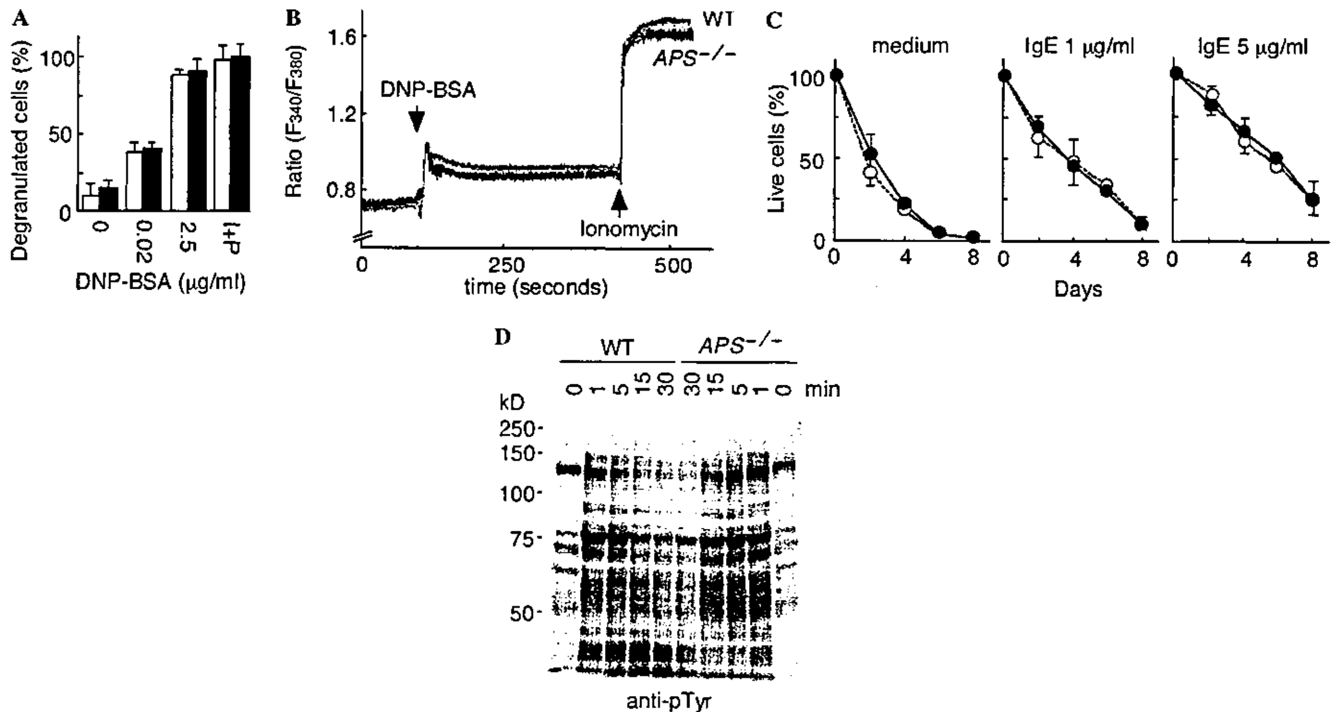

Fig. 3. Cellular responses of $APS^{-/-}$ BMMCs mediated through cross-linking FcεRI. (A) Proportion of degranulated cells after cross-linking FcεRI with various concentrations of antigens was determined by cytochemistry. Percentages of degranulated cells were comparable between $APS^{-/-}$ (closed bars) and wild-type control mice (open bars). The average ± SD of three independent experiments are shown. (B) Calcium influx induced upon cross-linking FcεRI in $APS^{-/-}$ (lower line) and wild-type (upper line) BMMCs. After IgE sensitization, BMMCs were loaded with Fura PE3 and stimulated with 5 μg/ml DNP-BSA and 10 μg/ml ionomycin at the indicated time points (arrows), and fluorescence intensity ratio at 340–380 nm was measured. Representative results of two independent experiments are shown. (C) Survival of $APS^{-/-}$ (closed circles) and wild-type (open circles) BMMCs by binding of monomeric IgE to FcεRI. Cells were cultivated in the absence or in the presence of various concentrations of monomeric IgE and percentages of live cells were measured. The average ± SD of three independent experiments are shown. (D) Tyrosine phosphorylation of total cellular proteins after cross-linking FcεRI. Sensitized BMMCs were stimulated with 2.5 μg/ml DNP-BSA for the indicated times. Total cell lysates were separated through SDS–PAGE and subjected to immunoblot using anti-phosphotyrosine mAb (4G10). Representative results of three experiments are shown.

Discussion

We investigated functions of Lnk, SH2-B or APS in mast cells, since possible regulatory roles of Lnk-family adaptor proteins in signaling through c-Kit or FcεRI had been suggested. We established BMMCs lacking either Lnk, SH2-B or APS and examined their cellular responses. None of those mutant BMMCs showed altered responses against IL-3 or SCF, the c-Kit ligand. *APS*-deficiency resulted in enhanced FcεRI-mediated degranulation, while both $lnk^{-/-}$ and $SH2\text{-}B^{-/-}$ BMMCs did not show any abnormal responses induced by cross-linking FcεRI.

We have shown that Lnk negatively regulates c-Kit signaling in B cell precursors and hematopoietic progenitor cells [8,10]. We did not observe significant enhancement in SCF-dependent growth of $lnk^{-/-}$ BMMCs in contrast to a previous report by Velazquez et al. [11]. SCF-dependent adherence was also comparable to normal cells. Expression levels of *lnk* transcripts are rather low in BMMCs compared to B-lineage cells or hematopoietic progenitor cells (unpublished data). It is likely that *lnk*-deficiency alone hardly affects mast cell function because of low expression of Lnk in mast cells.

APS had been cloned as a possible candidate substrate for the c-Kit [7]. However, $APS^{-/-}$ BMMCs did not show any altered responses upon stimulation with SCF. Instead, they showed enhanced FcεRI-mediated degranulation. $APS^{-/-}$ BMMCs showed reduced actin assembly at steady state compared to normal BMMCs. Inhibition of actin assembly in normal BMMCs by latrunculin resulted in enhanced degranulation similar to $APS^{-/-}$ BMMCs. In $APS^{-/-}$ mice, B-1 cells in peritoneal cavity increased and showed reduced F-actin contents. Conversely, in transgenic mice overexpressing APS in lymphocytes, B cells were reduced and showed enhanced actin assembly [17]. These results suggest that APS may negatively regulate degranulation process by controlling actin dynamics in mast cells. In RBL-2H3 mast cells, F-actin assembly induced by cross-linking FcεRI negatively controls degranulation as well as calcium signaling [22,23]. Oka et al. [24] recently reported that monomeric IgE binding induced actin assembly and that inhibition

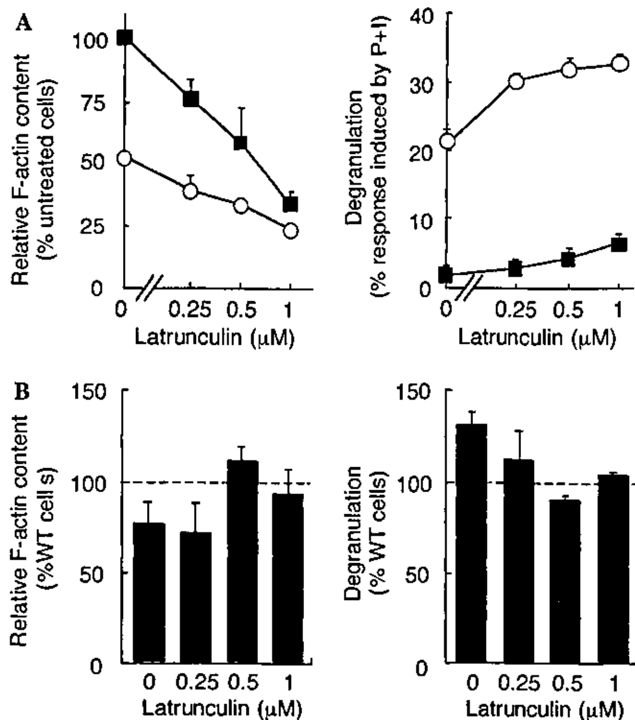

Fig. 4. Enhanced degranulation correlated with reduced F-actin contents in BMMCs treated with inhibitor of actin assembly, latrunculin or by APS-deficiency. (A) Treatment with latrunculin inhibited actin assembly and resulted in reduced F-actin content in BMMCs. Sensitized wild-type BMMCs were incubated with the various concentrations of latrunculin, kept unstimulated (squares) or stimulated with 2.5 μg/ml DNP-BSA (circles). F-actin contents of cells were then analyzed by rhodamine–phalloidin staining and flow cytometry, and the results are shown as relative F-actin contents compared with that of unstimulated cells in the absence of latrunculin (left). Degranulation was determined by measuring β-hexosaminidase activity released into culture supernatants, and results were shown as percent maximal responses induced by PMA and ionomycin treatment (right). (B) F-actin content of *APS*$^{-/-}$ BMMCs in the absence or the presence of various concentrations of latrunculin was measured and relative F-actin contents compared with those of control cells treated with the same concentrations of latrunculin were shown (left). Degranulation from *APS*$^{-/-}$ BMMCs treated with latrunculin was measured, and shown as percent reaction compared with those from wild-type control cells in the same conditions (right). Results shown are means ± SE of values obtained from three independent experiments.

of IgE-induced actin assembly by cytochalasin D initiates calcium influx and degranulation. Although enhancement of calcium influx in *APS*$^{-/-}$ BMMCs was not observed, reduction of actin assembly in *APS*$^{-/-}$ BMMCs may lead to augmented degranulation in analogy with those observed in RBL-2H3 mast cells. The molecular mechanisms for APS-mediated actin assembly as well as APS function downstream of cross-linking FcεRI remain to be elucidated.

APS function in insulin-R signaling has been also indicated in various experiments using cell lines [15,16,25–27]. *APS*$^{-/-}$ mice exhibited increased sensitivity to insulin and enhanced glucose tolerance [28]. It is intriguing to examine whether effect of *APS*-deficiency on insulin sensitivity is also mediated by actin dynamics. Regulation of actin cytoskeleton seems one of the common functions of Lnk-family adaptor proteins. Lnk associates with an actin binding protein ABP-280 [19] and facilitates actin assembly in overexpressed fibroblasts by activating Vav and Rac (S.M.K. and S.T., unpublished data). SH2-B is required for actin reorganization and regulates cell motility induced by GH-R activation [20,21].

SH2-B has been identified as a possible adaptor binding to ITAMs of FcεRI γ chain [6]. However, all examined responses induced by FcεRI ligation were normal with *SH2-B*$^{-/-}$ *BMMCs*. It seems *SH2-B*-deficiency do not affect mast cell function. However, it should be notified that interaction of SH2 domains of Lnk-family proteins with c-Kit or ITAM of FcεRI γ chain had been demonstrated in overexpression systems with different combinations, for example, SH2-B with FcεRI γ chain, APS with c-Kit. *SH2-B*$^{-/-}$ mice showed mild growth retardation and infertility due to impaired maturation of gonad organs [12]. Thus, SH2-B seemed to have a true target except FcεRI, worked as a positive regulator of signal transduction in contrast to Lnk and APS that function as negative regulators as shown in previous studies and in this study. Despite the significant structural similarities between APS, Lnk, and SH2-B, their functions appear to be quite different from each other. However, possible common functions of those adaptor proteins in vivo should be examined by generating mutant mice lacking APS, Lnk or SH2-B in various combinations.

In conclusion, our studies describe roles of Lnk family adaptor proteins on BMMCs. Both Lnk and SH2-B were dispensable for various mast cell responses mediated through c-Kit, FcεRI as well as IL-3-R. APS plays a role in controlling FcεRI-induced degranulation response but not in c-Kit-mediated proliferation or adhesion. APS may regulate degranulation by controlling actin dynamics in mast cells.

Acknowledgments

We thank our colleagues for helpful discussions, technical advices, and critical reading of the manuscript. This work was performed through Special Coordination Funds for Promoting Science and Technology, and Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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International Immunology, Vol. 15, No. 10, pp. 1183–1194
doi: 10.1093/intimm/dxg115

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Immunogenicity of Peptide-25 of Ag85B in T_h1 development: role of IFN-γ

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Keywords: Ag85B, mycobacterium, subunit vaccine, TCRVβ11, tuberculosis

Abstract

Ag85B (also known as α antigen or MPT59) is immunogenic, and induces expansion and differentiation of TCRVβ11⁺CD4⁺ T cells to IFN-γ-producing cells in C57BL/6 (I-A^b) mice. We reported that Peptide-25 (amino acids 240–254) of Ag85B is a major T cell epitope, and its amino acid residues at position 244, 247, 249 and 252 are I-A^b contact residues. Here we examined roles of IFN-γ in the generation of Peptide-25-reactive CD4⁺ TCRVβ11⁺ T cells and the efficacy of mutant peptides of Peptide-25 for T_h1 development in mice other than C57BL/6 mice. Immunization of C57BL/6 mice with Peptide-25 included in incomplete Freund's adjuvant led to preferential induction of CD4⁺ TCRVβ11⁺ IFN-γ- and tumor necrosis factor-α-producing T cells. Compared with other I-A^b-binding peptides such as Peptide-9 of Ag85B, 50V of pigeon cytochrome *c* and ovalbumin $(OVA)_{265-280}$ peptide, only Peptide-25 was capable of inducing enormous expansion of TCRVβ11⁺ IFN-γ-producing T cells. Treatment of C57BL/6 mice with anti-Vβ11 antibody before Peptide-25 immunization reduced the development of CD4⁺ IFN-γ-producing T cells. Furthermore, B10.A(3R) mice, I-A^b-positive and TCRVβ11⁻ strain, showed remarkably lower response to Peptide-25 immunization than C57BL/6 mice. Peptide-25-primed IFN-$\gamma^{-/-}$ cells showed significantly decreased expansion of CD4⁺ TCRVβ11⁺ T cells as compared with wild-type cells. Interestingly, Peptide-25-primed cells from MyD88-deficient mice responded to Peptide-25 and differentiated into IFN-γ-producing cells to a similar extent as wild-type mice, indicating Toll-like receptor-independent IFN-γ production. These results imply that IFN-γ plays important roles for the generation and expansion of CD4⁺ TCRVβ11⁺ T cells in response to Peptide-25. Although Peptide-25 was non-immunogenic in C3H/HeN mice, a substituted mutant of Peptide-25, 244D247V, capable of binding to I-A^k, induced T_h1 development. These results clearly demonstrate important roles of IFN-γ in the expansion of CD4⁺ TCRVβ11⁺ T cells, and will provide useful information for delineating the regulatory mechanisms of T_h1-cell development and for analyzing mechanisms on T_h1-dominant immune responses.

Introduction

After encountering antigen, T_h cells undergo differentiation to effector cells that can produce IFN-γ, IL-4, IL-10 and other cytokines/chemokines. Naive T_h cells can differentiate to at least two functional classes of cell during an immune response—T_h1 and T_h2 (1,2). T_h1 cells produce IFN-γ and lymphotoxin [tumor necrosis factor (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 mycobacteria, viruses and parasites (1–4). T_h1 cells also regulate IgG2a and IgG3 antibody production via IFN-γ production that is involved in the opsonization and phagocytosis of particulate microbes. T_h2 cells secrete IL-4 and IL-5 as effector cytokines, and are responsible for extracellular immunity (3).

The process by which an uncommitted T_h cell develops into a mature T_h1 or T_h2 is a matter of fact for regulating immune response to various antigens. There is a body of evidence to indicate that the cytokines IL-12 and IL-4 are key determinants

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Transmitting editor: S. Koyasu

Received 29 October 2002, *accepted* 14 July 2003

of the T_h1 and T_h2 response respectively (4). The strength of interaction mediated through the TCR and MHC–peptide complex directly affects the lineage commitment of T_h cells to T_h1 and clonal expansion (5–7). Antigen doses, co-stimulators, genetic modifiers and other non-cytokine factors have crucial roles in determining the polarization of a T_h cell response. Critical cytokines that promote the appearance of T_h1 cells include IL-12 and IL-18. The canonical T_h1 cytokine IFN-γ has also been implicated in T_h1 cell development through its ability to optimize IL-12 production from macrophages and IL-12 receptor expression on activated T cells. As naive T cells do not produce IFN-γ until after activation, the contribution of T-cell-derived IFN-γ in T_h1 development is still obscure.

T_h1 cells play an important role in the development of resistance to *Mycobacterium tuberculosis* (8–17), primarily through the production of macrophage-activating cytokines, such as IFN-γ, TNF-α or granulocyte macrophage colony stimulating factor. Although specific antigens eliciting T_h1 cell responses are not yet known for tuberculosis, one of the major protein antigens secreted from *M. tuberculosis* is Ag85B (also known as α antigen or MPT59) (18–24). Purified Ag85B or recombinant forms of Ag85 can elicit strong T_h1 responses *in vitro* from purified protein derivative-positive asymptomatic individuals (18–20). We have shown that *in vitro* stimulation of lymph node cells from *M. 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 the TCR (TCRVβ11) in an $I\text{-}A^b$-restricted manner (25,26). Using peptides covering the entire mature 285-amino-acid-long Ag85B protein as 15mer molecules overlapping by 5 amino acids, we identified the 15mer peptide (Peptide-25), covering amino acid residues 240–254 of Ag85B, as the major epitope for Ag85B-specific TCRVβ11$^+$ T cells (25). Peptide-25 contains the motif that is conserved for $I\text{-}A^b$ and requires processing by antigen-presenting cells (APC) to trigger Ag85B-specific TCRVβ11$^+$ T cells (25). We also have reported that amino acid residues of Peptide-25 at positions 244, 247, 249 and 252 (P1, P4, P6 and P9 sites respectively) are $I\text{-}A^b$ contact residues (26). Active immunization of C57BL/6 mice with Peptide-25 can induce protective immunity against subsequent infection with live *M. tuberculosis* H37Rv (26).

MyD88 is an adaptor molecule essential for signaling via the Toll-like receptor (TLR)/IL-1 receptor family. Both TLR2 and TLR4 have been shown to mediate *M. tuberculosis*-induced intracellular signaling *in vitro* (27). Takeuchi *et al.* examined the responsiveness of MyD88-deficient mice to various bacterial cell wall components and demonstrated that MyD88 is essential for the cellular response to bacterial cell wall components including mycobacterial whole-cell lysates (28). However, it remains unclear whether MyD88 is involved in the T_h1 development induced by proteins or peptide secreted by *M. tuberculosis*.

In this report, we have examined roles of TCRVβ11$^+$ T cells in the expansion of IFN-γ-producing T cells in response to Peptide-25 and of IFN-γ in the development of TCRVβ11$^+$ T cells. Our results clearly demonstrate that immunization of TCRVβ11$^+$ C57BL/6 mice, but not TCRVβ11$^-$ B10.A(3R) mice, with Peptide-25 is capable of inducing remarkably potent oligoclonal development of IFN-γ-producing T cells including TCRVβ11$^+$ T cells. This is not observed in C57BL/6 mice immunized with Peptide-9 of Ag85B, 50V of pigeon cytochrome *c* and ovalbumin $(OVA)_{265-280}$, all of which can bind to $I\text{-}A^b$. The use of anti-Vβ11 mAb is effective in the prevention of the development of T_h1 cells specific for Peptide-25. Reduced proportions of TCRVβ11$^+$ T cells are observed in Peptide-25-immunized IFN-γ-deficient mice after Peptide-25 stimulation, indicating an important role of IFN-γ. A mutant Peptide-25, 244D247V, whose amino acids critical for $I\text{-}A^b$-binding are substituted for amino acids capable of binding to $I\text{-}A^k$ molecules, can induce the T_h1 development in C3H/HeN mice. We will discuss the mechanisms on T_h1-inducibility of Peptide-25.

Methods

Mice

C57BL/6, BALB/c, C3H/HeN and C3H/HeJ mice were obtained from Japan SLC (Hamamatsu, Japan). B10.A(3R) mice were kindly provided by Dr T. Yokochi (University of Tokyo Graduate School of Medicine). MyD88-deficient ($MyD88^{-/-}$) mice and IFN-γ-deficient ($IFN\text{-}\gamma^{-/-}$) mice were generated as described (29,30). All mice were maintained in the animal facility at the Institute of Medical Science, University of Tokyo under specific pathogen-free conditions and used at 8–15 weeks of age.

Antigens and reagents

Ag85B was purified as described (31) and used at selected concentrations. Peptide-25 and its substituted mutant, Peptide-25 (244D247V), Peptide-9 of Ag85B (25), 50V of pigeon cytochrome *c* (32) and $OVA_{265-280}$ (33) were synthesized by Sawaday Chemicals (Tokyo, Japan) using Fmoc chemical strategies and purified using HPLC. Amino acid sequences of each peptide are shown in Fig. 1. The following mAb were used: GK1.5 (34) which recognizes CD4 (ATCC, Rockville, MD), 53-6.72 (35) which recognizes CD8 (ATCC) and 2.4G2 (36) which recognizes FcγR (ATCC). B20.6 [anti-Vβ2 (37)], 44-22-1 [anti-Vβ6 (38)] and F23.1 [anti-Vβ8 (39)] were kindly provided by Dr Y. Yoshikai (Kyushu University, Fukuoka). KJ-25 [anti-Vβ3 (40)], KT4 [anti-Vβ4 (41)], B21.5 [anti-Vβ10 (42)], MR11-1 [anti-Vβ12 (43)], MR12-3 (anti-Vβ13) and 14-2 [anti-Vβ14 (44)] were purchased from PharMingen (San Diego, CA). MR9-4 [anti-Vβ5 (45)], MR10.2 [anti-Vβ9 (46)] and RR3-15 [anti-Vβ11 (47)] were kindly provided by Dr O. Kanagawa (Washington University, St Louis, MO) and Dr H. Nakauchi (University of Tokyo, Institute of Medical Science). The following anti-cytokine mAb were used: 11B11 (48) which neutralizes IL-4 and was kindly provided by Dr W. E. Paul (National Institutes of Health, Bethesda, MD), BVβ6-24G2 (49) which recognizes IL-4 (PharMingen), RA4-6A2 (PharMingen) which neutralizes IFN-γ and XMG1.2 [anti-IFN-γ (49)] which was kindly provided by Dr. M. Howard (DNAX Research Institute, Palo Alto, CA). For intracellular cytokine staining, we used the following phycoerythrin-conjugated mAb (PharMingen): TRFK5 which recognizes IL-5, C.15.6 which recognizes IL-12, MP6-XT22 which recognizes TNF-α, S4B6 which recognizes IL-2 and R3-34 for rat IgG1 isotype control.

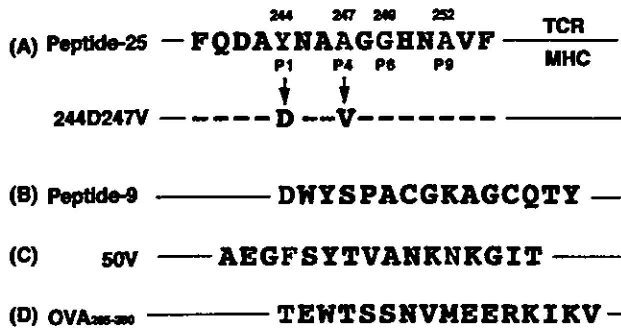


Fig. 1. Amino acid sequence of peptides. (A) Peptide-25 and its substituted mutant, 244D247V, (B) Peptide-9 of Ag85B, (C) 50V of pigeon cytochrome *c* and (D) $OVA_{265-280}$. We searched for the prediction of Peptide-9 and $OVA_{265-280}$ binding to the I-A^b molecule by using the RANKPEP program (Molecular Immunology Foundation). Amino acid residues shown in red are predicted binders to the I-A^b molecule.

Immunization

Each strain of mice was immunized by s.c. injection at the base of the tail with 1–10 μg of each peptide in incomplete Freund's adjuvant (IFA). In some experiments, mice were boosted with 10 μg of relevant peptide 7 days before *in vitro* culture.

Cell culture and ELISA

A single-cell suspension was prepared from inguinal lymph nodes of peptide-immunized mice. In some experiments, $CD4^+$ T cell clones specific for Peptide-25 were used as described (26). For proliferation assay, cells (5×10^5) were cultured in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in the presence of selected concentrations of synthetic peptides. Each well was filled with 200 μl of complete medium consisting of RPMI 1640 medium (Gibco) supplemented with 8% FCS, penicillin (100 IU/ml), streptomycin (50 μg/ml) and 5×10^{-5} M 2-mercaptoethanol. Cultures were set up in triplicate. The cells were pulse labeled with [^{3}H]thymidine (1 μCi/well) during the last 8 h of a 72 h culture period and the incorporation of radioactivity was measured as previously described (26). The amounts of IFN-γ secreted in supernatants of 3-day culture were quantitatively analyzed by ELISA. For FACS analysis, cells (5×10^6 in 2 ml) were cultured in the presence of peptides in 24-well flat-bottom microtiter plates. Representative results are shown from a series of three separate experiments.

Flow cytometric analysis of intracellular cytokine production

$CD4^+$ cloned T cells (2×10^5/ml) specific for Peptide-25 were stimulated with plate-coated anti-CD3. Various peptide-primed lymph node cells were stimulated with each peptide for 4 days. In both cases, 4 h before cell harvest, GolgiStop (PharMingen) was added to block intracellular transport processes. Cells were harvested, washed and stained with FITC-labeled anti-CD4, anti-CD8 or biotin-labeled anti-Vβ mAb. Anti-FcγR mAb (10 μg/ml) was added over the duration of the incubation period for cell staining to block non-specific binding of labeled mAb. Then the cells were stained with streptavidin–allophycocyanin in 7-amino-actinomycin D buffer (2 μg/ml) to distinguish viable cells from dead cells. After washing, cells were fixed with 4% formaldehyde. Then the cells were washed and stained with phycoerythrin-labeled anti-cytokine mAb. For intracellular staining by anti-cytokine mAb, we added 1% FCS and 0.1% saponin to all staining reagents and washing buffer, and kept them at 4°C. Fluorescence intensity of blastic T cells was analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA). To assess particular TCRVβ11$^+$ cells, we calculated the percentage of TCRVβ11$^+$ cells from total $CD4^+$ cells.

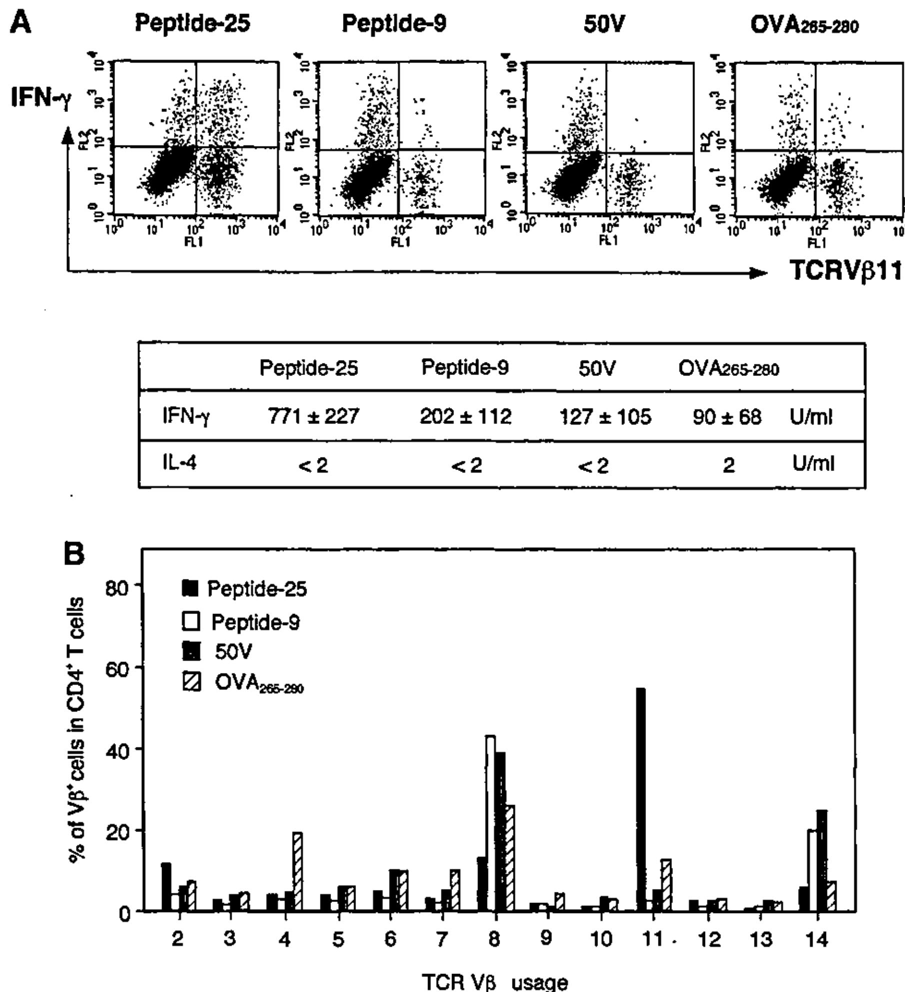
Results

Production of T_h1 cytokines by Peptide-25-primed $CD4^+$ TCRVβ11$^+$ T cells upon Peptide-25 stimulation

To set up conditions for intracellular staining and cytometric analysis of intracellular cytokine production, three different Peptide-25-reactive T cell clones (BP1, 2501 and BM2) were stimulated with plate-coated anti-CD3 mAb. Each Peptide-25-reactive T cell clone produced both IFN-γ and TNF-α intracellularly upon anti-CD3 stimulation, but did not produce IL-12 and IL-4 (data not shown). Production of IL-2 and IL-5 was variable clone by clone. For example, cloned TCRVβ11$^+$ BP1 and 2501 cells showed no significant production of IL-2 and IL-5, but cloned TCRVβ14$^+$ BM2 cells produced IL-2 and IL-5 in addition to IFN-γ and TNF-α.

We then investigated intracellular production of IFN-γ, TNF-α, IL-4 and IL-5 in Peptide-25-stimulated, Peptide-25-primed lymph node cells by cytometric analysis. Lymph node cells of Peptide-25-primed C57BL/6 mice were stimulated *in vitro* with Peptide-25 and intracellular cytokine production was examined after the culture. As controls, lymph node cells stimulated with Peptide-25 from non-primed C57BL/6 mice were also stained under the same conditions. Within 5 h of stimulation of Peptide-25-immunized cells with Peptide-25 a significant proportion of IFN-γ- and TNF-α-producing $CD4^+$ T cells, and to a lesser extent IL-2-producing $CD4^+$ T cells, became detectable (data not shown). Proportions of IFN-γ- and TNF-α-producing $CD4^+$ T cells were increased by day 4. Cells producing IL-4 or IL-5 were rarely detected. We could not detect IFN-γ-producing cells in Peptide-25-stimulated non-primed cells (data not shown). These results indicate that large proportions of IFN-γ-producing cells are $CD4^+$ T cells.

To compare immunogenicity of Peptide-25 with other I-A^b-binding peptides regarding the development of TCRVβ11$^+$ IFN-γ-producing cells, we immunized C57BL/6 mice with Peptide-25, Peptide-9 of Ag85B, 50V or $OVA_{265-280}$ included in IFA, and their lymph node cells were re-stimulated *in vitro* with relevant peptide. Results revealed that all of peptides tested induced development of IFN-γ-producing cells to various extents (Fig. 2A). Among peptides, Peptide-25 showed the most potent ability to induce IFN-γ-producing cells. Levels of IFN-γ produced by Peptide-25-primed cells were 6- and 8-times higher than these produced by 50V-primed cells and $OVA_{265-280}$-primed cells, respectively. Interestingly, large proportions of Peptide-25-reactive IFN-γ-producing cells were TCRVβ11$^+$ T cells and a significant proportion, but to a lesser extent, of IFN-γ-producing cells were TCRVβ11$^-$ T cells (Fig. 2B). In contrast, IFN-γ-producing



	Peptide-25	Peptide-9	50V	OVA265-280	
IFN-γ	771 ± 227	202 ± 112	127 ± 105	90 ± 68	U/ml
IL-4	< 2	< 2	< 2	2	U/ml

Fig. 2. (A) Induction of TCRVβ11⁺ IFN-γ-producing cells by Peptide-25. Four groups of C57BL/6 mice were immunized with 10 μg of Peptide-25, Peptide-9, 50V or $OVA_{265-280}$ in IFA. Lymph node cells from each group of mice were re-stimulated for 4 days *in vitro* with relevant peptides (10 μg/ml) in the presence of GolgiStop and stained for the intracellular IFN-γ as described in Methods. IFN-γ-producing TCRVβ11⁺ T cells were analyzed by a FACSCalibur flow cytometer. IFN-γ and IL-4 in cultured supernatants were titrated by ELISA. (B) TCRVβ usage. The proportion of CD4⁺ T cells expressing TCRVβ was examined post-culture by staining the cells with anti-Vβ mAb followed by a FACS analysis. Results were expressed as percentages of TCRVβ⁺ cells in CD4⁺ T cell populations.

cells reactive to Peptide-9, 50V or $OVA_{265-280}$ were TCRVβ11⁻ T cells. Peptide-9-reactive T cells and 50V-reactive T cells showed an increase in TCRVβ8⁺ and TCRVβ14⁺ populations respectively, while $OVA_{265-280}$-reactive T cells displayed an increase in TCRVβ4⁺ and TCRVβ11⁺ populations (Fig. 2B).

CD4⁺ TCRVβ11⁺ T cells of Peptide-25-immunized mice produce IFN-γ and TNF-α

As we described, large proportions of Peptide-25-stimulated, Peptide-25-immunized cells in C57BL/6 mice are TCRVβ11⁺ T cells (Fig. 2). To examine the role of TCRVβ11⁺ T cells in the development of IFN-γ-producing T cells, we immunized C57BL/6 and B10.A(3R) mice, both of which are I-A^b-positive strains. B10.A(3R) mice are TCRVβ11⁻. Lymph node cells of each group of Peptide-25-immunized mice were re-stimulated *in vitro* with Peptide-25 for 4 days. As shown in Fig. 3(A), Peptide-25-primed C57BL/6 cells responded to Peptide-25 in a dose-dependent manner for proliferation and differentiation into IFN-γ-producing cells. The response peaked at 1.0 μg/ml of Peptide-25 stimulation. In contrast, Peptide-25-immunized cells from B10.A(3R) mice showed a significant proliferation, but marginal IFN-γ production was observed upon Peptide-25 stimulation even at higher doses (10 μg/ml) whose level was much lower than that produced by C57BL/6 mice (Fig. 3A). TCRVβ11⁺ T cells play a role in the development of Peptide-25-reactive precursors for IFN-γ-producing cells.

More than 85% of IFN-γ-producing cells in the culture of Peptide-25-stimulated, Peptide-25-primed cells of C57BL/6 mice were CD4⁺ T cells (Fig. 3B, upper panel) and >65% of CD4⁺ T cells expressed TCRVβ11 (data not shown). About 55 and 30% of IFN-γ-producing CD4⁺ cells were assumed to be TCRVβ11⁺ and TCRVβ11⁻ T cells respectively. Indeed, as shown in Fig. 3(B), ~57 and 43% of IFN-γ-producing cells were TCRVβ11⁺ and TCRVβ11⁻ T cells respectively. Intracellular

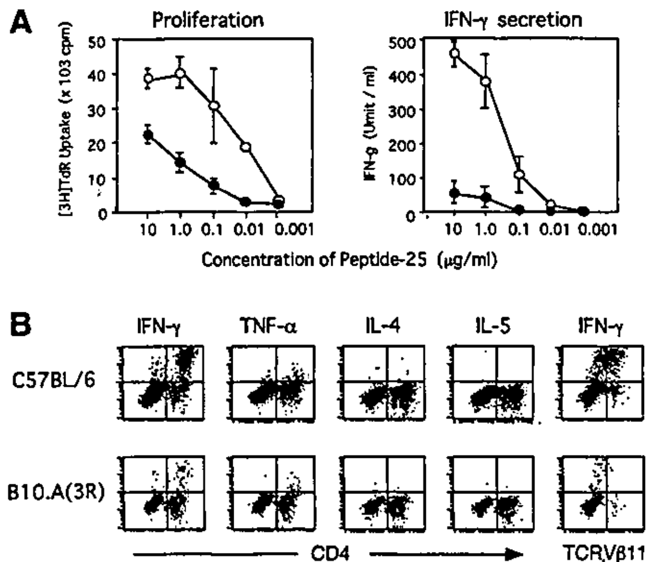


Fig. 3. TCRVβ11$^-$ B10.A(3R) mice weakly respond to Peptide-25 for T_h1 development. (A) Proliferation and IFN-γ secretion. Groups of C57BL/6 (open circles) or B10.A(3R) (closed circles) mice were immunized with 10 μg of Peptide-25 in IFA. Lymph node cells from each group of mice were stimulated *in vitro* with various concentrations of Peptide-25. Peptide-25-induced proliferation was monitored post-culture by [^{3}H]thymidine uptake. IFN-γ production was titrated by ELISA. (B) Analysis of CD4$^+$ cytokine-producing cells by intracellular cytokine staining. After the culture, cells were stained intracellularly with anti-cytokine mAb and analyzed by a FACSCalibur.

staining analyses also revealed that a small proportion of Peptide-25-primed CD4$^+$ T cells in B10.A(3R) mice produced IFN-γ upon Peptide-25 stimulation (Fig. 3B, lower panel). We infer from these results that Peptide-25-primed TCRVβ11$^+$ T cells may be required for differentiation of Peptide-25-primed TCRVβ11$^-$ T cells into IFN-γ-producing cells in response to Peptide-25.

We then examined the effect of passive administration of anti-Vβ11 antibody on the development of IFN-γ-producing T_h1 cells specific for Peptide-25. Both anti-Vβ11 antibody-treated and non-treated groups of C57BL/6 mice were immunized with Peptide-25. Peptide-25-primed cells from each group of mice were stimulated with Peptide-25. In some experiments, anti-Vβ11 antibody was added during the *in vitro* culture. Results revealed that >60% of TCRVβ11$^+$ T cells were detected in the cells cultured with Peptide-25 from control groups of mice. In contrast, <1% of TCRVβ11$^+$ T cells were detected in the cells from the anti-Vβ11 treated mice (Fig. 4A). CD4$^+$ T cell populations expressing TCRVβ4, TCRVβ6, TCRVβ12 and TCRVβ14 were expanded; however, no dominant TCRVβ usage was observed. Proliferation of and IFN-γ production by Peptide-25-primed cells from anti-Vβ11-treated mice was ~50% less than these of the control group (Fig. 4B and C). When anti-Vβ11 antibody was added to the culture of Peptide-25-primed cells of the control mice, IFN-γ production was suppressed by 50% of control response (Fig. 4B) whose levels were comparable to those produced by Peptide-25-stimulated cells of Peptide-25-primed and anti-Vβ11 treated mice. Taking these results together, the Peptide-25-reactive T cell population expressing TCRVβ11 is a major population of IFN-γ-producing cells in Peptide-25-immunized C57BL/6 mice, although TCRVβ11$^-$ T cells are capable of differentiating into IFN-γ-producing cells.

Role of IFN-γ in the expansion of Peptide-25-reactive CD4$^+$ T cells

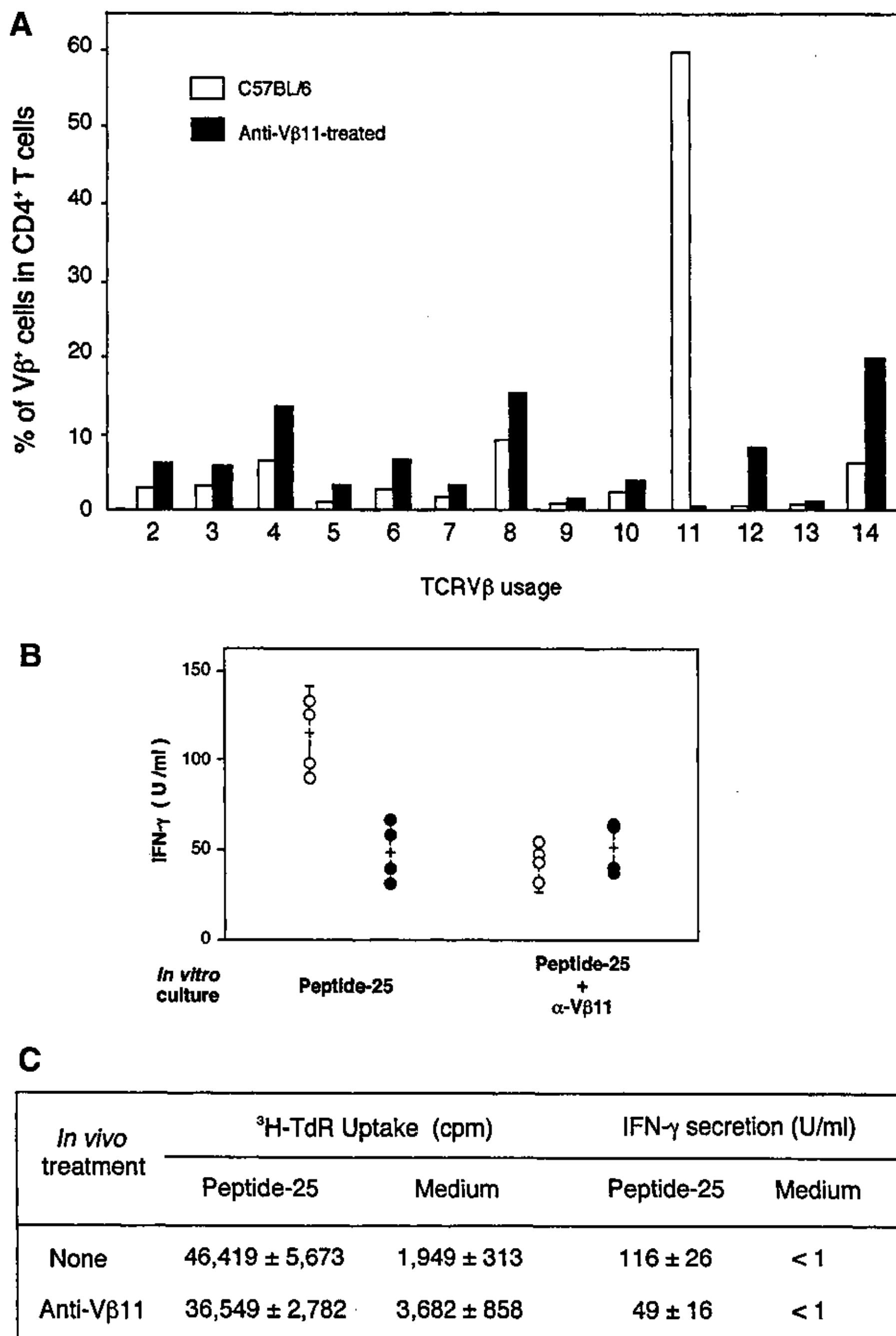
There is a body of evidence that IFN-γ plays a pivotal role in the polarization of naive T cells towards T_h1 cells (50). To examine the role of IFN-γ in the development of TCRVβ11$^+$ T cells in Peptide-25-primed C57BL/6 mice, we immunized wild-type and IFN-γ$^{-/-}$ C57BL/6 mice with Peptide-25, and their lymph node cells were cultured with Peptide-25. Results revealed that Peptide-25-primed cells from IFN-γ$^{-/-}$ mice displayed proliferation and IL-2 production in response to Peptide-25 to a lesser extent than wild-type mice (Fig. 5A and B). As expected, IFN-γ production was not observed in cultures of Peptide-25-primed cells from IFN-γ$^{-/-}$ mice (Fig. 5C). While ~41% of CD4$^+$ T cells expressed TCRVβ11 in Peptide-25-primed wild-type cells after Peptide-25 stimulation, Peptide-25-primed IFN-γ$^{-/-}$ cells contained ~22% of TCRVβ11$^+$ cells. In addition, CD4$^+$ T cell populations expressing TCRVβ5, TCRVβ6, TCRVβ9 and TCRVβ12 were increased slightly; however, no dominant TCRVβ usage was observed (data not shown). We analyzed the cell cycle number of TCRVβ11$^+$ cells by labeling Peptide-25-primed cells with CFSE before culture. FACS analysis revealed that significant proportions of both TCRVβ11$^+$ T and TCRVβ11$^-$ T cells experienced several cell divisions in Peptide-25-primed wild-type cells for 4 days upon Peptide-25 stimulation at two different concentrations (1 and 10 μg/ml). In contrast, Peptide-25-primed IFN-γ$^{-/-}$ T cells showed less cell divisions than wild-type cells (Fig. 5D).

Role of MyD88 in vivo induction of T_h1 cells by Peptide-25

Various cell types and molecules have been proposed to affect T_h1 development from naive T cells. As TLR- and MyD88-dependent signaling pathways are in involved in the activation of dendritic cells and T_h1 development (29,51), we compared T_h1 and T_h2 development in Peptide-25-immunized MyD88$^{-/-}$ mice with their development in Peptide-25-immunized wild-type mice. Re-stimulation of Peptide-25-primed cells of MyD88$^{-/-}$ mice with Peptide-25 induced IFN-γ production and expansion of TCRVβ11$^+$ T cells to a similar extent to cells of wild-type mice (Fig. 6A). Proportions of IL-4- and IL-5-producing T cells in Peptide-25-primed cells of MyD88$^{-/-}$ mice were also similar to those of wild-type mice. TCRVβ usage of Peptide-25-primed cells of MyD88$^{-/-}$ mice was unaltered from that of wild-type cells (Fig. 6B).

Substitution mutant of Peptide-25, 244D247V, induces T_h1 cells in vivo in C3H/HeN mice

In our previous analysis, P1 (at position 244), P4 (at position 247), P6 (at position 249) and P9 (at position 251) sites of Peptide-25 were important for I-A^b binding (26). Peptide-25 was not immunogenic in C3H/HeN mice because APC of C3H/HeN (I-A^k) did not bind to Peptide-25. To develop an immunogenic peptide for T_h1 development in C3H/HeN mice, we prepared several mutant peptides by substituting amino acid residues of the I-A^b-binding motif of Peptide-25 to the I-A^k-binding motif. We synthesized substituted mutants



In vivo treatment	^{3}H-TdR Uptake (cpm)		IFN-γ secretion (U/ml)	
	Peptide-25	Medium	Peptide-25	Medium
None	46,419 ± 5,673	1,949 ± 313	116 ± 26	< 1
Anti-Vβ11	36,549 ± 2,782	3,682 ± 858	49 ± 16	< 1

Fig. 4. Effect of anti-Vβ11 antibody treatment on the development of CD4$^+$ IFN-γ-producing cells. A group of C57BL/6 mice was injected i.p. with 1 mg of anti-Vβ11 and immunized with 10 μg of Peptide-25 at 1 day post-injection. Another group of non-treated mice was also immunized with Peptide-25. Lymph node cells from each group of mice were stimulated *in vitro* with Peptide-25 (10 μg/ml) for 4 days. (A) TCRVβ usage was analyzed according to procedures described in Fig. 3. Results are expressed as percentages of TCRVβ$^+$ cells in CD4$^+$ cell populations. Open and closed columns represent control and anti-Vβ11-treated mice respectively. (B) Effect of anti-Vβ11 treatment *in vivo* and *in vitro* on the development of IFN-γ-producing cells. Groups of non-treated (open circles) or *in vivo* anti-Vβ11-administered (solid circles) C57BL/6 mice (four mice for each group) were immunized with Peptide-25. Lymph node cells of each group of mice were cultured *in vitro* with Peptide-25 7 days after the immunization in the presence or absence of 10 μg/ml of anti-Vβ11 mAb. Each open and closed circle represents levels of IFN-γ produced by the cells of an individual mouse. Mean value of each group is also shown. (C) Peptide-25-induced proliferation and IFN-γ production. Peptide-25-induced proliferation and IFN-γ production were monitored according to procedures described in Fig. 3.

(244D247V, 244D or 247V) of Peptide-25 whose P1 and P4 sites were aspartic acid (244D) and valine (247V) respectively. A group of C3H/HeN mice was immunized with 244D247V (Fig. 1), 244D or 247V. Lymph node cells from each group of immunized mice were stimulated with the relevant peptide, and proliferative response and IFN-γ secretion were examined. As shown in Fig. 7, stimulation of 244D247V-immunized cells with 244D247V induced a significant proliferative

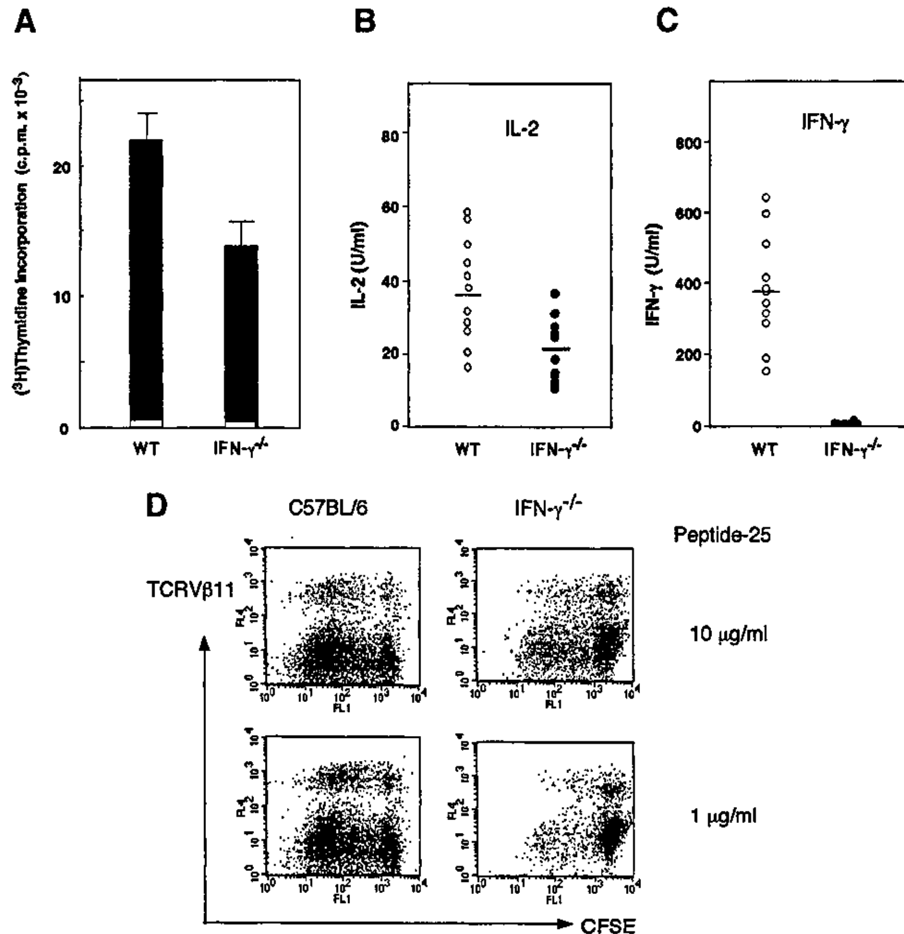


Fig. 5. Role of IFN-γ in the development of TCRVβ11$^+$ T cells. Either wild-type mice or IFN-$\gamma^{-/-}$ mice were immunized with 10 μg of Peptide-25 in IFA. Lymph node cells from each group of mice were stimulated *in vitro* with Peptide-25 (10 μg/ml) for 4 days. (A) Peptide-25-induced proliferation was monitored post-culture by [^{3}H]thymidine uptake. The black column represents Peptide-25-induced [^{3}H]thymidine uptake. The white column represents [^{3}H]thymidine uptake in the absence of Peptide-25. Peptide-25-induced IL-2 (B) and IFN-γ production (C) were monitored by ELISA. Each open and closed circle represents the results of an individual mouse. The horizontal bar represents the mean value of 12 mice. (D) Cell division number analysis. Lymph node cells from Peptide-25-immunized wild-type mice and IFN-$\gamma^{-/-}$ mice were labeled with CFSE (Molecular Probes, Eugene, OR) and cultured *in vitro* with Peptide-25 (1 or 10 μg/ml) for 4 days. To analyze cell division number, proportions of CFSE$^+$ TCRVβ11$^+$ T cells were analyzed by a FACSCalibur after 4-day culture. Representative results of a series of three independent experiments are shown.

response and remarkable IFN-γ production in a dose-dependent manner, but it did not induce either IL-4 or IL-5 (data not shown). All IFN-γ-producing T cells were TCRVβ11$^-$ T cells. The 244D247V-primed cells showed no significant response to 244D or 252R (data not shown). Neither 252R nor 244D was immunogenic (Fig. 7).

Discussion

After engagement of the TCR by the appropriate peptide–MHC complex, which triggers clonal expansion, T_h cells rapidly undergo programmed differentiation. T_h subsets are distinguished by their ability to produce distinct cytokine patterns and promote specific immune responses. The IFN-γ produced by T_h1 cells amplifies T_h1 development and inhibits the expansion of T_h2 cells (7). Conversely, IL-4 and IL-10 produced by T_h2 cells antagonizes the production of IFN-γ by T_h1 cells (3). Dysregulation of T_h cell responses can result in immunopathology in that aberrant T_h1 responses can be responsible for organ-specific autoimmunity and exaggerated T_h2 responses have been associated with allergic diseases. Thus, understanding the factors and microenvironments that influence the development of naive T cells into different effector subsets has important implications for the development of new strategies to induce beneficial immune responses.

Considerable progress has being made in identifying and analyzing regulatory function to specify cell fate decisions in the lymphoid system during the generation of T_h1 versus T_h2 cells. While various microenvironments including cytokines, co-stimulatory molecule expression and transcription factors regulate the development of T_h1 cells, other mechanisms such as the type of APC, antigen dose and peptide ligand density can also be involved in the differentiation of naive T cells into T_h1 and T_h2 cells (4,5,52). A number of studies have attempted to define the T cell determinants of various

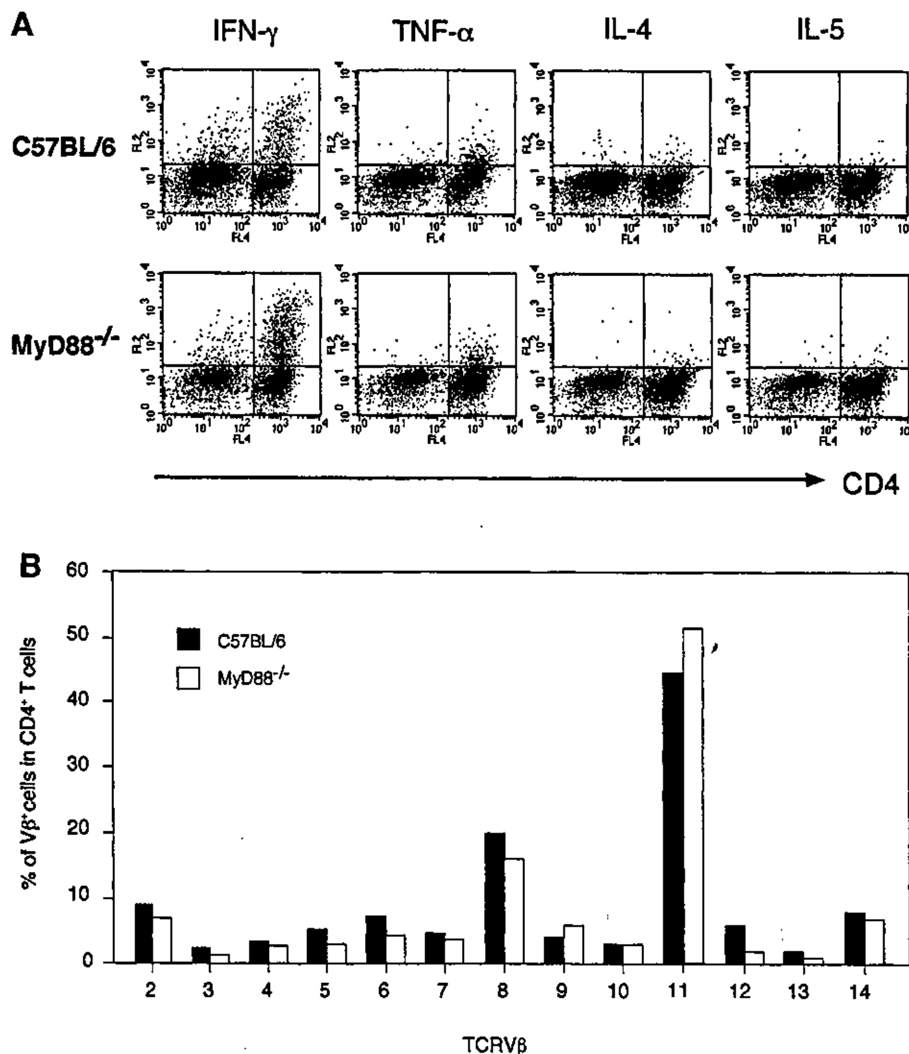


Fig. 6. Induction of TCRVβ11+ IFN-γ-producing cells in MyD88-deficient mice. Either wild-type mice or MyD88-/- mice were immunized with 10 μg of Peptide-25. Lymph node cells from each group of mice were re-stimulated *in vitro* with Peptide-25 (10 μg/ml) for 4 days. (A) IFN-γ-, TNF-α-, IL-4- and IL-5-producing CD4+ T cells were determined by intracellular staining and FACSCalibur analysis. (B) Proportion of TCRVβ usage was analyzed post-culture by FACSCalibur as described in Fig. 3. Results are expressed as percentages of TCRVβ+ cells in CD4+ cell populations. Closed and open columns represent wild-type mice and MyD88-/- mice respectively.

antigens, which are common structures or specific alignments of amino acids within peptides (motifs), required for binding to certain MHC molecules. In contrast to MHC class I-bound peptides, antigenic peptides comprised of 10–12 amino acid residues are able to bind MHC class II molecules (53,54). However, the role of protein and peptides in the polarized development of T_h1 versus T_h2 cells remains elusive.

The major focus of our study is to understand mechanisms underlying preferential induction of T_h1 cells by peptides derived from *M. tuberculosis*. As we reported, Peptide-25 corresponding to amino acid residues 240–254 of Ag85B is a major T cell epitope of Ag85B-specific TCRVβ11+ T_h1 cells (26). Antigen processing of Peptide-25 is required for triggering TCRVβ11+ T_h1 cells specific for Peptide-25 in an I-A^b-restricted manner (26). Ramachandra *et al.* found $Ag85B_{241-256}$–I-A^b complexes in *M. tuberculosis* phagosomes that later appeared on the plasma membrane (55). Thus Ag85B–MHC class II complexes are formed within and expressed on *M. tuberculosis*-infected macrophages.

In vitro stimulation of lymph node cells from Peptide-25-immunized C57BL/6 mice with Peptide-25 induces massive expansion of both TCRVβ11+ and TCRVβ11− CD4+ T cells that produce IFN-γ (Figs 2A and 3B). As far as we examined, development of IL-4- and IL-5-producing T cells was hardly detectable. Peptide-25 was not immunogenic for C3H/HeN (I-A^k) or BALB/c (I-A^d) mice (data not shown). We have also examined immunogenicity of three different I-A^b-binding peptides (Peptide-9, 50V and $OVA_{265-280}$) in C57BL/6 mice. Results revealed that all peptides were immunogenic and induced significant IFN-γ production (Fig. 2A). Among them, only Peptide-25 could induce remarkable development and expansion of TCRVβ11+ IFN-γ-producing cells. Moreover, Peptide-25 was the most potent immunogen to induce the development of the IFN-γ-producing cells. A significant

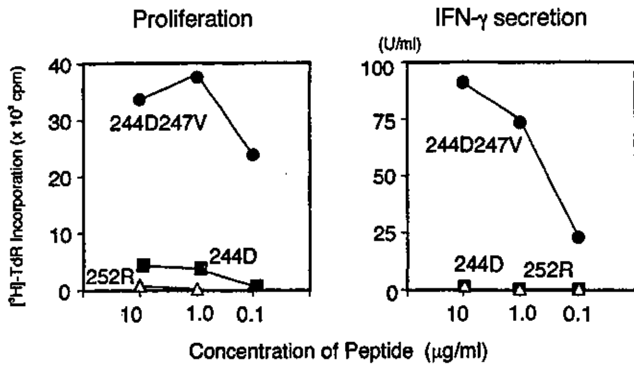


Fig. 7. 244D247V is immunogenic *in vivo* in C3H/HeN mice. C3H/HeN mice were immunized with 10 μg of 244D247V, 244D or 252R in IFA, and boosted 7 days before the experiment. Lymph node cells from each group of mice were stimulated *in vitro* with various concentrations of relevant peptide for 4 days. Proliferative response and IFN-γ secretion in culture supernatants were monitored post-culture according to procedures described in Fig. 3.

proportion of Peptide-25-reactive IFN-γ-producing cells were TCRVβ11⁻ T cells. IFN-γ-producing cells reactive to Peptide-9, 50V and $OVA_{265-280}$ were TCRVβ11⁻ T cells (Fig. 2A). Although passive administration of anti-Vβ11 at 1 day before Peptide-25 immunization fully suppressed the development of $TCRV\beta11^+$ T cells (Fig. 4A), development of IFN-γ-producing T cells was inhibited partially, up to 50% of untreated control, by anti-Vβ11 mAb administration (Fig. 4B). It was demonstrated that IFN-γ derived from $CD4^+$ T cells is sufficient to mediate T_h1 cell development (56). We do not know whether IFN-γ is produced by cells other than T cells in Peptide-25-immunized mice.

There is a core sequence (P1 to P9) of 9 amino acids in the MHC class II binding motif (53). Using substitution mutants of Peptide-25 and T_h1 clones specific for Peptide-25, we demonstrated that the amino acid residues of Peptide-25 at positions 244, 247, 249 and 252 are $I\text{-}A^b$ contact residues, and are essential for stimulatory activity (26). Alanine-substituted mutant Y at position 244 of Peptide-25 significantly reduced the stimulatory activity of Peptide-25. Thus, the Y residue at position 244 of Peptide-25 appears to be the P1 site. The amino acid residues of Peptide-25 at positions 244, 247, 249 and 252 are at P1, P4, P6 and P9 sites respectively. Substitution mutant of Peptide-25, 244D247V, at P1 and P4 sites that are capable of binding to $I\text{-}A^k$ was immunogenic in C3H/HeN mice for T_h1 development, while neither of 244D or 247V was immunogenic (Fig. 7). The cells responding to 244D247V were exclusively $TCRV\beta11^-$ T cells (data not shown). Similarly, a mutant peptide 1431 of Peptide-25 capable of binding to $I\text{-}A^d$ was immunogenic in BALB/c mice to induce solely T_h1 cell development (data not shown). This further supports the notion that Peptide-25 and its substituted mutants can induce T_h1 cells preferentially in various strains of mice.

It has been shown that the immunodominant encephalitogenic T cell epitope of major basic protein (MBP), in mice of the $H\text{-}2^u$ haplotype (PL/J or B10.PL), is the acetylated N-terminal 9 or 11mer (NAc1–9 or NAc1–11) peptide (57). T cell recognition of the epitope is restricted by MHC class II ($I\text{-}A^u$) molecule. The MBPNAc1–11 epitope stimulates $CD4^+$ T cells expressing Vβ8.2 TCR that are capable of causing experimental allergic encephalomyelitis (EAE) (58–60). The MBPNAc1–11 epitope has been characterized to have a post-translational modification to the MBP peptide, N-terminal acetylation, which is required for induction of EAE (58). Both $TCRV\beta8^+$ and $TCRV\beta8^-$ T cell clones were shown to recognize MBPNAc1–9. The non-acetylated MBP1–11 peptide is incapable of inducing EAE. Interestingly, not all of the $I\text{-}A^u$-restricted T cell clones specific for MBPNAc1–11 induce EAE with paralysis. A strong positive correlation between levels of lymphotoxin and TNF-α activity, their capacity to induce paralysis, and the clinical hallmarks of EAE has been elucidated (61). No correlation was found between IL-2 or IFN-γ production and encephalitogenicity. Although Peptide-25 is derived from Ag85B secreted from *M. tuberculosis* and is able to induce T_h1 development, Peptide-25 by itself is not pathogenic and is actually capable of enhancing anti-tuberculosis immunity. It is not clear whether Peptide-25 is modified in APC during antigen process.

Considerable progress has being made in identifying and analyzing regulatory functions that specify cell fate decisions at various nodal points in the lymphoid system, during the generation of T_h1 versus T_{h2} cells. It remains unclear why Peptide-25 can induce potent T_h1 response preferentially with particular TCR usage. There are several possibilities to account for our observations. First, Pepetide-25 might activate dendritic cells to enhance expression of co-stimulatory molecules or transcription factors such as T-bet that lead to prompt production of IFN-γ, IL-12 or an unidentified 'master cytokine' for T_h1 development (62,63). Expansion of not only $TCRV\beta11^+$ T cells, but also $TCRV\beta11^-$ T cells, was observed when Peptide-25-immunized cells of wild-type mice were stimulated with Peptide-25. We examined the contribution of IFN-γ in the development of Peptide-25-reactive T cells using $IFN\text{-}\gamma^{-/-}$ mice. In $IFN\text{-}\gamma^{-/-}$ mice, both $TCRV\beta11^+$ T cell and $TCRV\beta11^-$ T cell populations were significantly decreased (Fig. 5D). Taken together, IFN-γ production is necessary for full development of $TCRV\beta11^+$ and $TCRV\beta11^-$ T cells in response to Peptide-25. Reduced IL-2 production in Peptide-25-primed $IFN\text{-}\gamma^{-/-}$ cells compared with wild-type cells (Fig. 5A and 5B) may account for the reduced proportions of Peptide-25-reactive $TCRV\beta11^+$ and $TCRV\beta11^-$ T cells in $IFN\text{-}\gamma^{-/-}$ mice. Second, the avidity of Peptide-25 for its specific TCR may be strong enough to induce T_h1 development. It is quite intriguing that >40% of $CD4^+$ T cells express a predominant TCRβ11 gene. $TCRV\beta11^+$ cells are necessary for full activation and expansion of T_h1 development in response to Peptide-25 (Figs 3 and 4). Third, Peptide-25-reactive T cells or their products indirectly stimulate $CD4^+$ T cells that enhance T_h1 cell development. Other possibilities are not exclusive.

In our preliminary data, co-immunization of OVA with Peptide-25 in C57BL/6 mice induced the augmented generation of OVA-specific cytotoxic T cells compared with immunization of OVA alone (unpublished observations). These results suggest that Peptide-25 enhances the development of a concomitant T_h1 response by unknown mechanisms. Taking all the results together, Peptide-25 has unique features represented by expansion of T_h1 cells with a high proportion of $TCRV\beta11^+$ T cells and oligoclonal response as

well. To gain further insight in to the site of action and mechanisms on induction of preferential T_h1 development by Peptide-25, transgenic mice expressing TCR that specifically recognize Peptide-25 together with $I\text{-}A^b$ would provide a useful model. We recently succeeded in generating such TCR-Tg mice and found that T cells of the TCR-Tg mice could differentiate only to T_h1 in response to Peptide-25 within 3 days in primary culture in the absence of IL-12 and IL-18 (unpublished observation).

Recently, TLR have been shown to regulate T_h1 responses in addition to innate immune responses in response to relevant stimuli (64–67). Although most TLR recognize carbohydrate or lipid of bacteria, some TLR, e.g. TLR5, recognize protein antigen such as flagellin (68). Stimulation of lymph node cells from Peptide-25-immunized $MyD88^{-/-}$ mice with Peptide-25 showed similar patterns of IFN-γ production to those observed in wild-type mice (Fig. 6). These results indicate that at least the MyD88-dependent pathway is not involved in major steps of Peptide-25-induced T_h1 development.

In conclusion, this study demonstrates that immunization of C57BL/6 mice with Peptide-25 leads to the preferential induction of development of TCRVβ11$^+$ T_h1 and oligoclonal response. The IFN-γ production is required for full development and activation of TCRVβ11$^+$ T cells and TCRVβ11$^-$ T cells in response to Peptide-25. Cytokines other than IFN-γ may also be involved in Peptide-25-induced T_h1 development from naive T cells. These experimental systems will provide a useful tool for delineating the regulatory mechanisms of antigenic peptides for T_h1-dominant immune response.

Acknowledgements

We thank Drs T. Yokochi, Y. Yoshikai, H. Nakauchi, W. E. Paul, O. Kanagawa and M. Howard for providing mice and mAb, and Dr S. Takaki and Dr Yuji Kikuchi for their valuable suggestions throughout this study. We are indebted to Drs S. Taki and Yoko Kikuchi for their critical review of this manuscript. This work was supported in part by a Research Grant from the Human Frontier Science Program (K. T.), by Special Coordination Funds for Promoting Science and Technology, and by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, in Japan.

Abbreviations

APC	antigen-presenting cell
EAE	experimental allergic encephalomyelitis
IFA	incomplete Freund's adjuvant
MPB	myelin basic protein
OVA	ovalbumin
TNF	tumor necrosis factor

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The Laryngoscope
Lippincott Williams & Wilkins, Inc.
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Rhinological and Otological Society, Inc.

The Role of Nuclear Factor-κB in Interleukin-8 Expression by Human Adenoidal Fibroblasts

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Objectives/Hypothesis: The production of cytokines by adenoids is known to be associated with inflammation of nasopharynx and the pathogenesis of otitis media with effusion. However, the role of adenoids in producing inflammatory cytokines such as interleukin-8 (IL-8) is not yet clear. In the present study, expression of IL-8 in adenoidal fibroblasts was investigated at the level of transcription factors. Further, the effects of clarithromycin, a 14-member ring macrolide, on IL-8 gene expression and nuclear factor-κB (NF-κB) activation in adenoidal fibroblasts were evaluated. _Study Design:_ In vitro study for the production of inflammatory cytokine from human adenoidal fibroblasts. _Methods:_ Adenoidal fibroblasts were incubated with nontypeable _Haemophilus influenzae_ endotoxin or interleukin-1β. Then the expression of IL-8 and the influence of NF-κB inhibitor and clarithromycin were evaluated. Interleukin-8 protein production was assessed by ELISA, and IL-8 messenger RNA production was measured by Northern blot analysis and reverse transcriptase–polymerase chain reaction. Activation of NF-κB and inhibition of its activation were determined by electrophoretic mobility shift assay. _Results:_ The expression of both IL-8 protein and messenger RNA in adenoidal fibroblasts was enhanced by _Haemophilus influenzae_ endotoxin and interleukin-1β and was positively correlated with increases in NF-κB activity. Treatment of cells with the NF-κB inhibitor _N_-tosyl-$_L$-phenylalanine chloromethyl ketone, as well as with clarithromycin, reduced expression of IL-8 and NF-κB activity in a dose-dependent manner. _Conclusions:_ Results suggest that adenoidal fibroblasts produce IL-8 in response to endotoxin through NF-κB activation. The inhibitory effects of clarithromycin on NF-κB activation and IL-8 production in adenoidal fibroblasts might explain, in part, the mechanism of this drug in improving otitis media with effusion. _Key Words:_ Adenoid, fibroblast, interleukin-8, nuclear factor-κB, clarithromycin.

***Laryngoscope*, 113:1378–1385, 2003**

INTRODUCTION

Otitis media with effusion (OME) is a prevalent infectious disease of the upper respiratory tract characterized by persistent middle ear effusions.[1] Eustachian tube dysfunction and microbial infection are considered the chief cause of this disease.[2] Pathogenesis of OME is associated with the adenoids and their ability to disturb eustachian tube function.[3] Further, the adenoids harbor pathogens such as nontypeable *Haemophilus influenzae* (NTHi) linked to nasopharyngeal colonization and OME,[3,4] suggesting that NTHi pathogens present in the adenoids play an important role in inflammatory and immune responses in the nasopharynx.

Recently, inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) were detected in nasopharyngeal secretions.[5,6] Lindberg et al.[5] reported that local production of IL-1β, IL-6, and TNF-α in the nasopharynx is related to nasopharyngeal bacterial colonization, as well as the pathogenesis of recurrent OME. The concentrations of IL-6 and IL-8 in nasopharyngeal secretions obtained from children with respiratory syncytial virus (RSV) infection were significantly greater than those in samples obtained from control children.[6] Those inflammatory cytokines are produced mainly from fibroblasts, epithelial cells, and macrophages in response to inflammatory stimulants such as endotoxin.[7,8] Endotoxin is contained in outer membrane of NTHi and the other Gram-negative bacteria. These findings indicate that inflammatory cytokines such as IL-8 in the nasopharynx might be produced from adenoidal fibroblasts and epithelial cells by the stimulation with NTHi harbored in the adenoids. However, the role of adenoids in producing inflammatory cytokines has not yet been clari-

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Supported by a Grant-in-Aid for General Scientific Research (B) (2) from the Ministry of Education, Science and Culture of Japan (grant 14370548).

Editor's Note: This Manuscript was accepted for publication April 3, 2003.

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fied. In addition to the inflammatory cytokines, immunoregulatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-10 (IL-10) are also produced in the adenoids, mainly from adenoidal lymphocytes.[9] Bernstein et al.[9] investigated the production of T helper cell type 1 (Th_1) cytokines (IFN-γ and IL-2) and T helper cell type 2 (Th_2) cytokines (IL-4 and IL-10) in adenoidal lymphocytes obtained from children with recurrent otitis media. They found that these cytokine profiles are different from those of peripheral blood lymphocytes. These Th_1 and Th_2 cytokines produced in adenoidal lymphocytes induce mucosal immune responses in the nasopharynx that are responsible for the clearance of NTHi from the nasopharynx.[10]

Interleukin-8 is a potent neutrophilic chemoattractant and is associated with the initiation and maintenance of inflammatory processes in a variety of tissues.[11] In children with OME, IL-8 has been found in a high percentage of middle ear effusions, concentrations of which correlate positively with the total number of neutrophils.[12,13] Further, IL-8 gene expression in nasal mucosa is increased in patients with chronic rhinosinusitis, and the level of IL-8 gene expression correlates with disease severity.[14] In addition, concentrations of IL-8 in nasopharyngeal secretions are remarkably increased during RSV infection.[6] These findings, as well as data showing that experimental OME can be induced in mice by transtympanic injection of recombinant IL-8,[15] indicate that IL-8 plays a crucial role in the inflammatory responses in upper respiratory tract tissues.

It has been reported that IL-8 gene expression is regulated by transcription factors such as nuclear factor-κB (NF-κB).[16,17] Nuclear factor-κB was originally identified as a transcriptional factor bound to the enhancer element of the κ light chain gene in B lymphocytes.[18] Clarithromycin (CAM), a new macrolide used for the treatment of various infectious diseases, suppresses the production of IL-8 in human monocytes and bronchial epithelial cells by inhibiting activation of NF-κB.[19,20] In recent years, several studies have demonstrated that low-dose, long-term administration of 14-membered ring macrolides is effective for OME complicated with sinobronchial syndrome,[21,22] suggesting that macrolides might affect the production of inflammatory cytokines through modulating NF-κB activation. However, the precise mechanisms regulating production of IL-8 in the nasopharynx and middle ear and the effects of macrolides on its production are still unknown.

In the present study, expression of IL-8 and its relationship with NF-κB activation are investigated in adenoidal fibroblasts following stimulation with endotoxin isolated from NTHi to further understand regulation of IL-8 production in the nasopharynx. Further, the influence of CAM on the production of IL-8 was evaluated to elucidate the mechanisms of macrolide-induced reductions in chronic upper respiratory tract inflammation.

MATERIALS AND METHODS

Reagents

Recombinant IL-1β was purchased from Genzyme (Cambridge, MA). *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and dexamethasone (DEX) were purchased from Sigma Chemical Company (St. Louis, MO). *Haemophilus influenzae* endotoxin (HIE) was prepared from NTHi isolated from the nasopharynx of patients with otitis media as previously described.[7] Clarithromycin was provided by Taisho Pharmaceutical (Tokyo, Japan).

Preparation of Human Adenoidal Fibroblasts

Human adenoidal tissues were obtained from the patients who received adenoidectomies, and adenoidal fibroblasts were isolated from the specimens after regular passages as previously reported.[8,23] Briefly, the specimens of adenoids were thoroughly washed with phosphate-buffered saline, minced, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with 100 mg/mL penicillin/streptomycin under a humidified atmosphere of 95% air and 5% CO_2. Because it has been shown that the optimal response elicited by endotoxin in leukocytes, endothelial cells, and nasal fibroblasts requires the presence of serum, the cells were cultured and stimulated with HIE in the medium containing 10% fetal bovine serum.[24,25] After adenoidal fibroblasts had achieved a state of confluence, they were harvested with 0.25% trypsin and 0.02% ethylenediamine tetra-acetic acid (EDTA) and divided into two dishes. Fibroblasts were subcultured under the same conditions and used for experiments after six to eight passages. These multiple passages are known not to impact on the function and viability of fibroblasts.[23,25,26] Then, characterization and purity of cultured fibroblasts were identified by the use of inverted phase-contrast microscopy. Cell viability as assessed by trypan blue exclusion was greater than 95% in all experiments.

Electrophoretic Mobility Shift Assay

Activation of NF-κB was assessed by electrophoretic mobility shift assay (EMSA). Adenoidal fibroblasts were stimulated with 1 ng/mL HIE or 100 pg/mL IL-1β for 30 minutes, then nuclear proteins were isolated as previously described.[7] In brief, the harvested cells were lysed in buffer A (0.6% Nonidet-P40, 10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 0.1 mmol/L EDTA, 3.5 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 1 mmol/L dichlorodiphenyltrichloroethane [DTT]) on ice for 15 minutes with occasional vortexing. The nuclei of cells were collected by centrifugation at 10,000*g* for 4 minutes and washed once with buffer A. The pellets were suspended in buffer C (20 mmol/L HEPES [pH 7.9], 0.4 mol/L KCl, 1 mmol/L EDTA, 1 mmol/L ethyleneglycol-bis-[β-aminoethylether]-N,N,N',N'-tetra-acetic acid [EGTA], 1 mmol/L DTT, and 1 mmol/L PMSF) and incubated on ice for 30 minutes with occasional vortexing. After centrifugation at 10,000*g* for 30 minutes, the supernatants containing nuclear protein were collected and stored at −80°C. Protein concentrations were measured with a protein assay kit (Bio Rad, Hercules, CA). Equivalent amounts of nuclear proteins (1–3 μg) were run in a gel shift assay system (Promega, Madison, WI) according to the manufacturer's protocol, with a few modifications. A double-stranded NF-κB oligonucleotide probe included in the assay system was end-labeled with [γ-^{32}P] adenosine triphosphate (ATP) using T4 polynucleotide kinase. Nuclear proteins were incubated with 30,000 cpm NF-κB probe at room temperature for 30 minutes. DNA-protein complexes were resolved by electrophoresis on non-denaturing 6% polyacrylamide gels in tris-boric acid/EDTA buffer. Gels were then dried under vacuum and exposed to Kodak x-ray film overnight at −70°C.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA from cultured fibroblasts was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). One microgram of total RNA was reverse-transcribed with 200 units of reverse transcriptase (SuperScript II, Invitrogen Corp., Carlsbad, CA), 500 μg oligo (dT)12–18 (Invitrogen Corp., Carlsbad, CA), 0.5 mol/L 2′-deoxyribonucleoside-5′-triphosphates (dNTPs; Pharmacia, Piscataway, NJ), and 10 mmol/L DTT in a total volume of 20 μL according to manufacturer's instructions. Reverse transcriptase–polymerase chain reaction (RT-PCR) for the detection of IL-8 mRNA was performed as described by Brenner et al.[27] In brief, PCR was performed using 1 μL template complementary DNA (cDNA), 0.5 U Taq polymerase (Stratagene, La Jolla, CA), 1.25 μmol/L oligomer primers, and 0.75 mmol/L dNTPs in a total volume of 20 μL. Interleukin-8 message was amplified by 30 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds in an automatic thermocycler (Pekin-Elmer Cetus DNA thermocycler, Newwalk, CA). The PCR products were resolved by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. β-actin was used as internal control. The primer sequences were as follows: IL-8 sense, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′; IL-8 antisense, 5′-TCTCAGCCCTCTTCAA -AAACTTCTC-3′; β-actin sense, 5′-GTGGGGCGCCCCAGGCACCA-3′; and β-actin antisense, 5′-CTCCTTAATGTCACGCACGATTTC-3′. These primers gave 292 and 540 bp products, respectively.

Northern Blot Analysis

Northern blot analysis to detect IL-8 mRNA was performed as previously described.[7] In brief, total RNA was electrophoresed on a 1% agarose formaldehyde gel, transferred onto a Nytran nylon membrane (Schleicher and Schvell, Dassel, Germany), and fixed by exposure to ultraviolet light for 1 minute. After prehybridization, hybridization was performed overnight at 45°C with a human IL-8 or β-actin probe that was digoxigenin-labeled by random primer method (DIG DNA Labeling Kit; Boehringer Mannheim, Mannheim, Germany). Plasmids containing human IL-8 and β-actin were prepared in our laboratory. After stringent washing, digoxigenin-labeled probes were detected with the DIG Luminescent Detection Kit (Boehringer Mannheim).

Enzyme-Linked Immunosorbent Assay

Interleukin-8 production from adenoidal fibroblasts was stimulated with 1 ng/mL HIE and 100 pg/mL IL-1β. Measurements of IL-8 secretion into cell culture media by ELISA were performed with a Quantikine human IL-8 kit (R&D Systems, Minneapolis, MN) according to manufacturer's protocol.

Statistical Analysis

The data of IL-8 concentrations measured by ELISA were expressed as means ± SE. One-way analysis of variance (ANOVA) was used to calculate differences between experimental groups pretreated with TPCK and controls without TPCK pretreatment. Values of $P < .05$ were considered to be significant.

RESULTS

Activation of NF-κB With Interleukin-1β or Haemophilus influenzae *Endotoxin in Human Adenoidal Fibroblasts*

To test NF-κB activation in human adenoidal fibroblasts, dose–response and time–response studies were performed with HIE or IL-1β stimulation. For the dose–response study, cultured adenoidal fibroblasts were stimulated for 30 minutes with different concentrations of HIE (1, 10, 100, and 1000 pg/mL) or IL-1β (0.1, 1, 10, 100, and 1000 pg/mL) and the activation of NF-κB was examined by EMSA. The EMSA revealed increased binding of NF-κB probe following stimulation with at least 1 pg/mL HIE or 0.1 pg/mL IL-1β, and both HIE and IL-1β induced NF-κB activation in a dose-dependent manner (Fig. 1A and B).

For the time–response study, human adenoidal fibroblasts were stimulated with 1 ng/mL HIE or 100 pg/mL IL-1β and nuclear proteins were extracted 15 and 30 minutes and 1, 2, 6, 24, and 48 hours after stimulation with HIE or IL-1β. The EMSA revealed initiation of NF-κB activation occurring at least 15 minutes after stimulation with HIE or 5 minutes after IL-1β treatment (Fig. 2A and B). Peak NF-κB activation occurred approximately 60 minutes after stimulation with either HIE or IL-1β and remained high after 48 hours. Thus, these results indicate that human adenoidal fibroblasts are capable of inducing NF-κB activation in response to HIE or IL-1β.

Effects of NF-κB Inhibitor TPCK on the Expression of IL-8 in Human Adenoidal Fibroblasts

To investigate the role of NF-κB in IL-8 expression, we examined the effects of an NF-κB inhibitor, TPCK, on the expression of IL-8 in human adenoidal fibroblasts. As expected, the EMSA revealed suppression of NF-κB activation in adenoidal fibroblasts by TPCK (Fig. 3A and B). Interleukin-8 gene expression, as assessed by RT-PCR, was enhanced both by HIE and IL-1β, and that increase was strongly suppressed by TPCK (Figs. 4A and 5A). Further, on ELISA it could be seen that TPCK significantly suppressed HIE-induced and IL-1β–induced IL-8 production in a dose-dependent manner (Figs. 4B and 5B). These results indicate that expression of IL-8 induced by HIE or IL-1β is strongly associated with NF-κB activation in human adenoidal fibroblasts.

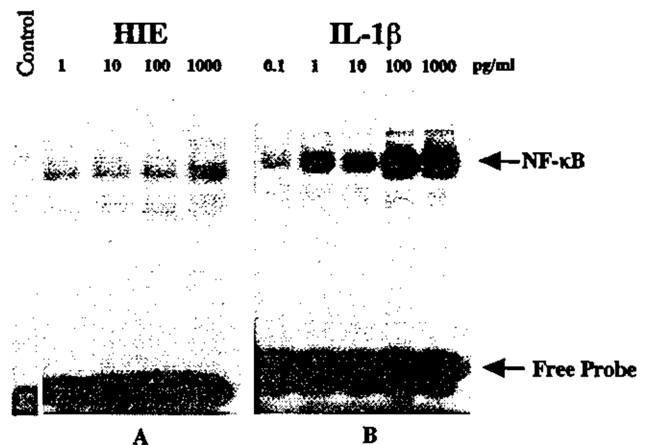


Fig. 1. (A and B) Electrophoretic mobility shift assay for dose-response study of nuclear factor-κB (NF-κB) activation following stimulation with *Haemophilus influenzae* endotoxin (A) or interleukin-1β (B) in human adenoidal fibroblasts. Control indicates the NF-κB binding activity without any stimulation. The arrows indicate specific NF-κB DNA complexes and free probe. Results are representative of three separate experiments.

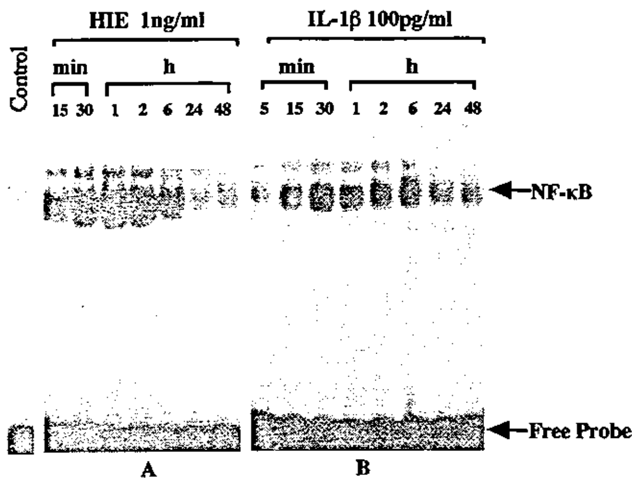


Fig. 2. (A and B) Electrophoretic mobility shift assay for time-response study of nuclear factor-κB (NF-κB) activation following stimulation with *Haemophilus influenzae* endotoxin (A) or interleukin-1β (B) in human adenoidal fibroblasts. Control indicates the NF-κB binding activity without any stimulation. The arrows indicate specific NF-κB DNA complexes and free probe. Results are representative of three separate experiments.

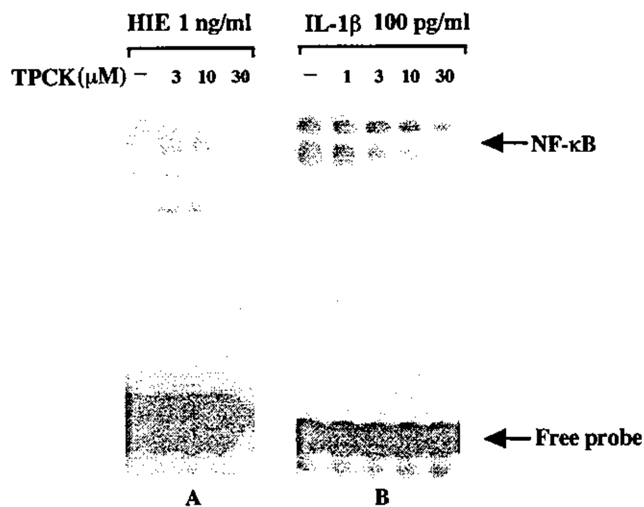
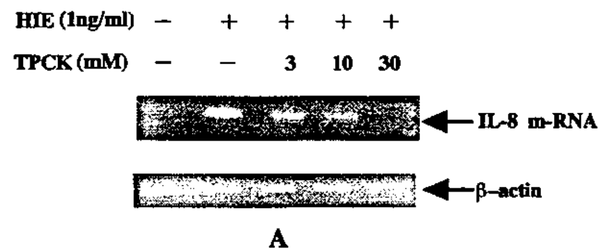


Fig. 3. (A and B) Inhibitory effect of nuclear factor-κB (NF-κB) inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) on NF-κB activation with *Haemophilus influenzae* endotoxin (HIE) (A) or inteleukin-1β (IL-1β) (B). Cultured adenoidal fibroblasts were pretreated with different concentrations of TPCK (1, 3, 10, and 30 μmol/L) for 30 minutes before stimulation with 1 ng/mL HIE (A) or 100 pg/mL IL-1β (B). Nuclear proteins were extracted 30 minutes after stimulation; then electrophoretic mobility shift assay was performed. The arrows indicate specific NF-κB DNA complexes and free probe. Results are representative of three separate experiments.

Effects of Clarithromycin on Expression of Interleukin-8 and NF-κB Activation

Clarithromycin has been reported to suppress the production of IL-8 and NF-κB activation in human monocyte and bronchial cells.[19,20] However, the effects on human adenoidal fibroblasts were not yet investigated. Dexamethasone, which has been demonstrated to inhibit the activation of NF-κB,[7] was used as positive control. In



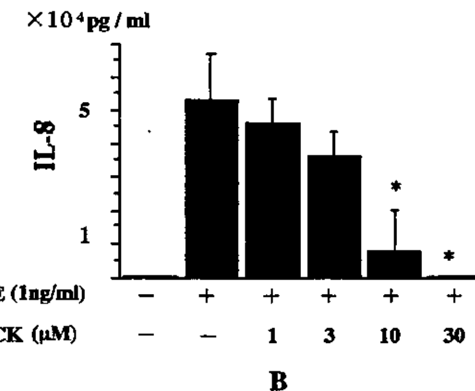


Fig. 4. (A and B) Effect of nuclear factor-κB (NF-κB) inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) on expression of interleukin-8 (IL-8) induced by *Haemophilus influenzae* endotoxin (HIE). (A) Cultured adenoidal fibroblasts were pretreated with different concentrations of TPCK (1, 3, 10, and 30 μmol/L) for 30 minutes, then stimulated with 1 ng/mL HIE. Total RNA was isolated 8 hours after stimulation, and IL-8 messenger RNA (mRNA) was detected by reverse-transcriptase–polymerase chain reaction. The arrows indicate specific amplified products for IL-8 (292 bp) and β-actin mRNA (540 bp). Results are representative of three separate experiments. (B) Cells were pretreated with TPCK, and the concentrations of IL-8 in cell culture media 24 hours after stimulation with 1 ng/mL HIE were examined by ELISA. Values are expressed as means ± SE of three separate experiments. *$P < .05$ compared with control samples stimulated with HIE without TPCK pretreatment (one-way analysis of variance).

preliminary experiments, no IL-8 signal was detected by Northern blot analysis without prior stimulation with IL-1β (data not shown), but both CAM and DEX reduced the expression of IL-8 induced by IL-1β in human adenoidal fibroblasts (Fig. 6A). The NF-κB stimulatory activity of IL-1β was suppressed by CAM in a dose-dependent manner as shown by EMSA (Fig. 6B). Thus, these results indicate that CAM has suppressive effects on IL-8 expression and NF-κB activation in human adenoidal fibroblasts.

DISCUSSION

The present study shows that both HIE and IL-1β induce expression of both IL-8 protein and mRNA in adenoidal fibroblasts. Endotoxin is the major component of the outer surface of Gram-negative bacteria such as NTHi and is a potent activator of the immune system and inflammatory cytokine production.[7] Recently, molecular biological studies have demonstrated that toll-like receptor 4 (TLR4), a member of human TLR family, is a receptor for endotoxin and that its cytoplasmic domain is homologous to that of the IL-1 receptor.[28,29] The binding of endotoxin

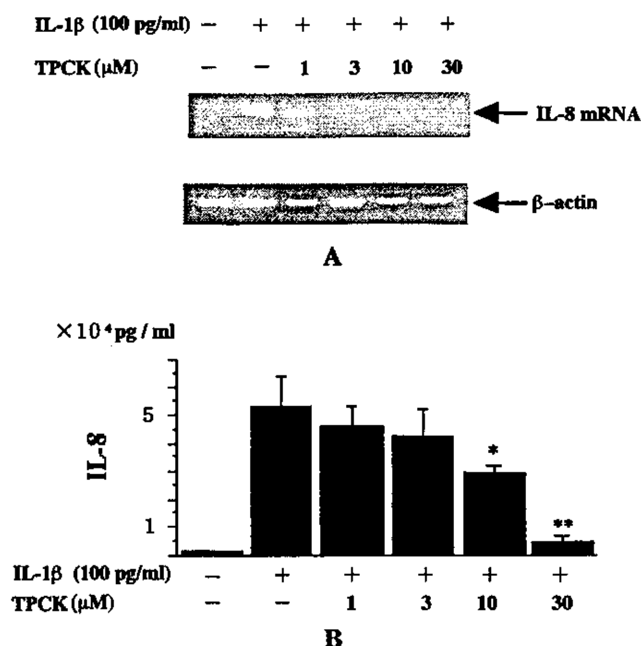


Fig. 5. (A and B) Effect of nuclear factor-κB (NF-κB) inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) on expression of interleukin-8 (IL-8) induced by interleukin-1β (IL-1β). (A) Interleukin-8 (IL-8) messenger RNA was examined by reverse transcriptase–polymerase chain reaction when TPCK-pretreated adenoidal fibroblasts were stimulated with 100 pg/mL IL-8. Results are representative of three separate experiments. (B) Cells were pretreated with TPCK, and concentrations of IL-8 in cell culture media 24 hours after stimulation with 100 pg/mL IL-1β were examined by ELISA. *$P < .05$ and **$P < .01$ compared with control samples stimulated with IL-1β without TPCK pretreatment (one-way analysis of variance).

by TLR4 and IL-1 receptor recruits IL-1 receptor-associated kinase (IRAK) and IL-1 receptor-associated kinase-2 (IRAK-2) with adapter protein MyD88. IRAK and IRAK-2 interact subsequently with adapter molecule tumor necrosis factor receptor–associated factor 6 (TRAF6), which in turn attracts two more protein tyrosine kinases, transforming growth factor-β–activated kinase 1 (TAK-1) and TAK-1–binding protein 1 (TAB-1). These events eventually lead to the activation of NF-κB and activator protein-1 (AP-1), which induce IL-8 and the other inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α).[16,17,29,30] Those findings explain why the responses of adenoidal fibroblasts after the stimulation with HIE were similar to that with IL-1β.

Nuclear factor-κB is a transcriptional factor regulating the gene expression of inflammatory cytokines and adhesion molecules such as TNF-α, IL-1, IL-6, and vascular cell adhesion molecule (VCAM).[31] Inactive NF-κB is sequestered in the cytoplasm by IκB, but stimulation with endotoxin or IL-1β induces proteolytic degradation of IκB allowing translocation of NF-κB into the nucleus where it binds to the promoter regions and drives the transcriptional induction of target genes of several inflammatory cytokines.[31] In the present study, NF-κB was found to be activated gradually after stimulation with HIE and IL-1β, and activation was maintained for more than 48 hours. Further, HIE and IL-1β enhance activation of NF-κB in a dose-dependent manner. These results are consistent with the findings reported by Chow et al.[32] demonstrating endotoxin-induced NF-κB–mediated gene expression in cells transfected with the TLR4 gene in a time- and dose-dependent fashion. The present study also demonstrates that release of IL-8 protein and the expression of IL-8 mRNA were remarkably reduced by the treatment with the NF-κB inhibitor TPCK. The results indicate that production of IL-8 from adenoidal fibroblasts in response to stimulation with HIE or IL-1β is primarily dependent on activation of NF-κB.

Interleukin-8 is consistently present in middle ear effusions.[12,13] The levels of IL-8 in middle ear effusions are highest among the inflammatory cytokines measured and correlate with IL-1β levels.[12] Moreover, a strong, statistically significant correlation exists between concentrations of endotoxin and IL-1β,[33] suggesting that production of IL-8 in the middle ear is associated with presence of IL-1β and endotoxin. Further, concentrations of IL-8 are elevated in nasopharyngeal secretions of children with RSV disease.[6] Preincubation or simultaneous incubation of RSV with recombinant IL-1 receptor antagonist almost entirely blocked the production of IL-8 by respiratory epithelial cells.[34] Lindberg et al.[5] found substantial amounts of IL-1β in nasopharyngeal secretions and significant increases in these levels in children whose nasopharynx was colonized with NTHi. Further, the present study demonstrates that adenoidal fibroblasts produced IL-8 in response to stimulation with endotoxin or IL-1β. By viewing these findings in toto, we speculate that the adenoids are one of the important sources of IL-8 in the nasopharynx, the production of which is induced by endotoxin and IL-1β, as observed in the middle ear.

The NTHi pathogen has been isolated from adenoid tissues as well as nasopharyngeal secretions, and the incidence is significantly higher in the adenoids of children with middle ear disease and rhinosinusitis symptoms compared with patients without that disease or those symptoms.[4,35] Forsgren et al.[36,37] demonstrated that NTHi is found in twice as many cases in the adenoid hypertrophy group as in the OME group. They further showed that NTHi pathogens reside in macrophage-like cells located subepithelially in the crypts of the adenoids. The NTHi pathogen contains endotoxin capable of inducing the production of a variety of inflammatory cytokines such as IL-1 and IL-8 from macrophages and fibroblasts.[7,8] Moreover, outer membrane proteins of NTHi stimulate the production of immunoregulatory cytokines such as IL-2, IL-4, IL-10, and IFN-γ from lymphocytes that are known to modulate adenoidal immune responses.[9,38] Those findings suggest that NTHi harbored within the adenoids may chronically stimulate the local inflammatory and immune responses, which might be associated with the pathogenesis of adenoidal inflammation and hypertrophy.

Clarithromycin remarkably suppresses IL-1β-induced expression of IL-8 and mRNA from adenoidal fibroblasts in a dose-dependent manner, although the effects were slightly lower than those of DEX. Macrolides such as erythromycin and CAM are known to be effective