag85B of *Mycobacterium tuberculosis* and is immunogenic in I-A^b mice. To elucidate the role of the TCR and IFN-γ/IL-12 signals in Th1 induction, we generated TCR transgenic mice (P25 TCR-Tg) expressing TCR α- and β-chains of Peptide-25-reactive cloned T cells and analyzed Th1 development of CD4$^+$ T cells from P25 TCR-Tg. Naive CD4$^+$ T cells from P25 TCR-Tg differentiate into both Th1 and Th2 cells upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg preferentially develop Th1 cells upon Peptide-25 stimulation in the presence of I-A^b splenic antigen-presenting cells under neutral conditions. In contrast, a mutant of Peptide-25 can induce solely Th2 differentiation. Peptide-25-induced Th1 differentiation is observed even in the presence of anti-IFN-γ and anti-IL-12. Furthermore, naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg also differentiate into Th1 cells upon Peptide-25 stimulation. Moreover, Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells induce Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg in the absence of IFN-γ or IL-12. These results imply that interaction between Peptide-25/I-A^b and TCR may primarily influence determination of the fate of naive CD4$^+$ T cells in their differentiation towards the Th1 subset.

Introduction

Naive CD4$^+$ Th cells recognize an antigenic peptide through their TCR in the context of MHC class II molecules on antigen-presenting cells (APC) and undergo differentiation to effector cells that can produce cytokines and chemokines. During this process, naive CD4$^+$ T cells can differentiate to at least two functionally distinct subsets of cells, represented by Th1 and Th2 (1). Th1 cells produce IFN-γ and lymphotoxin (TNF-β) in addition to IL-2 and are responsible for directing cell-mediated immune responses leading to the eradication of intracellular pathogens such as *Mycobacterium*, viruses and parasites (1–4). Th1 cells also regulate IgG2a and IgG3 antibody production via IFN-γ production, which is involved in the opsonization and phagocytosis of particulate microbes. Th2 cells secrete IL-4, IL-5 and IL-13 as effector cytokines and are responsible for humoral immune responses for the eradication of helminths. Th2 cells also cause inflammatory damage during allergic diseases, such as asthma and atopic dermatitis. The process by which an uncommitted Th cell develops into a mature Th1 or Th2 subset is a matter of fact for regulating the immune response to various antigens.

Considerable progress has been made in identifying the factors that govern the progression of cell differentiation during the generation of Th subsets (2–4). Using T cells stimulated with polyclonal activators or T cells from mice expressing transgenic antigen receptors of known specificities, it has become clear that Th1 and Th2 subsets develop

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from the same T cell precursor (5–7), which is a naive $CD4^+$ T cell. There is a body of evidence to indicate that the cytokines IL-12 and IL-4 are key determinants of the Th1 and Th2 response, respectively (4). For example, IL-12 directs Th1 development from antigen-stimulated naive $CD4^+$ T cells and activates STAT4 in Th1 cells (8,9). In terminally differentiated Th1 cells, successive IFN-γ production can occur through TCR ligation or IL-12 and IL-18 stimulation. Using mice deficient in either cytokines or STAT, it has been shown that activation of the IFN-γR/STAT1 is also important for the differentiation of $CD4^+$ T cells into Th1 cells (10,11). The IL-4R/STAT6 signaling pathway plays a central role in the differentiation of naive $CD4^+$ T cells into Th2 cells (12–14). The balance of IFN-γ and IL-4 levels present during T cell activation is considered to be the major influence on Th1 versus Th2 differentiation. Although the strength of the interaction mediated through TCR and MHC/peptide complex is suspected to affect the lineage commitment of Th cells to Th1 cells and clonal expansion (15–17), it remains unclear whether Th1 cells can develop from naive $CD4^+$ T cells upon antigenic peptide stimulation in the presence of APC under neutral conditions.

Ag85B (also known as α antigen or MPT59) is the most potent antigen species yet purified for both humans and mice (18). Ag85B can elicit strong Th1 response *in vitro* from PPD^+ asymptomatic individuals (19–21). We have shown that *in vitro* stimulation of lymph node cells from *Mycobacterium tuberculosis*-primed C57BL/6 mice with Ag85B induces the production of IFN-γ and IL-2 and expansion of $CD4^+$ T cells expressing Vβ11 of TCR (TCRVβ11) in an $I\text{-}A^b$-restricted manner (22,23). We identified the 15-mer peptide (Peptide-25), covering amino acids residues 240–254 (FQDAYNAAGGHNAVF) of Ag85B, as the major epitope for Ag85B-specific $TCRV\beta11^+$ T cells (22). Using Peptide-25-reactive $V\beta11^+$ T cell clones (BP1, BP4, BM5, BM7 and BM12) and substituted Peptide-25 mutants, we determined which amino acid residues within Peptide-25 were critical for TCR recognition (23,24). Peptide-25 contains the motif that is conserved for $I\text{-}A^b$ binding and requires processing by APC to trigger Ag85B-specific $TCRV\beta11^+$ T cells (22). Active immunization of C57BL/6 mice with Peptide-25 can induce the differentiation of $CD4^+$ TCR $V\beta11^+$ Th1 that produce IFN-γ and TNF-α and protect against subsequent infection with live *M. tuberculosis* H37Rv (23).

Here we generate transgenic mice (P25 TCR-Tg) expressing functional TCR that interacts with Peptide-25 in conjunction with $I\text{-}A^b$. We report that naive $CD4^+$ T cells in the spleen of P25 TCR-Tg mice respond specifically to Peptide-25 in the presence of APC from $I\text{-}A^b$ mice and differentiate to Th1 cells in the absence of IFN-γ or IL-12 under neutral conditions.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). STAT1 deficient mice were kindly provided by Dr R. D. Schreiber, Center for Immunology, Washington University School of Medicine. These mice were maintained under specific pathogen-free conditions in our animal facility according to our Institute's guidelines, and used at 8–15 weeks of age.

Cell lines

Five different Peptide-25-reactive $CD4^+$ Th1 clones (BP1, BP4, BM5, BM7 and BM12) were established *in vitro* by culturing lymph node cells from C57BL/6 mice immunized with heat-killed *M. tuberculosis* H37Rv as described (23). TG40 is a variant T cell hybridoma cell line lacking the expression of surface TCR-α and -β chains that has been used a recipient cells for TCR (25). PLAT-E is a packaging cell line that produces retroviruses (26). Chinese hamster ovary cells expressing $I\text{-}A^b$ ($I\text{-}A^b$-CHO) (27) were kindly provided by Dr Y. Fukui (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan).

Reagents and antibodies

All peptides including Peptide-25 and its substituted mutants were synthesized by Funakoshi Co. Ltd (Tokyo, Japan). Anti-IFN-γ–FITC (XMG1.2), anti-IL-4–allophycocyaninn (11B11), anti-Vβ11–PE (RR3-15), anti-CD4–FITC or -PE (GK1.5), anti-CD8–PE (53.6.72), anti-CD25–FITC (7D4), anti-CD28–FITC (37.51), anti-CD69–FITC (H1.2F3), anti-CD44–FITC (IM7), anti-CD45RB–PE (16A) and anti-LFA1–FITC (2D7) were purchased from BD Biosciences PharMingen (San Diego, CA). Purified anti-CD3 ε (2C11), anti-IFN-γ (R4-6A2) and anti-IL-12 (C17.8) were purchased from BD Biosciences PharMingen.

Subcloning of TCR

Total cellular RNA was isolated from BP1 by using acid guanidinium–phenol–chloroform method. cDNA was synthesized with random hexamer primers and superscript II cDNA kit (GIBCO BRL, Grand Island, NY). 5′-Rapid amplification of cDNA end (5′-RACE) was performed using the 5′-RACE System Ver.2.0 (Life Technologies, Rockville, MD) according to the manufacturer's instructions. The first strand of cDNA was synthesized with gene-specific primer 1 (5′-ATCCATAGCTTTCATGTCCA for TCR α-chain and 5′-GCCATTCACCCACCAGCTCA for TCR β-chain). The first PCR amplification was carried out by using gene-specific primer 2 (5′-GCGAATTCTGAGACCGAGGATCTTTTAACTGGTAC for TCR α-chain and 5′-GCGTCGACTCTGCTTTTGATGGCTCAAAC for TCR β-chain). The second PCR amplification was carried out with nested gene-specific primer (5′-GCGTCGACACAGCAGGTTCTGGGTTCTGGAT for TCR α-chain and 5′-GCGTCGACAAGGAGACCTTGGGTGGAGTCAC for TCR β-chain). The PCR fragment was subcloned in Bluescript SK^+ and sequences by automatic DNA sequencer (ABI PRISM 3700 DNA analyzer, Applied Biosystems, Foster City, CA).

Retrovirus-mediated gene transfer

Full length cDNAs genes encoding the TCR α- and β-chains of BP1 were inserted into a retroviral vector pMX-IRES-GFP vector, pMX-BP1-α and pMX-BP1-β, respectively, and were transfected into a retroviral packaging cell line, PLAT-E with LipofectAMINE Plus Reagent (GIBCO BRL) (28) The cultured supernatant of PLAT-E after 24 h culture was collected, and added to TG40 cells together with DOTAP Liposomal Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) (29). Transfection was monitored by the cell surface expression of TCR by FACS analysis. TG40 cells were transfected with each of plasmids or in their combinations

and selected TG40 cells expressing TCR-αβ (TG40-BP1). TG40-BP1 cell line for expression of CD4 (TG40-BP1/CD4) was established by electroporation of the expressible constructs of full length CD4 cDNA into TG40-BP1 cells by Gene Pulser (Bio-Rad laboratories, Hercules, CA).

Establishment of transgenic mice

The transgenic TCR-α and -β genes were isolated from BP-1 as described in the previous session. The DNA sequences of the PCR products revealed that BP1-TCR-α was composed of Vα5, Jα15 and Cα1, and the TCR-β chain of Vβ11, Jβ2.3 and Cβ2. The pHSE3' plasmid contains the H-2K^b promoter (provided by H. Pircher), a poly(A) signal from β-globin and the immunoglobulin heavy chain enhancer (30). The full-length BP1 TCR α and β cDNAs were subcloned into the *Sal*I and *Bam*HI sites of the expression vector pHSE3' plasmid under control of the H-2K^b promoter. The constructs were excised from these plasmids by *Xho*I cleavage for TCR-α chain and *Apa*LI cleavage for TCR-β, and purified by using QIAEX II gel extraction system (Qiagen Inc., Valencia, CA). The purified expression constructs for TCR α and β cDNAs were co-injected into fertilized eggs of C57BL/6 mice. We finally obtained a TCR-Tg line of mice expressing TCR-Vα5-Vβ11 (P25 TCR-Tg). P25 TCR-Tg mice were bred to STAT1 deficient mice (STAT1 deficient P25 TCR-Tg) on a C57BL/6 background in our animal faculty under specific pathogen-free conditions.

Preparation of naive CD4$^+$ T cells and APC

Splenic T cells from either P25 TCR-Tg or littermate C57BL/6 mice were enriched by passing splenocytes through a nylon wool column. To further purify primary CD4$^+$ T cells, the splenic T cells were incubated with a mixture of Microbead-bound monoclonal antibodies that were specific for CD8 (53-6.72), CD49b (DX5), B220 (RA3-6B2) and I-A^b (M5/114.15.2) (Miltenyi Biotec, Bergisch Gladbach, Germany). MEL-14^{high} T cells were purified from splenic CD4$^+$ T cells by positive sorting using MACS after treatment with anti-CD62L (MEL-14)-Microbeads (Miltenyi Biotec) and were used as naive CD4$^+$ T cells. The purity of CD4$^+$ naive T cells was >98%. Splenocytes from wild-type (WT) C57BL/6 mice were incubated with a mixture of anti-Thy1 (30-H12)-Microbeads and anti-CD49b-Microbeads (Miltenyi Biotec) to deplete T cells and NK cells. Cells were then recovered by passage through a MACS column according to the manufacturer's instructions. Recovered cells were irradiated with a total of 3500 Rad, and used as APC. I-A^b-CHO was incubated with 10 μg/ml Peptide-25 for 12 h and extensively washed and incubated with 50 μg/ml mitomycin C for 15 min in 37°C and used as APC in some experiments.

Cell culture

Stably transfected TG40-BP1 or TG40-BP1/CD4 cells (1 × 10^4/culture) were stimulated with various concentrations of peptide in the presence of irradiated spleen cells (5 × 10^5/culture) from various strains of mouse in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The cultured supernatants were collected and subjected to ELISA.

To examine Th differentiation *in vitro*, two-step cultures were employed. For the first culture, purified splenic naive CD4$^+$ T cells (5 × 10^5/culture) were activated for 6 days with 10 μg/ml of anti-CD3 or 10 μg/ml Peptide-25 or its substituted mutant in the presence of T- and NK cell-depleted C57BL/6 splenic APC (2.5 × 10^6/culture) in a 48-well plate. In some experiments, we used Peptide-25 loaded I-A^b-CHO (2.5 × 10^5/culture) as APC. For the second culture, the cells collected from the first culture were extensively washed and dead cells were removed by centrifugation through Ficol–Hypaque gradients. The viable primed CD4$^+$ T cells were re-stimulated with 10 μg/ml of anti-CD3 or 10 μg/ml of Peptide-25 in the presence of splenic APC or 1 μg/well of immobilized anti-CD3.

Intracellular cytokine staining and FACS analysis

We identified cytokine-producing cells by cytoplasmic staining with anti-cytokine antibody as previously described (24). First, 2 μM of Monensin (BD Biosciences PharMingen) was added to the secondary culture for the last 4 h of each stimulation. The cells were harvested at 24 h of the secondary culture and stained with 7-amino-actinomycin D and with anti-Vβ11–PE or anti-CD4–PE. The cells were fixed with 4% formaldehyde after washing with 0.05% azide–1% FCS–PBS, permeabilized with 0.1% saponin, and stained with both anti-IFN-γ–FITC and anti-IL-4–allophycocyanin. Isotype-matched control antibodies were also used. The cells stained were gated on live Vβ11- or CD4-positive cells and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

ELISA

Amounts of IL-2, IL-4 and IFN-γ in the culture supernatant were measured by ELISA. All monoclonal antibodies specific for mouse IL-2, IL-4 and IFN-γ used for capture and detection of cytokines were purchased from BD Biosciences PharMingen. ELISA was performed following the instruction of BD Biosciences PharMingen.

ELISPOT assay

Cytokine producing cells were identified by ELISPOT assay, using the IFN-γ and IL-4 ELISPOT assay kits (R&D Systems, Minneapolis, MN). After naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO for 20 h in a 96-well plate coated with capture antibodies, ELISPOT assay was performed following the manufacturer's instructions. Spots were analyzed by KS ELISPOT compact (Carl Zeiss, Oberkochen, Germany).

Results

Analysis of Peptide-25 recognition by reconstituted TCR-αβ pairs

To investigate the functional TCR able to bind a Peptide-25/MHC complex at the clonal level, we first determined the usage of TCR-α and -β chains of Peptide-25-reactive Vβ11$^+$ Th1 clone (BP1) that was of C57BL/6 (I-A^b) mouse origin (23) with the use of 5'-RACE. BP1-TCR α-chain was found to be composed of Vα5 and Jα15 and Cα (Accession No.: AB183189). BP1-TCR β-chain was also identified to be Vβ11, Jβ2.3 and Cβ2 (Accession No.: AB183190).

In order to analyze Peptide-25-recognition by TCR dimers composed of the TCR α- and β-chains of BP1, TCR α- and

β-chain were subcloned into a retrovirus vector and then transfected by retrovirus-mediated gene transfer into a TCR-αβ- and CD4-deficient recipient T cell hybridoma cell line, TG40 (28), and the reconstruction and functional specificity of the TCR was assessed by measuring IL-2 production (Fig. 1). TG40-BP1 produced substantial amounts of IL-2 in response to Peptide-25 plus APC in a dose dependent manner. Enforced expression of CD4 molecules on TG40-BP1 (TG40-BP1/CD4) augmented IL-2 production even upon a lower dose of Peptide-25 stimulation (0.3 μg/ml). TG40-BP1/CD4 produced much more IL-2 than TG40-BP1 when stimulated with higher concentrations of Peptide-25 (10 μg/ml) (Fig. 1A). TG40 transfectants of TCR-α alone or TCR-β alone did not respond to Peptide-25 in the presence of splenic APC (data not shown). These results indicate that recombinant TCR α- and β-chains can reconstruct functional TCR and recognize Peptide-25/I-A^b complex to become IL-2-producing cells.

The specificity of BP1 TCR for Peptide-25 and splenic APC from C57BL/6 mice was examined by culturing TG40-BP1/CD4 with various I-A^b-binding peptides in the presence of APC from different strains of mice. Although we do not show data here, among the various peptides only Peptide-25 could induce IL-2 production by TG40-BP1/CD4 in the presence of splenic APC from C57BL/6 (I-A^b) mice. The 11-mer from Peptide-25 was stimulatory while the 8-mer from Peptide-25 was ineffective. We then stimulated TG40-BP1/CD4 cells with a mutant of Peptide-25 as an altered peptide ligand (APL). The APL preserves those amino acid residues within Peptide-25 essential for I-A^b binding, while one of TCR-binding amino acid residues, glutamic acid at position 248 of Peptide-25, was substituted to alanine, G248A. The APL stimulation at 10 μg/ml of TG40-BP1/CD4 induced marginal IL-2 production, and the stimulatory activity was much lower than with Peptide-25 (Fig. 1B).

We then determined the amino acid sequences for the TCR-α and -β chains of four other Peptide-25-reactive Th1 clones (BP4, BM5, BM7 and BM12). All these Th1 clones responded to Peptide-25 for proliferation and IFN-γ production (23). Analysis of the TCR-α and -β chain amino acid sequences for each clone revealed no obvious differences from BP1 except in the sequence and in the length of CDR3 regions of TCR α- and β-chain (Supplementary table 1, available at *International Immunology* Online). Taking all these results together, the TCR-α5 and -β11 can reconstitute a functional TCR complex that is able to recognize and respond to Peptide-25 when presented in the context of I-A^b. As BP1 is the best Peptide-25-reactive Th1 clone with respect to IFN-γ production in response to Peptide-25, we chose BP1 TCR cDNAs for generating P25 TCR-Tg mice.

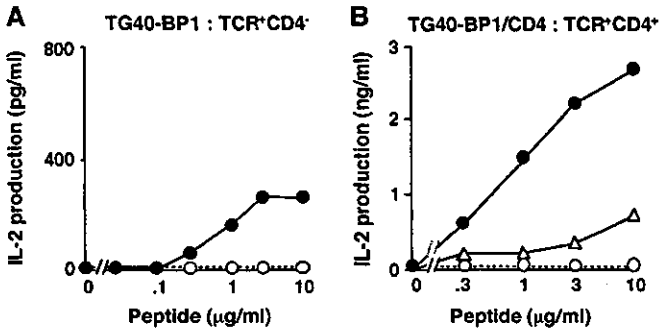

Fig. 1. IL-2 production of TG40 transfectants upon stimulation with Peptide-25. TG40 transfectants retrovirally introduced TCR-αβ of BP1, TG40-BP1 (TCR$^+$ CD4$^-$) and TG40-BP1 transfectants of CD4, TG40-BP1/CD4 (TCR$^+$ CD4$^+$) (1 × 10^4 cells/culture) were stimulated with various concentrations of Peptide-25 in the presence (closed circles) or absence (open circles) of irradiated C57BL/6 spleen cells (5 × 10^5 cells/culture) as APC in 96-well microplates. We also stimulated TG40-BP1/CD4 with APL (triangles) in the presence of C57BL/6 spleen cells (5 × 10^5 cells/culture) as APC. After incubation for 24 h, IL-2 in the cultured supernatants were titrated by ELISA.

Generation of Peptide-25-reactive TCR-Tg mice

We then analyzed the clonal basis of preferential Th1 development by single TCR-Tg mice line expressing TCR-α5 and -β11. We constructed transgenes for TCR α5- and β11-chains under the control of the H-2K^b promoter, the poly(A) signal from human β-globin gene and the immunoglobulin heavy chain enhancer. The transgenes were excised from the vector sequences and co-microinjected into fertilized eggs from C57BL/6 mice. Transgenic mice were screened by Southern blot analysis of tail DNA and by staining peripheral blood T cells with anti-Vβ11, followed by FACS analysis. We obtained founder mice expressing Vα5$^+$-Vβ11$^-$, Vα5$^-$-Vβ11$^+$ and Vα5$^+$-Vβ11$^+$ T cells. In the present study, we have mainly analyzed TCR transgenic (P25 TCR-Tg) mice expressing both TCR-Vα5 and -Vβ11.

FACS analysis revealed that >85% of splenic CD4$^+$ T cells from the P25 TCR-Tg mice expressed TCR β11-chain, while 5–7% of splenic CD4$^+$ T cells were Vβ11$^+$ in WT mice (22). Over 98% of splenic CD4$^+$ T cells from the RAG-2 deficient P25 TCR-Tg mice expressed TCR Vβ11-chain. Similar results were obtained by staining splenic CD4$^+$ T cells from P25 TCR-Tg mice with anti-idiotypic antibody (KN7) for the recombinant TCR αβ (A.K. and K.T., unpublished observation). We did not observe any significant KN7$^+$ lymph node cells from transgenic mice expressing TCR α-chain alone or β-chain alone. We compared the expression patterns of LFA-1, CD25, CD28, CD44, CD45RB and CD69 on splenic CD4$^+$ T cells from P25 TCR-Tg mice with those from WT mice. There were no significant differences in the expression pattern or mean fluorescence intensity of these cell surface molecules between the two groups. RT-PCR analysis revealed that T-bet and IFN-γ mRNA expressions were not detected in freshly prepared splenic CD4$^+$ cells of P25 TCR-Tg mice. Taking these results together, CD4$^+$ T cells from P25 TCR-Tg mice are not pre-activated *in vivo*.

Naive CD4$^+$ T cells from P25 TCR-Tg mice are able to differentiate into both Th1 and Th2

Naive CD4$^+$ T cells from P25 TCR-Tg and WT mice were purified from the spleen and stimulated *in vitro* with anti-CD3 in the presence of exhaustively T- and NK cell-depleted irradiated C57BL/6 splenocytes as APC. After 6 days in culture, the proliferated cells were harvested and re-stimulated for another day with anti-CD3 in the presence of APC. After culturing, IFN-γ- and IL-4-producing cells were analyzed by intracellular staining. The cultured supernatants were subjected to ELISA assay for cytokine titration. The results revealed that *in vitro* stimulation of naive CD4$^+$ T cells from

P25 TCR-Tg mice with anti-CD3 induced the propagation of both IFN-γ- and IL-4-producing cells to a similar extent as from WT mice (Fig. 2A). The IFN-γ and IL-4 production were confirmed by ELISA (Fig. 2B). It is also evident from Fig. 2 that P25 TCR-Tg T cells has a higher proportion of IFN-γ-producing cells and IFN-γ production upon anti-CD3 stimulation compared with T cells from WT mice. These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can differentiate into both Th1 and Th2 upon TCR cross-linking.

Induction of naive CD4$^+$ T cells from P25 TCR-Tg mice to Th1 differentiation upon Peptide-25 stimulation

To examine the differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon *in vitro* Peptide-25 stimulation, naive CD4$^+$ splenic T cells were purified from P25 TCR-Tg mice and stimulated *in vitro* for 6 days with Peptide-25 in the presence of T and NK cell-depleted irradiated C57BL/6 splenocytes as APC. The activated cells produced IL-2 and proliferated upon Peptide-25 stimulation in a dose dependent manner in the presence of APC, but they did not produce IL-2 in the absence of Peptide-25 or in the presence of APC from strains of mice other than C57BL/6 mice (data not shown).

In another set of cultures, we stimulated naive CD4$^+$ T cells from P25 TCR-Tg mice *in vitro* with Peptide-25. After 6 days in culture, the proliferated cells were re-stimulated for another day with immobilized anti-CD3. After culturing, IFN-γ- and IL-4-producing cells were analyzed by cytoplasmic staining, followed by FACS analysis. The cultured supernatants were subjected to ELISA for titration of cytokine levels. As a control, we also cultured the cells with APL or medium alone. Naive CD4$^+$ T cells stimulated with Peptide-25 in the presence of splenic APC became solely IFN-γ-producing cells under neutral conditions (Fig. 3A). IFN-γ production was detected on the first day of culture and increased for the rest of the culture period at day 5 (data not shown). IL-4 secretion was not detected even after 5 days of culture. Importantly, stimulation of the cells with APL, in place of Peptide-25, solely induced IL-4-producing cells (Fig. 3B). When we cultured naive CD4$^+$ T cells and splenic APC in the absence of Peptide-25 or APL in the primary culture, cells did not proliferate well (data not shown). These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can be activated leading to proliferation and differentiate solely into Th1 cells upon stimulation with Peptide-25 under neutral conditions.

Roles of IFN-γ/STAT1 and IL-12 signaling in the Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice

It is well known that in addition to the TCR signals IFN-γ and IL-12 play an important role in the Th1 development. To examine whether IFN-γ and IL-12 are required for Th1 development, we

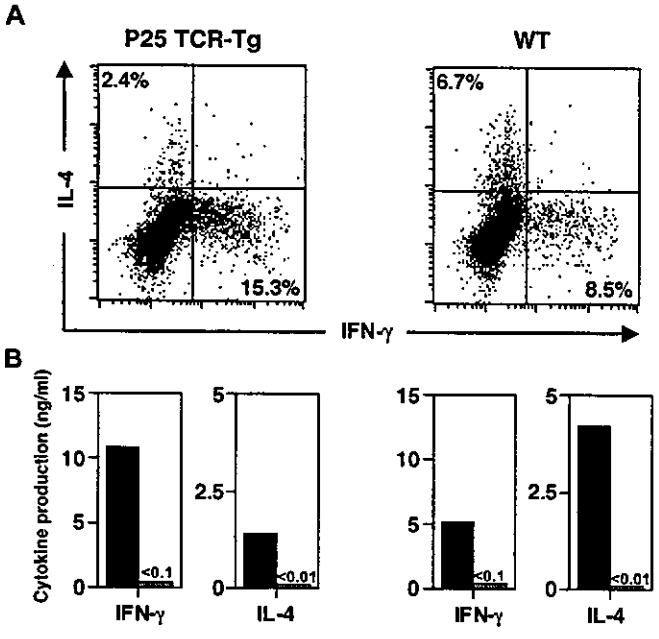

Fig. 2. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg and WT mice were purified and cultured with 10 μg of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for 6 days. (A) After the culture, the cells were washed extensively and re-stimulated with 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) After the culture, the cells were washed extensively and re-stimulated with (black bar) or without (hatched bar) 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

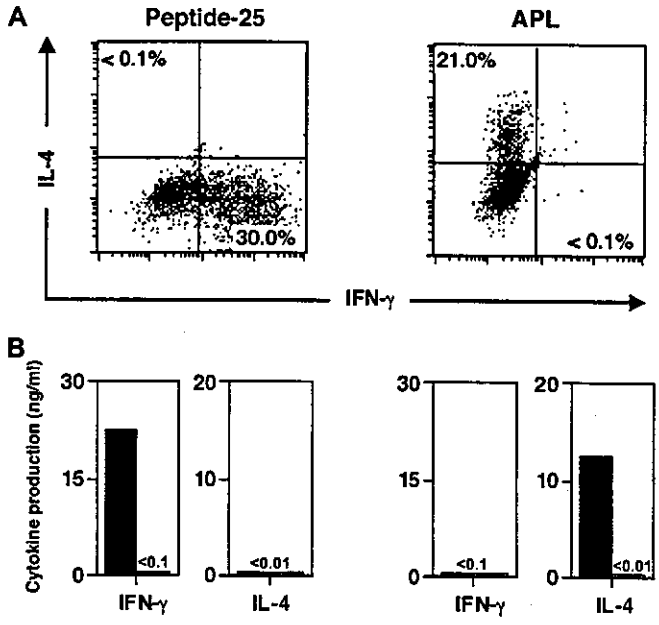

Fig. 3. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25 and APL, respectively. Naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 or APL for 6 days. (A) On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) On day 6, the cells were washed and re-stimulated with (black bar) or without (hatched bar) 1 μg/well of immobilized anti-CD3 for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

cultured naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25 and splenic APC in the presence of anti-IFN-γ, anti-IL-12 or anti-IFN-γ and anti-IL-12 for 6 days. Results revealed that IFN-γ-producing cells were predominantly observed even when cultured in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). It was also evident that addition of anti-IL-12 partially reduced the proportion of IFN-γ-producing cells without enhancing IL-4-producing cells, while the addition of anti-IFN-γ treatment slightly increased the frequencies of both IFN-γ- and IL-4-producing cells. These results imply that IFN-γ and IL-12 are not essential for Th1 development of CD4$^+$ T cells from P25 TCR-Tg mice in response to Peptide-25. To evaluate further the role of IFN-γ in the Th1 development, we examined the differentiation fate of naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice upon Peptide-25 stimulation. This result revealed that Peptide-25-stimulated naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice became solely IFN-γ-producing cells after 6 days of culture under neutral conditions (Fig. 5).

Induction of IFN-γ-producing cells upon stimulation of naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO

To elucidate the mechanism that ensures Th1 differentiation upon TCR stimulation with peptide/MHC, naive CD4$^+$ T cells were stimulated *in vitro* with Peptide-25-loaded I-A^b-CHO for 20 h and assayed for IFN-γ and IL-4 production by ELISPOT assay. IFN-γ-producing cells were induced upon treatment with Peptide-25-loaded I-A^b-CHO stimulation in a dose-dependent manner; however, IL-4-producing spots were not detected. Neither IFN-γ nor IL-4 spots were detected when naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured *in vitro* without Peptide-25-loaded I-A^b-CHO for 20 h. These results indicate that activated CD4$^+$ T cells stimulated with Peptide-25/I-A^b produced IFN-γ in primary culture within 24 h.

To evaluate the role of IFN-γ and IL-12 in Th1 development, naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence of anti-IFN-γ and anti-IL-12. At 24 h after the re-stimulation with immobilized anti-CD3, the frequency of IFN-γ producing cells was 14.5% for the live CD4$^+$ T cells (13% for the live TCRVβ11$^+$ T cells) (Fig. 6), indicating that naive CD4$^+$ T cells can differentiate into Th1 by TCR activation with Peptide-25/I-A^b stimulation even in the absence of IFN-γ and IL-12. In separate experiments, we confirmed IFN-γ-producing cells when CD4$^+$ naive T cells from RAG-2$^{-/-}$ P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO even in the presence of anti-IFN-γ or anti-IL-12. Therefore, direct interaction between Peptide-25/I-A^b and TCR may determine the fate of naive CD4$^+$ T cells for differentiating into Th1 subsets.

Discussion

Peptide-25 is the major antigenic epitope for Ag85B of *M. tuberculosis*, is immunogenic in C57BL/6 (I-A^b) mice, and preferentially induces Vβ11$^+$ Th1 cells. It remains unclear why Peptide-25 can preferentially induce Th1 immune responses in C57BL/6 mice. We approached this question by analyzing naive CD4$^+$ T cells from transgenic mice, whose T cells express functional TCR capable of recognizing Peptide-25 in the context with I-A^b molecules. In the present study we generated TCR-Tg mice for the Th1-inducing peptide, Peptide-25, to elucidate the role of TCR signals in the decision of CD4$^+$ T cells to development into either a Th1 or Th2 cell. Our data support the notion that TCR signals may play a role in the determination of Th1 development under neutral conditions in the absence of IFN-γ or IL-12.

We determined usage of TCR α-chain in five different Peptide-25-reactive Vβ11$^+$ Th1 clones. All Peptide-25-reactive Vβ11$^+$ Th1 clones expressed Vα5, while each clone showed slightly different amino acid sequences in CDR3 regions of both Vα5 and Vβ11 chains (Supplementary table 1). Although each Th1 clone responds to Peptide-25 to a similar extent with regard to proliferation and IFN-γ production, it responds differently to a mutant of Peptide-25 where an amino acid required for TCR-binding had been substituted to alanine (data not shown). However, this may be due to the heterogeneity of the CDR3 regions of both Vα5 and Vβ11 chain. TG40 transfectants (TG40-BP1) expressing α and β chains from the BP1 clone constructed functional TCRs that recognize Peptide-25 in the context of I-A^b on APC resulting in IL-2 production even in the absence of CD4 expression (Fig. 1A). Enforced expression of CD4 in TG40-BP1 enhanced IL-2 production along with a low dose of Peptide-25 stimulation (Fig. 1B), suggesting that the avidity of the TCR and Peptide-25/I-A^b complex is potent enough to trigger TG40-BP1

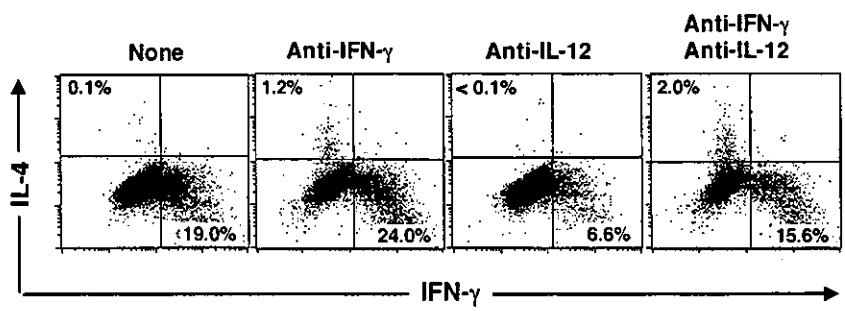

Fig. 4. Effect of anti-IFN-γ and anti-IL-12 on Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4$^+$ T cells in the spleen of P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. Anti-IFN-γ (10 μg/ml), anti-IL-12 (10 μg/ml) or anti-IFN-γ (10 μg/ml) plus anti-IL-12 (10 μg/ml) were added at the onset of culture. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day in the absence of antibodies. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

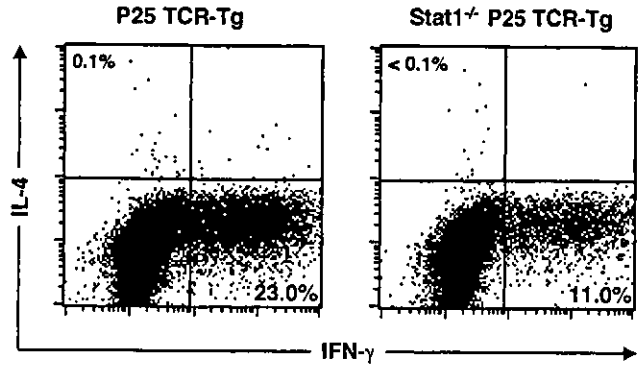

Fig. 5. Induction of Th1 differentiation of naive CD4+ T cells from STAT1 deficient P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4+ T cells in the spleen from STAT1 deficient P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

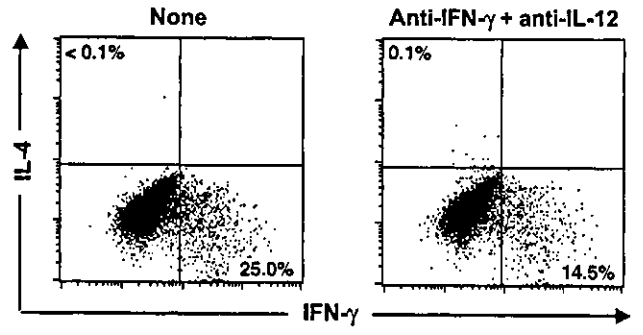

Fig. 6. Induction of Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25-loaded I-A^b-CHO. Naive CD4+ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence or absence of anti-IFN-γ and anti-IL-12. Six days after the culture, the proliferated cells were harvested and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h and subjected to cytoplasmic staining for IFN-γ and IL-4. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

transfectants. CD4 expression may facilitate the interaction between TG40-BP1 and APC, resulting in augmented IL-2 production. Intriguingly, the APL could stimulate TG40-BP1/CD4 IL-2 production to a much lesser extent even at higher peptide concentrations (Fig. 1B). As APL fully preserves the I-A^b-binding amino acids of Peptide-25, the APL/I-A^b complex may have lower avidity for the TCR compared with Peptide-25.

Expression profiles of cell surface activation markers on splenic T cells from P25 TCR-Tg mice were similar to these from WT mice, and mRNA expression of neither T-bet nor IFN-γ was observed, suggesting that CD4+ T cells in P25 TCR-Tg mice are not pre-activated. Naive CD4+ T cells from P25 TCR-Tg mice could differentiate into IFN-γ- and IL-4-producing cells upon anti-CD3 stimulation (Fig. 2), indicating that they keep their potential to differentiate into either Th1- or Th2-lineage cells upon TCR ligation. Interestingly, naive CD4+ T cells differentiated solely to IFN-γ-producing cells, but not to IL-4-producing cells upon Peptide-25 stimulation (Fig. 3). This preferential Th1 differentiation induced by Peptide-25 stimulation was also dependent on APC from C57BL/6 mice. As we described, stimulation of naive CD4+ T cells from P25 TCR-Tg mice with Peptide-25 at 10 μg/ml (6.0 μM) preferentially induces Th1 development. In contrast, when we stimulated the T cells with Peptide-25 at 0.1 μg/ml (0.06 μM), we observed a Th2-dominant response (data not shown). These observations are consistent with the published data (31) addressing that IFN-γ production is preferentially induced at 1.6–6.2 μM of OVA peptide in the OVA TCR-Tg mouse model. These results further support the notion that the Peptide-25 has an intrinsically highly potential to induce Th1. Intriguingly, stimulation with APL in place of Peptide-25 induced solely IL-4-producing cells (Fig. 3). When we analyzed APC cell surface marker expression after stimulation with either Peptide-25 or APL, we did not observe an activation-dependent alteration of cell surface marker expression such as CD80, CD86, or CD40 (data not shown). The differences between Peptide-25 and APL regarding Th1 and Th2 differentiation may be due to differences in avidity between Peptide-25/I-A^b and APL/I-A^b to TCR.

Differentiation of naive CD4+ Th precursors to Th1 and Th2 is affected by the manner and environment that they encounter (2,32,33). The strength of interaction between the TCR and MHC/peptide complex affects the lineage commitment of Th cells (15,17,31,34). It is well known that Th1 cell development involves IFN-γ signaling through STAT1 and IL-12 signaling through STAT4 activation (35,36). Peptide-25-induced Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg mice was observed even in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). We obtained similar results using T cells of STAT1 deficient P25 TCR-Tg mice (Fig. 5). This indicates that both IFN-γ/STAT1 and IL-12 signals are not essential for preferential induction of P25 TCR-Tg naive CD4+ T cells to Th1.

The activation and differentiation of naive CD4+ T cells appears to require at least three separate signals. The first signal is delivered through the TCR/CD3 complex after its interaction with MHC/peptide complex on APC. The second signal is provided by a number of co-stimulatory or accessory molecules on the APC that interact with their ligands on T cells such as CD28/CD80/86, CTLA-4/CD80/86, LFA-1/ICAM-1, OX40/OX40L or ICOS/B7h (37–43). The dose or antigen concentration is also important in determining the Th1-dominated immune response. Third, cytokines such as IFN-γ, IL-12 or IL-18 play a role in the expansion of the committed Th1 cells (10,11,44–46). Stimulation of naive CD4+ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO in primary culture lead to lower proliferation and cell recovery after culturing compared to stimulation with Peptide-25-loaded splenic APC (data not shown). Interestingly, anti-CD3 stimulation of the T cells, recovered from culture with Peptide-25-loaded I-A^b-CHO, could induce Th1 development preferentially as shown in T cells stimulated with Peptide-25 and splenic APC in primary culture (Fig. 6). As Chinese hamster ovary cells do not express detectable levels of CD80, CD86, ICAM-1, OX40L or B7h, we are in favor of the hypothesis that preferential induction of Th1 development in P25 TCR-Tg naive CD4+ T cells may be independent of these well-known co-stimulating signals from APC.

A complex network of gene transcription events is likely to be involved in establishing an environment that promotes Th1 development. T-bet, a recently discovered member of T-box transcription factor is expressed selectively in thymocytes and Th1 cells, and controls the expression of the hallmark Th1 cytokine, IFN-γ (47). T-bet expression correlates with IFN-γ expression in Th1 and NK cells. Ectopic expression of T-bet both transactivates the IFN-γ gene and induces endogenous IFN-γ production (47). T-bet appears to initiate Th1 lineage development from naive Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs (47). It has been reported that T-bet is regulated by IFN-γ signaling through STAT1 activation in the context of TCR ligation (10,11) and induces chromatin remodeling of the *ifn*-γ locus (48). As naive $CD4^+$ T cells are capable of differentiating into IFN-γ producing cells even in the presence of anti-IFN-γ, the interaction between Peptide-25/I-A^b and TCR may directly induce T-bet that leads to Th1 differentiation. We are currently investigating T-bet expression during Th1 differentiation in P25 TCR-Tg naive $CD4^+$ T cells in response to Peptide-25-loaded I-A^b-CHO.

There are several possibilities to account for the immunogenicity and adjuvant activity of Peptide-25 for Th1 development. First, Peptide-25 may activate DCs directly or indirectly through Th cells to enhance expression of co-stimulatory molecules leading to activate Th1 precursors by enhancing well-known transcription factors such as T-bet or unidentified 'master cytokine' for Th1 development. Second, the avidity of Peptide-25 to its specific TCR would be potent enough leading to Th1 development preferentially. Third, Peptide-25 might enhance activation or selection of unidentified T cell subpopulations that suppress GATA-3 leading to Th2 development.

In conclusion, we have presented data showing that naive $CD4^+$ T cells from P25 TCR-Tg mice stimulated with Peptide-25/I-A^b that polarize to Th1 differentiation preferentially in the absence of IFN-γ or IL-12. We propose the hypothesis that direct interaction of the specific antigenic peptide/MHC class II complex and TCR may primarily influence the determination of naive $CD4^+$ T cell fate in development towards the Th1 subset. Therefore, P25 TCR-Tg mice may provide us with new insights and help us understand how Th cell fate is determined.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Acknowledgements

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Abbreviations

APL	altered peptide ligand of Peptide-25
I-A^b-CHO	Chinese hamster ovary cells expressing I-A^b
P25 TCR-Tg	TCR-Tg line of mice expressing TCR-Vα5-Vβ11
TCRVβ11	Vβ11 of TCR
TG40-BP1	TG40 cells expressing TCR-αβ
TG40-BP1/CD4	TG40-BP1 cell line for expression of CD4

References

1 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
2 O'Shea, J. J. and Paul, W. E. 2002. Regulation of TH1 differentiation—controlling the controllers. *Nat. Immunol.* 3:506.
3 Murphy, K. M. and Reiner, S. L. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2:933.
4 Szabo, S. J., Sullivan, B. M., Peng, S. L. and Glimcher, L. H. 2003. Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* 21:713.
5 Rocken, M., Muller, K. M., Saurat, J. H., Muller, I., Louis, J. A., Cerottini, J. C. and Hauser, C. 1992. Central role for TCR/CD3 ligation in the differentiation of $CD4^+$ T cells toward A Th1 or Th2 functional phenotype. *J. Immunol.* 148:47.
6 Kamogawa, Y., Minasi, L. A., Carding, S. R., Bottomly, K. and Flavell, R. A. 1993. The relationship of IL-4- and IFN γ-producing T cells studied by lineage ablation of IL-4-producing cells. *Cell* 75:985.
7 Seder, R. A. and Paul, W. E. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol.* 12:635.
8 Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A. and Murphy, K. M. 1993. Development of TH1 $CD4^+$ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260:547.
9 Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G. and Romagnani, S. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* 177:1199.
10 Lighvani, A. A., Frucht, D. M., Jankovic, D. *et al.* 2001. T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. *Proc. Natl Acad. Sci. USA* 98:15137.
11 Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive $CD4^+$ T cells. *Nat. Immunol.* 3:549.
12 Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T. and Akira, S. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380:627.
13 Shimoda, K., van Deursen, J., Sangster, M. Y. *et al.* 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380:630.
14 Kaplan, M. H., Schindler, U., Smiley, S. T. and Grusby, M. J. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4:313.
15 Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. and Bottomly, K. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med.* 182:1591.
16 Constant, S. L. and Bottomly, K. 1997. Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. *Annu. Rev. Immunol.* 15:297.
17 Tao, X., Grant, C., Constant, S. and Bottomly, K. 1997. Induction of IL-4-producing $CD4^+$ T cells by antigenic peptides altered for TCR binding. *J. Immunol.* 158:4237.
18 Huygen, K., Content, J., Denis, O. *et al.* 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat. Med.* 2:893.
19 Silver, R. F., Wallis, R. S. and Ellner, J. J. 1995. Mapping of T cell epitopes of the 30-kDa alpha antigen of *Mycobacterium bovis* strain bacillus Calmette–Guerin in purified protein derivative (PPD)-positive individuals. *J. Immunol.* 154:4665.

20 Kamath, A. T., Feng, C. G., Macdonald, M., Briscoe, H. and Britton, W. J. 1999. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. *Infect. Immun.* 67:1702.

21 Mustafa, A. S., Shaban, F. A., Abal, A. T., Al-Attiyah, R., Wiker, H. G., Lundin, K. E., Oftung, F. and Huygen, K. 2000. Identification and HLA restriction of naturally derived Th1-cell epitopes from the secreted *Mycobacterium tuberculosis* antigen 85B recognized by antigen-specific human CD4$^+$ T-cell lines. *Infect. Immun.* 68:3933.

22 Yanagisawa, S., Koike, M., Kariyone, A., Nagai, S. and Takatsu, K. 1997. Mapping of Vβ11$^+$ helper T cell epitopes on mycobacterial antigen in mouse primed with *Mycobacterium tuberculosis*. *Int. Immunol.* 9:227.

23 Kariyone, A., Higuchi, K., Yamamoto, S., Nagasaka-Kametaka, A., Harada, M., Takahashi, A., Harada, N., Ogasawara, K. and Takatsu, K. 1999. Identification of amino acid residues of the T-cell epitope of *Mycobacterium tuberculosis* α antigen critical for Vβ11$^+$ Th1 cells. *Infect. Immun.* 67:4312.

24 Kariyone, A., Tamura, T., Kano, H., Iwakura, Y., Takeda, K., Akira, S. and Takatsu, K. 2003. Immunogenicity of Peptide-25 of Ag85B in Th1 development: role of IFN-γ. *Int. Immunol.* 15:1183.

25 Ohno, H., Ushiyama, C., Taniguchi, M., Germain, R. N. and Saito, T. 1991. CD2 can mediate TCR/CD3-independent T cell activation. *J. Immunol.* 146:3742.

26 Morita, S., Kojima, T. and Kitamura, T. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7:1063.

27 Gyotoku, T., Fukui, Y. and Sasazuki, T. 1998. An endogenously processed self peptide and the corresponding exogenous peptide bound to the same MHC class II molecule could be distinct ligands for TCR with different kinetic stability. *Eur. J. Immunol.* 28:4050.

28 Kitamura, T., Koshino, Y., Shibata, F., Nakajima, H., Nosaka, T. and Kumagai, H. 2003. Retrovirus-mediated gene transfer and expression cloning, powerful tools in functional genomics. *Exp. Hematol.* 31:1007.

29 Yokosuka, T., Takase, K., Suzuki, M., Nakagawa, Y., Taki, S., Takahashi, H., Fujisawa, T., Arase, H. and Saito, T. 2002. Predominant role of T cell receptor (TCR)-α chain in forming preimmune TCR repertoire revealed by clonal TCR reconstitution system. *J. Exp. Med.* 195:991.

30 Pircher, H., Mak, T. W., Lang, R., Ballhausen, W., Ruedi, E., Hengartner, H., Zinkernagel, R. M. and Burki, K. 1989. T cell tolerance to Mlsa encoded antigens in T cell receptor Vβ8.1 chain transgenic mice. *EMBO J.* 8:719.

31 Hosken, N. A., Shibuya, K., Heath, A. W., Murphy, K. M. and O'Garra, A. 1995. The effect of antigen dose on CD4$^+$ T helper cell phenotype development in a T cell receptor-$\alpha\beta$-transgenic model. *J. Exp. Med.* 182:1579.

32 Abbas, A. K., Murphy, K. M. and Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.

33 O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275.

34 Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A. and Bottomly, K. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation *in vivo*. *J. Exp. Med.* 181:1569.

35 Pernis, A., Gupta, S., Gollob, K. J., Garfein, E., Coffman, R. L., Schindler, C. and Rothman, P. 1995. Lack of interferon γ receptor beta chain and the prevention of interferon γ signaling in TH1 cells. *Science* 269:245.

36 Brombacher, F., Kastelein, R. A. and Alber, G. 2003. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol.* 24:207.

37 Rulifson, I. C., Sperling, A. I., Fields, P. E., Fitch, F. W. and Bluestone, J. A. 1997. CD28 costimulation promotes the production of Th2 cytokines. *J. Immunol.* 158:658.

38 Kato, T. and Nariuchi, H. 2000. Polarization of naive CD4$^+$ T cells toward the Th1 subset by CTLA-4 costimulation. *J. Immunol.* 164:3554.

39 Salomon, B. and Bluestone, J. A. 1998. LFA-1 interaction with ICAM-1 and ICAM-2 regulates Th2 cytokine production. *J. Immunol.* 161:5138.

40 Smits, H. H., de Jong, E. C., Schuitemaker, J. H., Geijtenbeek, T. B., van Kooyk, Y., Kapsenberg, M. L. and Wierenga, E. A. 2002. Intercellular adhesion molecule-1/LFA-1 ligation favors human Th1 development. *J. Immunol.* 168:1710.

41 Ohshima, Y., Yang, L. P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P. and Delespesse, G. 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4$^+$ T cells into high IL-4-producing effectors. *Blood* 92:3338.

42 Akiba, H., Miyahira, Y., Atsuta, M. *et al.* 2000. Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *J. Exp. Med.* 191:375.

43 Dong, C., Juedes, A. E., Temann, U. A., Shresta, S., Allison, J. P., Ruddle, N. H. and Flavell, R. A. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97.

44 Thierfelder, W. E., van Deursen, J. M., Yamamoto, K. *et al.* 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171.

45 Kaplan, M. H., Sun, Y. L., Hoey, T. and Grusby, M. J. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174.

46 Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 19:423.

47 Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655.

48 Mullen, A. C., High, F. A., Hutchins, A. S. *et al.* 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292:1907.

特集

粘膜免疫

経粘膜ワクチン

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要旨 生体防御の最前線である粘膜局所においては，物理・化学的および生物学的バリアーの形成とともに，病原微生物と常在細菌叢の認識・識別，あるいは粘膜を介して侵襲する様々な抗原に対する正の免疫応答と負の免疫寛容という相反する対応が厳格に行われている。しかし，その制御機構をつかさどる細胞や分子は，全身免疫システムとは随分異なっていることがしだいに明らかになってきた。ヒトの生命を脅かす病原微生物感染症の多くは，皮膚を介して感染するマラリアや新生児破傷風などを除いて，消化器，呼吸器，生殖器などの粘膜を介して感染するものである。このことからも粘膜という水際で致死的な病原細菌やウイルスによる感染症と対峙している粘膜免疫システムの重要性をうかがい知ることができる。したがって，全身系の免疫システムとは似て非なる粘膜免疫システムの特徴を生かした微生物感染症やアレルギー・免疫疾患の新たな制御法の開発が大いに期待されている。

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Mucosal Vaccine

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Summary

The mucosal immune system consists of molecules, cells, and organized lymphoid structures intended to provide immunity to pathogens that impinge upon mucosal surfaces. Mucosal infection by pathogens, such as bacteria, virus, and protozoa, results in the induction of cell mediated immunity, as manifested by T helper, as well as cytotoxic T lymphocytes. These responses are normally accompanied by the synthesis of secretory immunoglobulin A antibodies, which provide an important first line of defense against invasion of mucosal surfaces by these pathogens. A new generation of live, attenuated mucosal vaccines, such as the cold-adapted, recombinant nasal influenza, can optimize this form of mucosal immune protection. Despite these advances, emerging and re-emerging infectious diseases are tipping the balance in favor of the parasite; continued mucosal vaccine development will be needed to effectively combat these new threats.

Key words: Mucosal vaccine, Mucosal immune system, Gut-associated lymphoid tissue, Secretory immunoglobulin A, Emerging and re-emerging infectious diseases

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はじめに

わが国では戦後の高度経済成長期を経て，医療・保健・福祉を取り巻く社会・経済・行政的環境が格段に整備・改善された。したがって，結核やはしかなど，過去に猖獗を極めた感染症はもはや克服された病気として，これらに対する対応を軽視する風潮が産・官・学・民のいずれの次元においても見受けられた。しかし眼を世界に転ずると，エイズ（AIDS），サーズ（SARS），エボラ出血熱，病原性大腸菌O157など，感染症が原因となる新たな疾病の脅威は枚挙に暇がない。また，もはや地球上から消滅したと考えられた天然痘が，昨今バイオ・テロリズムの手段として悪用されるという新たな脅威が生じている。このようにウイルスや細菌などの微生物による感染症は，グローバルな視点に立てば未だに深刻な問題を投げかけている人類の脅威なのである。本稿ではこのような疾病に対する予防や治療の有効な手段として注目されている体にやさしい経粘膜ワクチン研究の動向をわれわれの研究成果を踏まえて紹介したい。

I. ワクチンとは

人類の英知により病の本質に対する理解が深まるにつれ，疾病にかからない手段としての予防医学の重要性が近年とみに増している。肉体的，精神的，経済的な負担など，多くの点で予防は治療に勝る疾病対策であり，とりわけ感染症対策としてのワクチン開発は優れた科学的予防法といえる。ワクチンとは病原微生物に対する能動的あるいは受動的な特異免疫を付与する目的で投与される製剤であり，1786年に英国のJennerが天然痘を予防する目的で牛痘の病原ウイルスをPhipps少年に接種したのが予防接種のはじまりであった。この予防接種，すなわち種痘に使用した牛痘の病原体はvacca（ウシ）から採取したことにちなんでvaccine（ワクチン）と呼ばれたが，後にPasteurはこの功績を顕彰してワクチンを予防接種に利用される製剤に対して広義に用いた。世界保健機関は1979年の天然痘の根絶に引き続き，ワクチンによるポリオ，麻疹（はしか）の根絶をめざしている。このようにワクチンは予防医学のもたらした優れた生物製剤の一つといえる。また，最近はバイオ・テロリズムに対する予防・治療法としてのワクチンの重要性が注目されている。

II. 現行のワクチン

わが国で予防接種として利用されている現行のワクチンは，大別すれば弱毒生ワクチン，あるいは不活化ワクチンのいずれかであり，ポリオの経口弱毒生ワクチンを除くすべてのワクチンにおいて経皮注射による接種が実施されている。弱毒生ワクチンは自然宿主以外の宿主（鶏卵やマウスなどの動物や培養細胞）において継代を重ねて選択した弱毒変異株を用いることが一般的で，比較的自然感染（野生株の感染）に近い特異感染防御免疫を引きだすことができる。したがって，血清IgGを中心とした体液性免疫応答のみならず，ウイルス感染防御に重要な細胞傷害性T細胞（CTL）を中心とした細胞性免疫応答や結核などの細胞内寄生性細菌の防御に重要なIFN-γ産生性Tヘルパー1型（Th1）細胞の誘導が期待できる。しかしながら，自然罹患に比べて軽いが症状を引き起こすこと，さらに弱毒生ワクチン株の病原性の復帰の問題，また免疫不全者への使用が禁忌であることなどの副作用が懸念される。一方，不活化ワクチンはホルマリンなどの化学物質で処理して感染力を消失させた，不活化した形で投与するワクチン製剤であり，細菌毒素を無毒化（トキソイド）したものや病原微生物の成分を精製分離したもの，また遺伝子工学の手法を応用した組換え型ワクチンなども不活化ワクチンとみなすことができる。弱毒生ワクチンとは異なり，症状の発現や毒力を復帰することはない。しかし，十分な感染防御能を有する特異免疫応答を誘導するためには，ある程度以上の抗原量の接種が必要であり，時に免疫応答強化のための水酸化アルミニウムゲルに代表されるアジュバント（後述）の併用が要求され，細胞性免疫の誘導は期待し難い。望ましくない免疫応答を避けるためには不純物を取り除き，かつ強い免疫原性が維持されているワクチンの精製が必要な場合がある。

III. 粘膜ワクチン

前項において紹介した実用化済みのワクチンのほとんどは急性の経過をとり，かつ，終生免疫の

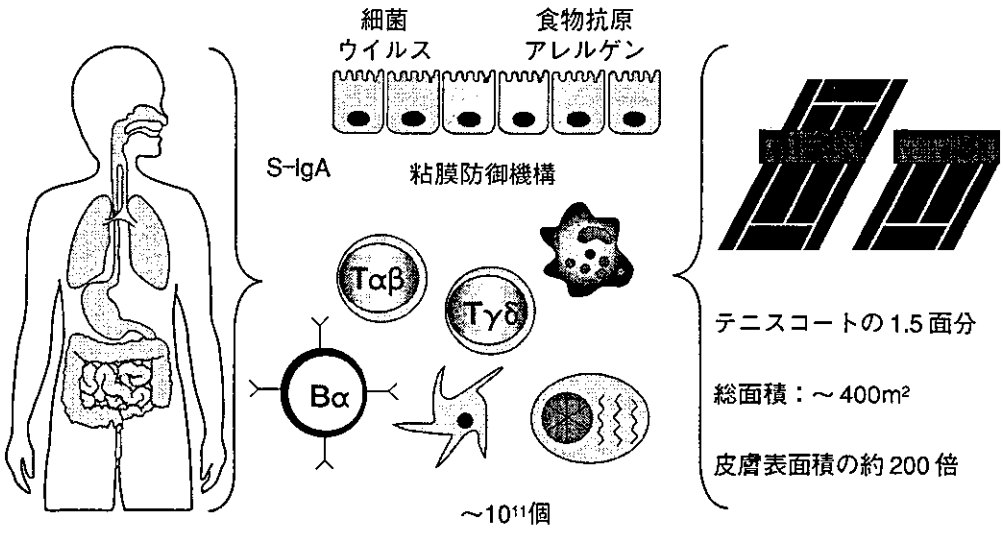

図 1　第一線の生体防御バリアーとしての粘膜免疫機構
ヒトの体は極論すればチューブ状構造物の集合体であり，その内腔を覆う粘膜の表面積はテニスコート1.5面分に相当する。天文学的数字にのぼる多様な病原微生物が侵襲する呼吸器・消化器の粘膜には人体の有する約50%の免疫担当細胞が集結している。

得られる微生物感染症に限られているが，現在世論が強く望んでいるのは，エイズや結核をはじめとする，反復再感染が常態である多くの粘膜標的感染症のワクチンの実用化である。また，理想的なワクチンが具備すべき特徴として，①被接種者すべてに効果があり，その効果の永続性が高いこと，②接種が容易で，副作用がなく安全であること，③製造や品質管理が容易で，かつコストが安いこと，などをあげることができる。マラリアや破傷風を除く，現在世界のレベルで人類の脅威となっている大部分の病原微生物感染症は，呼吸器，消化器，泌尿生殖器などを覆う粘膜面を介して感染が成立する。幸い粘膜組織には生体防御の最前線として前記病原微生物をはじめとする数多くの異物と対峙し，生体の免疫学的恒常性の維持に寄与する人体最大の免疫組織が配備されている（図1）[1,2]。

この粘膜での特異免疫誘導・制御の機構を介してワクチン抗原を投与し，粘膜組織を中心に感染防御免疫を付与するための製剤が，狭義の粘膜ワクチンである。粘膜ワクチンはその投与経路により，経口，点鼻，点眼ワクチンなどと呼称される。経口的に投与されたワクチンの多くは消化器粘膜に局在する gut-associated lymphoid tissue（GALT）において特異免疫応答を誘導する[3-6]。

わが国で実用に供されているポリオ生ワクチンを経口的に投与した場合は，小腸粘膜組織に分泌型IgA（S-IgA）を中心とした感染防御体液性免疫応答とCTLを中心とした感染防御細胞性免疫応答が誘導されることが知られている。また，流血中にもポリオウイルスに対するIgGを中心としたウイルス中和抗体が誘導され，結果的に粘膜組織のみならず全身末梢免疫系を含めた2段構えの防御免疫を誘導できる。すなわち，感染成立部位である粘膜面における水際での感染阻止に加えて，感染成立後のウイルス血症や小児麻痺の発症阻止まで期待できる（図2）。

さらに，粘膜免疫循環帰巣システム（common mucosal immune system）を介することで，ワクチンを投与した粘膜局所のみならず，遠隔の粘膜組織にまでS-IgAやCTLを中心とした特異免疫応答を誘導することが知られている。操作性，安全性などの観点でも従来の注射によるワクチン接種より格段に優れており，有効性，安全性に秀でた守備範囲の広い理想的な予防・治療ワクチンといえよう（表1）。米国ではすでに点鼻インフルエンザワクチンの実用化が進み，その使用が始まっている。

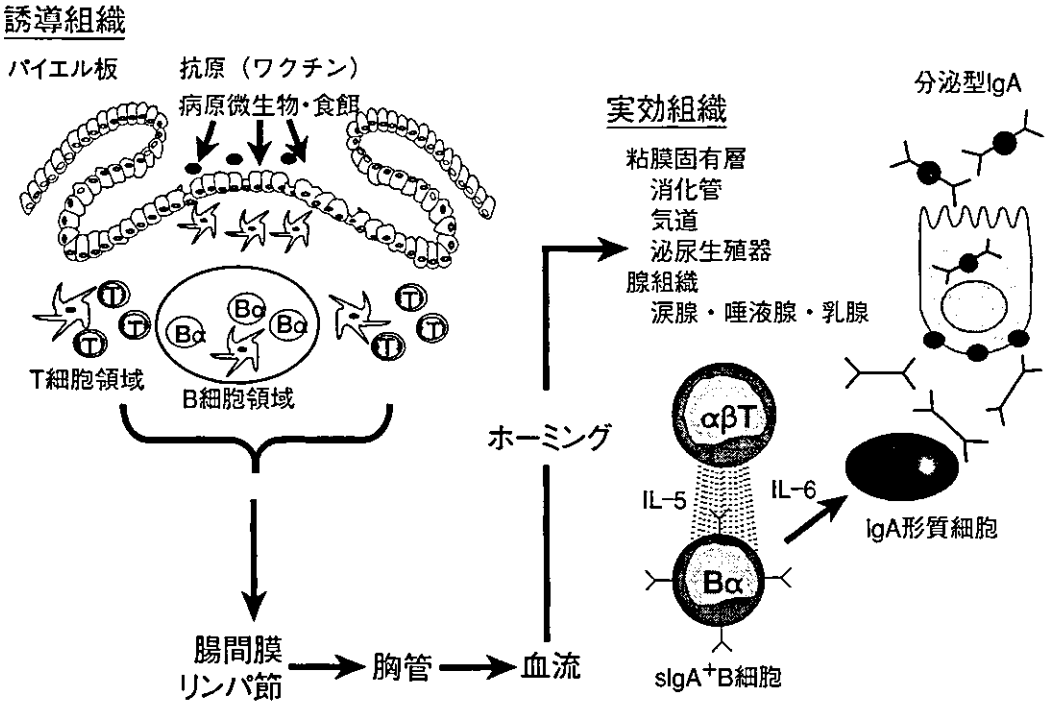

図 2 粘膜免疫機構：誘導組織と実効組織から構成された粘膜免疫誘導のための帰巣循環システム（CMIS）
消化管に取り込まれ，パイエル板に到達した粘膜ワクチンはM細胞と呼ばれる特殊な腸管の上皮細胞を介して，M細胞の直下に局在する粘膜系樹状細胞に送達される。粘膜系樹状細胞内での抗原の処理ならびに提示の過程，およびその帰結としての粘膜系樹状細胞の活性化の程度が，その後に生じるワクチン抗原特異的な感染防御免疫応答の運命を決定する。M細胞から取り込まれた抗原によって感作されたB細胞はリンパ濾胞内で分化成熟し，傍濾胞領域で感作されたT細胞とともに腸間膜リンパ節，胸管を経て血液循環に入り，再び近傍ないしは遠隔の粘膜固有層や腺組織に帰還（ホーミング）する。固有層に移動したB細胞は二量体のIgAを産生する形質細胞へ最終分化する。その後，上皮細胞を逆行輸送する際にpoly Ig受容体（分泌片）の付加を受け，最終的には二量体の分泌型IgAとして粘膜表面へ放出される。パイエル板樹状細胞によって活性化された$CD4^+$ T細胞から分泌されるサイトカインによってパイエル板 $sIgM^+$ B細胞は $sIgA^+$ B細胞に免疫グロブリンのアイソタイプを変換する。さらに粘膜固有層において，$sIgA^+$ B細胞はTh2型$CD4^+$ T細胞から分泌されるサイトカイン（IL-5, IL-6）によってIgA形質細胞へ最終分化する。また，Th1型$CD4^+$ T細胞は上皮細胞からのpoly Ig受容体（分泌成分）の産生に寄与する。

表 1 なぜ粘膜ワクチンなのか

利　点	実用化に向けて
・粘膜系と全身系免疫による2段構えの感染防御の誘導	・抗原量の検討
・分泌型IgAの誘導：病原体の粘膜への付着阻止・病原体の中和	・粘膜免疫調節因子・アジュバントの開発
・粘膜系傷害性T細胞（CTL）の誘導	・より効率的な抗原投与法・送達法の確立
・安全性が高い	
・操作性がよい（投与が簡便）	
・経済的である（費用対効果）	
・副作用が少ない	

IV.　粘膜免疫を非特異的に強化するアジュバント

経粘膜的にワクチン抗原を投与すると，粘膜のみならず脾臓に代表される全身系免疫担当組織において2段構えの抗原特異的免疫応答を誘導できること，さらに操作性，安全性などの観点においても注射による免疫より格段に優れていることが経験的に示されている。しかしながら不活化ワクチン・精製ワクチンを単独で粘膜を介して接種した場合は，粘膜組織固有の解剖学的ないしは生理学的な性状に起因する，ワクチン抗原の物理化学的な不安定性，免疫担当組織への不確実な抗原送達などの弱点により，期待したほどの特異免疫を粘膜組織に付与することができないのが実状であった。そこで，このような粘膜の特性に附随する弱点を克服し，粘膜を介した不活化・精製ワクチン抗原の送達の効率や免疫誘導効果を上げるために各種の工夫が試みられてきた。

最近，様々な細菌毒素由来の物質に粘膜免疫を介して粘膜局所のみならず全身系免疫をも非特異的に高める作用があることが明らかになってきた。その一つがコレラ毒素の粘膜アジュバントとしての利用である[7]。コレラ毒素をアジュバントとして粘膜ワクチンに併用すると，当該ワクチン抗原に対するS-IgAを主体とした粘膜系と血清IgGを主体とした全身系の両特異体液性免疫がワクチン抗原の単独投与に比べて著しく亢進することが示された。また，Th1型やCTLなどの細胞性免疫応答の亢進も粘膜組織のみならず全身組織においても確認された。このアジュバントとしての作用は蛋白質抗原のみならず，糖質，脂質，ウイルス，細菌など，幅広い抗原で認められた。

V.　粘膜アジュバントのヒトへの応用

ヒトへの応用に向けて，コレラ毒素に附随する毒素本来の活性を消失した安全な粘膜アジュバントの開発が積極的に試みられている[8]。コレラ毒素の毒素活性を担うADP-リボシルトランスフェラーゼの活性中心に変異を加えた変異型コレラトキシンは毒素活性を消失するものの，アジュバント活性は維持されていることが明らかになっている。

この無毒化変異型コレラトキシンの粘膜アジュバントとしての有用性については多種多様なワクチン，蛋白質抗原に対する免疫増強効果の検討が行われた。たとえば，肺炎双球菌次世代ワクチン抗原として期待される菌体表層蛋白質PsPAワクチンと無毒化変異型コレラトキシンを混合し，経鼻免疫する実験系で検証されている[9]。その結果，免疫動物の粘膜組織のみならず全身系において，PsPAに特異的な免疫応答の増強が観察された。さらに，その感染防御効果を致死量の肺炎双球菌の感染実験で評価したところ，変異毒素との混合ワクチン接種群において約80%のマウスが肺炎双球菌感染による致死を免れることが明らかになった。今後，この無毒化コレラトキシンのヒト粘膜アジュバントとしての実用化が期待される。さらに近年では，コレラトキシンと病原性大腸菌由来の易熱性毒素の有効成分を人為的に共存させたキメラ型粘膜アジュバントの開発が進められている。

VI.　新たな粘膜ワクチンキャリアー：膜融合リポゾーム

粘膜を介したワクチンの送達に関する前述した弱点を克服する新たなアプローチとして，センダイウイルスの膜融合能をリポゾームに付与したfusogenic liposome（膜融合リポゾーム）の応用が進められている。膜融合リポゾームは膜表面に存在するセンダイウイルス由来のエンベロープ蛋白質を利用し哺乳動物細胞に吸着・融合することで，リポゾームに封入した抗原を効率よく細胞質に導入することができる[10]。センダイウイルスの属すパラミキソウイルスは本来，上気道粘膜に感染するウイルスであることからも，この方法は経鼻ワクチン・吸入ワクチンの開発を考慮した場合，理にかなった抗原投与法といえる。モデル抗原として卵白アルブミン（OVA）を封入した膜融合リポゾームを経鼻免疫し，その後の免疫応答を検討したわれわれの実験成績によると，経鼻免疫マウスの粘膜関連リンパ組織ならびに脾臓の$CD4^+$ T細胞は試験管内における再度のOVA刺激によりIFN-γをはじめとするTh1型サイトカイン，IL-4，IL-5，IL-6などのTh2型サイトカインをバランスよく産生していること，また抗原特異的体液性免疫応答では上記の所見をよく反映して，

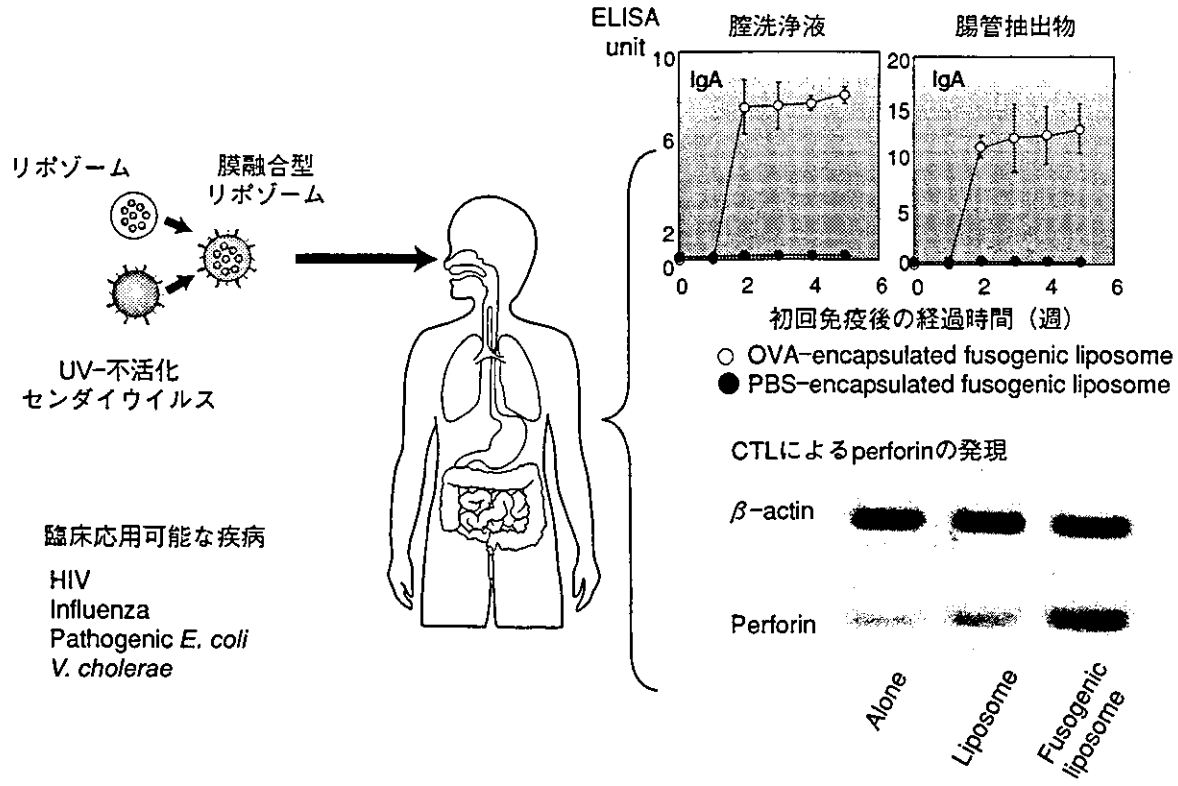

図 3 膜融合型リポゾーム経鼻粘膜ワクチンの開発
われわれは粘膜を介したより効果的なワクチン抗原の送達をめざして，センダイウイルスの膜融合能に着目し，これをリポゾームに付与した膜融合リポゾームを考案した。膜融合リポゾームは膜表面に存在するセンダイウイルス由来のエンベロープ蛋白質を利用し哺乳動物細胞に吸着・融合することで，リポゾームに封入した抗原を効率よく細胞質に導入することができる。この膜融合リポゾームに卵白アルブミン（OVA）を封入し，粘膜ワクチンとしての効果を評価した。その結果，ワクチン封入膜融合リポゾームを免疫した群では，エイズウイルスの感染が考えられる組織，腸管と生殖器などの遠隔の粘膜組織に抗原特異的な S-IgA の誘導と CTL 活性の誘導が認められた。

鼻腔洗浄液と糞便中に OVA 特異的 IgA 抗体の誘導が，また血清中では OVA 特異的 IgG 抗体産生の増強がみられた（図 3）[11]。さらに膜融合リポゾームワクチン経鼻免疫マウスでは，脾臓，腸間膜リンパ節，頸部リンパ節などの臓器・組織においてワクチン抗原特異的な細胞傷害活性を有する $CD8^+$T 細胞の誘導も観察された。

以上の結果より，膜融合リポゾームを用いて抗原を経粘膜的に投与することで粘膜面と全身の両組織において抗原特異的な細胞性ならびに体液性免疫応答を誘導できることから，膜融合リポゾームは新規の経粘膜ワクチンキャリアーとしての有用性が明確に示された。現在では，当該膜融合リポゾームは HIV ウイルスの感染予防と治療を目的とした粘膜ワクチンの投与媒体としての可能性についても検討が進んでいる。たとえば，エイズワクチン候補抗原の一つである HIV gp160 抗原を膜融合リポゾームに封入したものを経鼻免疫することで，効果的に HIV gp160 特異的免疫応答が粘膜系・全身系免疫の両方に誘導されることがわかってきた。

おわりに

近年，基礎免疫学と臨床免疫学が有機的に連携・融合した“粘膜免疫学”という新たな免疫学パラダイムの進展があり，生体防御の最前線における免疫応答の本質的な理解とその破綻に起因した疾病の病因・病態の解明，さらに合理的な治療・予防法の開発に関する研究が精力的に展開されている。この粘膜免疫学研究には既存の概念や方法論では未だに克服されていないエイズ，結核，新型インフルエンザなどの難治性の粘膜感染症の画期的な制御法の確立が託されている。

今後，新たな経口ワクチン・経鼻ワクチンに代

表される粘膜ワクチンが実用化されることによって，数多くの感染症の克服に限りない夢と希望を約束するかもしれない。たとえば米国では，噴霧式の体にやさしい経鼻インフルエンザワクチン（商品名 FluMist®）の実用化が進み，医療現場での使用が始まった。わが国でも粘膜を介して発症する感染症において注射によるワクチンから飲むワクチン・吸うワクチンへ転換することが期待されている。

さらに一歩進んで，食べるワクチンの研究も開始されている。たとえば，B型肝炎ワクチンを植物に寄生するウイルスを巧みに利用してバナナに作らせる研究や，病原性大腸菌ワクチンや齲蝕ワクチンを馬鈴薯に発現させる研究が欧米で進められている[12]。食べるワクチンは発展途上国などの経済的な事情により現行のワクチンを利用できない地域で威力を発揮するかもしれない。

文　献

1) 粘膜免疫（清野　宏，石川博通，名倉　宏・編），中山書店，東京，2001.
2) 清野　宏：粘膜免疫と経口免疫寛容．標準免疫学（谷口　克，宮坂昌之・編），医学書院，東京，2002, pp. 316-326.
3) 高橋一郎：消化管の意外な働きと経口ワクチン．粘膜免疫(清野　宏，石川博通，名倉　宏・編)，中山書店，東京，pp. 224-239, 2001.
4) 岩崎明子，高橋一郎：粘膜ワクチンは21世紀の感染症の救世主になりうるか？ アレルギー・免疫 **7** (12)：92-102, 2000.
5) 高橋一郎，朴　恩正，清野　宏：粘膜免疫担当組織細胞の特殊性と炎症性腸疾患．*Mol. Med.* **39**(9): 1000-1007, 2002.
6) Iijima, H., Takahashi, I. and Kiyono, H.: Mucosal immune networks in the gut for the control of infectious dieases. *Rev. Med. Virol.* **11**：117-133, 2001.
7) Elson, C.O. and Dertzbaugh, M.T.: Mucosal adjuvants. In Handbook of Mucosal Immunology (edited by Ogra, P.L., *et al.*), 2nd Ed, Academic Press, San Diego, 1999, pp. 817-838.
8) Yamamoto, S., Kiyono, H., Yamamoto, M., *et al.*: A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc. Natl. Acad. Sci. USA* **94**：5267-5272, 1997.
9) Yamamoto, M., Briles, D.E., Yamamoto, S., *et al.*: A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *J. Immunol.* **161**：4115-4121, 1998.
10) Nakanishi, M., Mizuguchi, H., Ashihara, K., *et al.*: Gene delivery systems using Sendai virus. *Mol. Membrane. Biol.* **16**：123-127, 1999.
11) Kunisawa, J., Nakanishi, T., Takahashi, I., *et al.*: Novel hybrid delivery system, fusogenic liposome, for the induction of mucosal and sytemic immune responses. *J. Immunol.* **167**：1406-1412, 2001.
12) Yuki, Y. and Kiyono, H.: New generation of mucosal adjuvants for the induction of protective immunity. *Rev. Med. Virol.* **13**：293-310, 2003.

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The role of allergic rhinitis in upper respiratory tract inflammatory diseases

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Summary

The number of patients with allergic rhinitis (AR) is increasing. Furthermore, patients with otitis media with effusion (OME) and chronic sinusitis (CS) are frequently complicated with AR. These findings suggest that AR has an impact on the pathogenesis of both OME and CS. The direct and indirect influence of AR on OME and CS was investigated by clinical and experimental studies to clarify the mechanism by which type I allergic reaction is associated with OME and CS. Clinical findings of patients with OME or CS complicated with AR were analysed and compared with those of nonallergic subjects. Samples such as sinus effusions and middle ear effusions (MEE) were collected from the patients and infiltration of inflammatory cells and concentrations of inflammatory cytokines determined. In addition, previous reports discussing the relationship between AR and OME or CS are reviewed. Eosinophil infiltration and oedema were remarkable in paranasal sinus mucosa of patients with CS complicated with AR, suggesting the presence of type I allergic reaction in sinus mucosa. However, there was little evidence of eosinophils in sinus effusions. Endotoxin was frequently detected in sinus effusions of patients with CS having AR as well as suppurative CS. Hypoxia was also considered an important factor inducing sinus pathology. Eosinophils and IgE antibodies in MEE were not increased in OME patients with AR. Anti-allergic medicine was effective in OME patients complicated with AR and improvement of nasal symptoms significantly correlated with that of ear symptoms. AR might be directly and indirectly associated with the pathogenesis of OME and CS.

Keywords allergic rhinitis, chronic sinusitis, eosinophil, eustachian tube, hypoxia, IgE, otitis media with effusion, vascular endothelial factor

Introduction

Otitis media with effusion (OME), chronic paranasal sinusitis (CS) and allergic rhinitis (AR) are the most common upper respiratory tract diseases in children [1]. Furthermore, AR has been implicated as a major causative factor of OME and CS due to the increased number of patients with AR complicated by OME and CS [2]. However, the precise role of AR in the pathogenesis of OME and CS is controversial.

Senturia et al. [3] reported that there is no direct scientific evidence to support the contention that middle ear effusion (MEE) is due to allergic reaction. Recent studies also demonstrated that IgE-mediated allergic reaction is not an aetiological factor but rather a persisting factor of OME due to disturbance of the clearance of MEE by the eustachian tube [4–6]. On the other hand, the presence of type I allergic reaction has been reported [7, 8] and anti-allergic drugs have proved efficacious in the successful treatment of OME in allergy-free patients [9].

Patients with AR frequently show a pathological shadow in X-ray examination of the sinuses despite a lack of infectious symptoms in nostrils such as purulent rhinorrhoea [10, 11]. Such patients are usually diagnosed as having allergic sinusitis (AS), although the definition and the pathogenesis of this disease have not been defined clearly [12, 13].

In the present study, we discuss the role of AR in the pathogenesis of OME and CS based on the results of our experiments and present a review of the literature on these issues.

Relation between AR and CS

Radiological opacity is frequently found in paranasal sinuses of patients with AR. A retrospective study of AR patients in our clinic showed that 67% of those aged < 16 years and 44% aged ≥ 16 years had some radiological opacity in maxillary sinuses [10]. Although the mechanism

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inducing sinus pathology in patients with AS is not fully understood, type I allergic response, infection and natural ostium blockade are considered the chief factors associated with the pathogenesis of this entity [14].

Type I allergic reaction in paranasal sinuses

Because remarkable eosinophilic accumulation can be observed in paranasal sinus mucosa as well as in nasal mucosa, the influence of type I allergic inflammation might be considered to be associated with the mucosal pathology of CS. Ogata et al. [15] investigated the histological differences in maxillary sinus mucosa between noninfectious CS complicated with AR and suppurative CS without AR and found that the numbers of goblet cells and eosinophils were significantly increased in the former condition. Basal membrane hyperplasia and oedema of lamina propria were also remarkable in noninfectious CS complicated with AR in comparison with suppurative CS without AR. Furthermore, the same histological findings were induced in the maxillary sinus mucosa of patients with AR caused by house dust by stimulating the mucosa with that antigen during sinus surgery [15]. These findings indicate that paranasal sinus is one of the target organs for type I allergic reaction and that type I allergic inflammation in paranasal sinuses might be associated with sinus pathology of patients with CS complicated with AR. However, the ostium connecting the nostril and paranasal sinus is too small for antigens such as mites and pollen to enter into the sinuses [16]. Moreover, autoradiographic study showed that sinus pathology does not change during the pollen allergy season, indicating that the sinus pathology was not associated with direct antigenic stimulation of sinus mucosa [13].

Infection in paranasal sinuses

Because neutrophils are frequently contaminated in nasal and sinus secretions obtained from patients with AS or suppurative CS, microbial infection seems to be involved in the pathogenesis of AS [17]. To investigate the characteristics of inflammatory cells infiltrating into paranasal sinuses of patients with AS, sinus secretions of child patients with AS complicated with OME were collected by aspiration during middle ear ventilation tube insertion under general anaesthesia and the cellular components examined. In all samples neutrophils but not eosinophils were observed (Table 1). In contrast, eosinophils were predominant in nasal secretions of patients with AR. Shirasaki et al. [18] developed an animal model of AR by sensitizing guinea pig with ovalbumin (OVA) and compared the inflammatory cells infiltrating into nasal and sinus mucosa. They found that mononuclear cells were predominant in the smears of sinus effusion from sensitized animals, although marked eosinophil infiltration and increased numbers of goblet cells were observed in nasal mucosa.

Table 1. Cell infiltration in maxillary sinus effusions obtained from child patients with allergic sinusitis

Case no.	Age (years)	Antigen	Eosinophils	Neutrophils
1	14	House dust	—	+++
2	5	House dust	—	++
3	6	House dust	—	+
4	6	House dust	—	+

Endotoxin is a cell wall component of Gram-negative bacteria and its presence in sinus secretion is considered indicative of infection of such bacteria into paranasal sinuses. In a preliminary study, we measured the concentration of endotoxin in sinus effusions and found that endotoxin was frequently detectable in sinus secretions of patients with CS. However, there was no significant difference in the concentrations of endotoxin in sinus secretion between AS and suppurative CS patients (Fig. 1). Moreover, when cultured human nasal fibroblasts isolated from nasal polyps were stimulated with endotoxin purified from nontypeable *Haemophilus influenzae*, the production of IL-8 and RANTES was remarkably enhanced (Fig. 2). As these cytokines act as chemokines specific for neutrophils and eosinophils, endotoxin present in sinus secretion of patients with AS may induce infiltration of not only neutrophils but also eosinophils into sinus mucosa.

Collectively, the above findings suggest that bacterial infection might be associated with the onset of sinus pathology in patients with AS as well as in those with suppurative CS.

Blockade of natural sinus ostium

If type I allergic reaction is related directly or indirectly to sinus pathology, the severity of AR might be correlated with the intensity of sinus opacity on X-ray examination. Pelikan et al. [19] investigated the role of AR in CS without an air–fluid level by nasal provocation tests with various inhalant allergens. Maxillary sinus radiographs performed

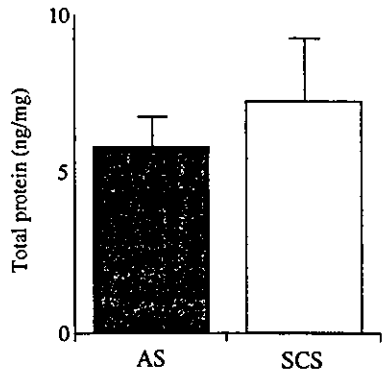

Fig. 1. Concentration of endotoxin in sinus effusion obtained from patients with allergic sinusitis (AS) and suppurative chronic sinusitis (SCS). Reprinted with kind permission from Kurono [37].