ELSEVIER

Available online at www.sciencedirect.com
ScienceDirect

Molecular Immunology 44 (2007) 2249–2256

Molecular Immunology

www.elsevier.com/locate/molimm

Chromatin remodeling at the Th2 cytokine gene loci in human type 2 helper T cells

Takaaki Kaneko[a,b], Hiroyuki Hosokawa[a], Masakatsu Yamashita[a], Chrong-Reen Wang[c], Akihiro Hasegawa[a], Motoko Y. Kimura[a], Masayuki Kitajiama[a], Fumio Kimura[b], Masaru Miyazaki[b], Toshinori Nakayama[a,]

[a] *Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*
[b] *Department of General Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*
[c] *Section of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Medical College, National Cheng Kung University, Tainan, Taiwan*

Received 25 August 2006; received in revised form 1 November 2006; accepted 6 November 2006
Available online 12 December 2006

Abstract

The differentiation of mouse naïve CD4 T cells into type 2 helper (Th2) cells is accompanied by chromatin remodeling at the nucleosomes associated with the IL-4, IL-13 and IL-5 genes. However, little is known about how chromatin remodeling of these Th2 cytokine gene loci occurs in human Th2 cells. We herein established an *in vitro* culture system in which both Th1 and Th2 cells are efficiently differentiated from human peripheral blood naïve CD4 T cells. This system allowed us to investigate the chromatin status at the Th2 cytokine gene loci and the IFNγ locus in human Th2 and Th1 cells, respectively. In typical individuals, the chromatin remodeling indicated by the induction of hyper-acetylation of histone H3 lysine 9 and hyper-methylation of histone H3 lysine 4 was induced at the whole Th2 cytokine gene loci in developing Th2 cells. We more precisely assessed the methylation status of histone H3 lysine 4 at the Th2 cytokine gene loci (IL-5 exon 3, IL-5 promoter, IL-5/RAD50 intergenic region, RAD50 promoter, CGRE, CNS1, IL-13 promoter, IL-4 promoter, and V_A enhancer regions) and the IFNγ locus in developing Th1 and Th2 cells prepared from 20 healthy volunteers. Th2-cell specific chromatin remodeling was induced at most of the Th2 cytokine gene loci. In parallel with the induction of chromatin remodeling, GATA3 mRNA was preferentially expressed in developing Th2 cells, whereas T-bet, HLX and ROG mRNA was selectively expressed in developing Th1 cells.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Th1/Th2 cells; Chromatin remodeling; Human Th2 gene locus

1. Introduction

CD4 helper T cell-dependent immune responses are controlled by the balance of antigen-specific Th1 and Th2 cells (Mosmann and Coffman, 1989; Reiner and Locksley, 1995; Seder and Paul, 1994). Th1 cells produce IFNγ and Th2 cells produce IL-4, IL-5 and IL-13. For Th2 cell differentiation, IL-4R-mediated signal transduction, including signal transducer and activator of transcription (STAT) 6 activation is required, while IL-12-mediated STAT4 activation plays an important role in Th1 cell differentiation (Constant and Bottomly, 1997; Gately et al., 1998; Murphy et al., 2000; Nelms et al., 1999; O'Garra, 1998). In addition to these cytokines, TCR-mediated signaling is also indispensable for Th1/Th2 cell differentiation (Constant and Bottomly, 1997). In particular, the fate of Th1/Th2 cell differentiation appears to be controlled by the TCR-mediated activation of the Ras/MAPK cascade (Shibata et al., 2002; Yamashita et al., 1999; Yamashita et al., 2005). Both c-Jun NH_2-terminal kinase (JNK) and the p38 MAPK cascade have also been reported to play an important role in Th1 cell differentiation and Th1 cytokine production (Dong et al., 1998; Rincon et al., 1998; Yang et al., 1998). The role for NF-κB activation in Th1/Th2 cell differentiation has also been suggested (Barnes and Karin, 1997; Das et al., 2001; Inami et al., 2004). Several transcription factors that control Th1/Th2 cell differentiation have been identified. Among them, GATA3 appears to be a key factor for

Corresponding author. Tel.: +81 43 226 2200; fax: +81 43 227 1498.
E-mail address: tnakayama@faculty.chiba-u.jp (T. Nakayama).

0161-5890/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2006.11.004

Th2 cell differentiation (Lee et al., 2000; Ouyang et al., 1998; Zhang et al., 1997; Zheng and Flavell, 1997), and T-bet was recently identified to be a key transcription factor for Th1 cell differentiation (Szabo et al., 2000).

Changes in the chromatin structure, chromatin remodeling of the murine Th2 cytokine (IL-4/IL-5/IL-13) gene loci occur during Th2 cell differentiation (Ansel et al., 2006; Lee et al., 2006; Lohning et al., 2002). Various modifications of the N-terminal tail of histones play critical roles in the epigenetic regulation of transcription. Among them, the acetylation of lysine 9 of histone H3 (H3-K9) and the methylation of lysine 4 of histone H3 (H3-K4) are typically associated with the transcriptionally active regions of chromatin (Turner, 2002). We and others recently demonstrated that histone H3-K9 hyper-acetylation of the Th2 cytokine gene loci occurs in developing Th2 cells in a Th2-specific and STAT6/GATA3-dependent manner (Avni et al., 2002; Yamashita et al., 2002). In addition, the long-range histone hyper-acetylation regions within the IL-13/IL-4 and IL-5 gene loci in developing Th2 cells and Tc2 cells were revealed (Inami et al., 2004; Omori et al., 2003; Yamashita et al., 2002).

In human, the promoter region of the IFNγ gene locus was reported to be hypermetylated in Th2 cells but hypomethylated in Th1 cells (Yano et al., 2003). In addition, histone acetylation of the IFNγ gene locus was associated with Th1 cell differentiation (Morinobu et al., 2004). The induction of DNase I hypersensitive sites and CpG demethylation at the IL-4 and IL-13 gene loci occurred during human Th2 cell differentiation (Santangelo et al., 2002). Subsequently, histone H3-K9 hyper-acetylation at the IL-4 promoter region in human memory Th2 cells was reported (Messi et al., 2003). However, it has not been reported how histone modifications at the various Th2 cytokine gene loci occur in developing human Th2 cells. We herein established an *in vitro* Th1/Th2 cell differentiation system using peripheral blood human naïve CD4 T cells, and extensively investigated the histone modifications at the various regions within the Th2 cytokine gene loci. Our results indicate that histone H3-K9/14 hyper-acetylation and H3-K4 hyper-methylation occurred throughout the Th2 cytokine gene loci in human developing Th2 cells.

2. Materials and methods

2.1. Purification of naïve CD4 T cells and differentiation of the naïve CD4 T cells into Th1 and Th2 cells

The protocol was approved by the Institutional Ethics Committee, Graduate School of Medicine, Chiba University. Human PBMC were isolated from healthy volunteer by Ficoll-Paque density gradient centrifugation, and naïve CD4 T cells ($CD4^+$, $CD45RA^+$) were purified with human naïve CD4 T cell isolation kits (Mylteni Biotec Inc.) and an auto-MACS sorter. The purity ($CD4^+$, $CD45RA^+$) was more than 95% (Supplemental Figure 1). We used these cells for naïve T cells. For Th1 and Th2 cells, the enriched naïve CD4 T cells (1×10^6) were stimulated for 2 days with 20 μg/ml immobilized anti-CD3 mAb (Orthoclone OKT3 Injection, Raritan, NJ) in the presence of 3 μg/ml anti-CD28 mAb (BD-555725; Pharmingen), 50 units/ml IL-2 (Imunace 35, Shionogi & Co. Ltd., Osaka, Japan), 1 ng/ml IL-12 (no. 200-12; R&D systems), and 5 μg/ml anti-IL-4 mAb (BD-554481; Pharmingen) for Th1 culture conditions. For Th2 conditions, the cells were stimulated with 20 μg/ml immobilized anti-CD3 mAb in the presence of 3 μg/ml anti-CD28 mAb, 50 units/ml IL-2, 1 ng/ml IL-4 (no. 204-IL; R&D systems), and 5 μg/ml anti-IFNγ mAb (BD-554547; Pharmingen). The cells were then transferred to new plates, and cultured for another 5 days in the presence of cytokines and antibodies used in the original culture. Another cycle of the stimulation was performed. The cultured T cells were re-stimulated with 10 ng/ml phorbol myristate acetate (PMA) and 1 μM ionomycin for 4 h in the presence of 2 μM monensin. The Th1/Th2 cell differentiation was assessed by intracellular cytokine staining (Yamashita et al., 2002). Anti-IL-4 mAb (no. 340451; Becton Dickinson) and anti-IFNγ mAb (no. 340449; Becton Dickinson) were used.

2.2. ELISA

The cultured human Th1/Th2 cells were re-stimulated with 10 ng/ml PMA and 1 μM ionomycin in 48-well flat bottom plates for 24 h. The production of IL-2, IL-4, IL-5, IL-13 and IFNγ was measured by ELISA as previously described (Ishikawa et al., 2005; Shirai et al., 2003).

2.3. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using histone H3 ChIP assay kits (no. 17-245; Upstate Biotechnology) with antibodies specific for acetylated histone H3 (at both lysine residues 9 and 14; upstate #06-599) and dimethylated histone H3 (at lysine residues 4; abcam ab7766) as described previously (Yamashita et al., 2002, 2006). In brief, 2×10^6 CD4 T cells were fixed with paraformaldehyde (1%) at 37 C for 15 min. The cells were sedimented, washed, lysed with a SDS lysis buffer (50 mM Tris–HCl, 1% SDS, 10 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin A). The lysates were sonicated to reduce DNA lengths to between 200 and 1000 bp. The soluble fraction was diluted, precleared with salmon sperm DNA/protein A agarose, and then incubated with antibody (5 μg/ml) specific for the acetylated and dimethylated forms of histones H3. Next, immune complexes were precipitated with protein A agarose. The precipitated DNA was eluted with an elution buffer (0.1 M $NaHCO_3$ containing 1% SDS). The eluted material was incubated at 65 C for 6 h to reverse the formaldehyde cross-links, and DNA was extracted with phenol and chloroform. Ethanol-precipitated DNA was solubilized in water (2×10^6 cell equivalent/100 μl). Semi-quantitative PCR was performed with 3 and 1 μg of DNA samples (a three-fold dilution) at 31 cycles. PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide. The images were recorded and quantified using ATTO L&S analyzer (ATTO, Tokyo, Japan). Semi-quantitative PCR was performed using the primers listed in Supplemental Table 1.

2.4. Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (GIBCO BRL). Reverse transcription was done using Superscript II (Gibco-BRL). For quantitative real time PCR, a TaqMan universal PCR master mix was used for all reactions (Applied Biosystems), and the ABI Prism 7000 Sequence Detection System was employed (Kimura et al., 2005). The primers and TaqMan probes for the detection of human GATA3, T-bet, Eomesodermin, HLX, ROG and 18S were purchased from Applied Biosystems. The data are shown as the relative expression to the 18S signal.

3. Results

3.1. Histone H3-K9/14 hyper-acetylation and H3-K4 hyper-methylation at the Th2 cytokine gene loci in human Th2 cell

In order to examine the chromatin status of the human Th2 cytokine gene loci during the differentiation of naïve CD4 T cells into Th1 and Th2 cells, we established an *in vitro* differentiation system using freshly isolated human peripheral blood T cells. Naïve CD4 T cells ($CD4^+$, $CD45RA^+$) were cultured under Th1/Th2 culture conditions as described in the Materials and methods. The intracellular IFNγ/IL-4 profiles (Fig. 1A) and the levels of cytokine production (IL-4, IL-5, IL-13, and IFNγ) assessed by ELISA are shown (Fig. 1B). The developing Th2 cells produced substantial amounts of IL-4, IL-5 and IL-13 upon restimulation, and Th1 cells produced IFNγ selectively.

In order to examine the acetylation status of H3-K9/14 and the methylation status of H3-K4 in naïve CD4 T, *in vitro* differentiated Th1 and Th2 cells, we prepared a series of primer pairs throughout the Th2 cytokine gene loci (Fig. 1C). The ChIP assay was performed with antibodies specific for acetylated H3-K9/14 and dimethylated H3-K4. The real PCR bands (Fig. 1D) and the relative intensity of PCR bands (anti-acetyl H3-K9/14 or anti-dimethyl H3-K4/input DNA) are shown in Fig. 1E. The levels of histone H3-K9/14 acetylation associated with the Th2 cytokine gene loci (IL-5p, CGRE, IL-13p, CNS1, IL-13 intergenic, IL-4p, IL-4 exon 3, V_A enhancer, and CNS2 regions) were substantially higher in Th2 cells than in either naïve CD4 T or Th1 cells (Fig. 1E, left panel). In addition, the Th2-specific histone H3-K4 hyper-methylation at the Th2 cytokine gene loci (IL-5p, CGRE, IL-13p, CNS1, IL-13 intergenic, IL-4p, IL-4 exon 3, V_A enhancer, CNS2 and IL-4 intergenic regions) was observed (Fig. 1E, right panel). The acetylation of histone H3-K9/14 and methylation of histone H3-K4 at the RAD50 gene was equiva-

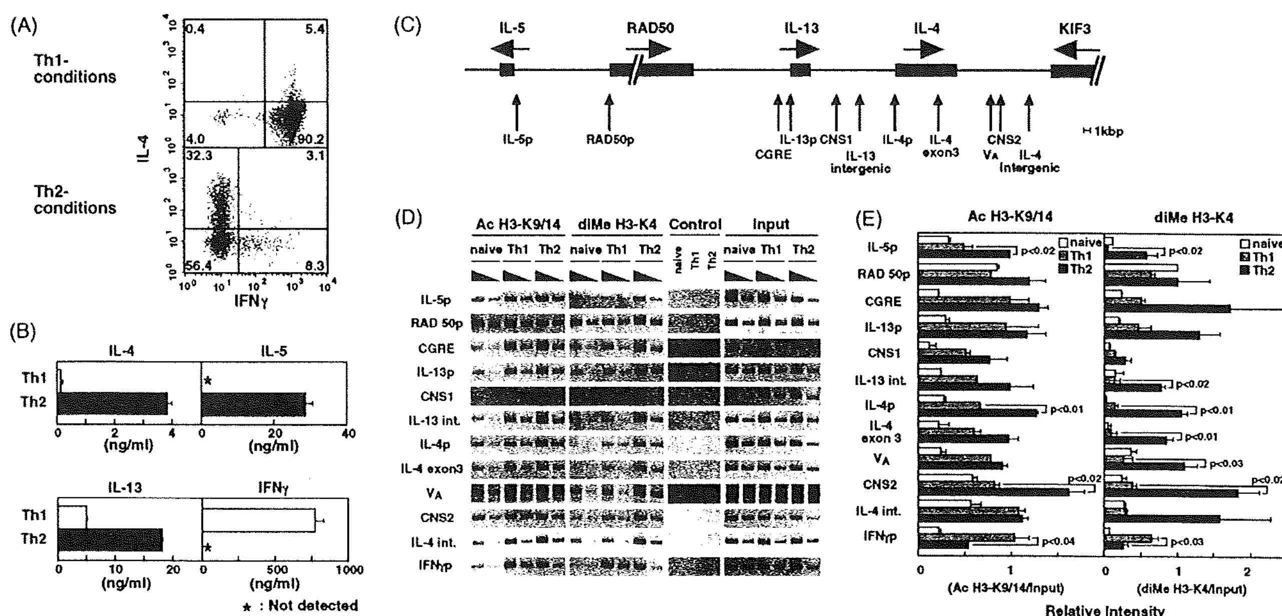

Fig. 1. Acetylation and methylation status of histone H3 at the Th2 cytokine gene loci. Human naïve CD4 T cells ($CD4^+$, $CD45RA^+$) purified from peripheral blood mononuclear cells were stimulated under Th1/Th2 conditions. (A) The levels of Th1/Th2 cell generation were assessed by intracellular cytokine staining. Representative IFNγ/IL-4 profiles are shown. (B) *In vitro* cultured Th1 and Th2 cells were stimulated with PMA and Ionomycin for 24 h, and the amounts of IL-4, IL-5, IL-13 and IFNγ in the culture supernatant were assessed by ELISA. The mean values with standard deviation of the triplicate cultures are shown. (C) Schematic representation of the human Th2 cytokine gene loci on chromosome 5q, and the locations of PCR primer pairs used in the ChIP assay are shown. (D) Acetylation status of histone H3-K9/14 and methylation status of H3-K4 at the Th2 cytokine gene loci. An isotype-matched control Ab (normal rabbit IgG, Santa Cruz sc-2027) was used. The ChIP assay was performed with naïve CD4T, Th1 and Th2 cells using the indicated primer pairs. Before immunoprecipitation, aliquots (6×10^2 cell equivalents) were removed for PCR to determine the relative levels of input DNA. To quantify the reactive levels of acetylation and methylation, a three-fold serial dilution was made with both the input DNA and immunoprecipitated DNA samples before PCR, and the PCR product intensities were then assessed. Three independent experiments with different T cell preparations were performed with similar results. (E) Quantitative representations of the bands measured at the highest concentration by densitometry, and the relative intensities (immunoprecipitates with anti-acetyl H3-K9 or anti-dimethyl H3-K4/input DNA) in each primer pair were calculated. The mean values with standard deviation are shown. The Student *p* values are shown where the difference is statistically significant.

lent in naïve CD4 T, Th1 and Th2 cells. As expected, increased histone H3-K9/14 hyper-acetylation and histone H3-K4 hyper-methylation on the IFNγ promoter region was preferentially observed in Th1 cells.

3.2. Histone H3-K9/14 hyper-acetylation and H3-K4 hyper-methylation around the IL-5 gene locus in developing Th2 cells

We previously reported that the intergenic region between the IL-5 and RAD50 gene locus was hyper-acetylated in a long range in mouse Th2 cells (Inami et al., 2004). No conserved GATA binding sequences between mouse and human was found around the downstream border of the long-rage acetylation region. This is in contrast to CGRE (conserved GATA3 response element) which existed in the upstream region of the IL-13 gene locus, and at the border of the long range hyperacetylation region within the IL-13 and IL-4 gene loci (Yamashita et al., 2002). Consequently, we more precisely examined the histone modifications at the intergenic region of IL-5 ad RAD50 by preparing a series of primer pairs between the IL-5 and RAD50 gene loci (Fig. 2A). A representative result of a ChIP assay of naïve CD4 T cells and *in vitro* differentiated Th1 and Th2 cells from a HV are shown in Fig. 2B. The relative band intensities (anti-acetyl H3-K9 or anti-dimethyl H3-K4/input DNA) of 19 primer pairs are depicted in Fig. 2C. The long-range Th2-specific histone H3-K9/14 hyper-acetylation and H3-K4 hyper-methylation were observed between the IL-5 and the RAD50 gene locus. Th1 cells showed a slightly increased acetylation as compared with that of naïve CD4 T cells, whereas no increased H3-K4 methylation was detected in Th1 cells.

3.3. Histone H3-K4 hyper-methylation in in vitro differentiated Th2 cells prepared from 20 healthy volunteers

In order to generalize the observation above, we next assessed the levels of histone H3-K4 methylation at the selected regions (IL-5 exon 3, IL-5p, IL-5 intergenic, RAD50p, CGRE, IL-13p, CNS1, IL-4p, V_A, and IFNγp) in developing Th1/Th2 cells prepared from 20 healthy volunteers (HV). The intracellular IFNγ/IL-4 profiles of the *in vitro* cultured developing Th1/Th2 cells from 20 HV are shown in Supplemental Figure 2. A ChIP assay using antibodies specific for dimethylated histone H3-K4 was performed. The real PCR bands are shown in Supplemental Figure 3, and the relative intensity (anti-dimethyl histone H3-K4/input DNA) is shown in Supplemental Figure 4. The mean values with standard deviations of the relative intensity for 20 HV are shown in Fig. 3B. The mean values of the methylated histones at the IL-5 exon 3, IL-5 promoter, IL-5 intergenic, CGRE, IL-13 promoter and IL-4 promoter regions were significantly higher in Th2 cells than those in Th1 cells. The levels of methylation of RAD50 promoter and CNS1 and V_A enhancer regions were not significantly different between Th1 and Th2 cells. The mean value of the methylated histones on IFNγ promoter was statistically higher in Th1 cells than in Th2 cells.

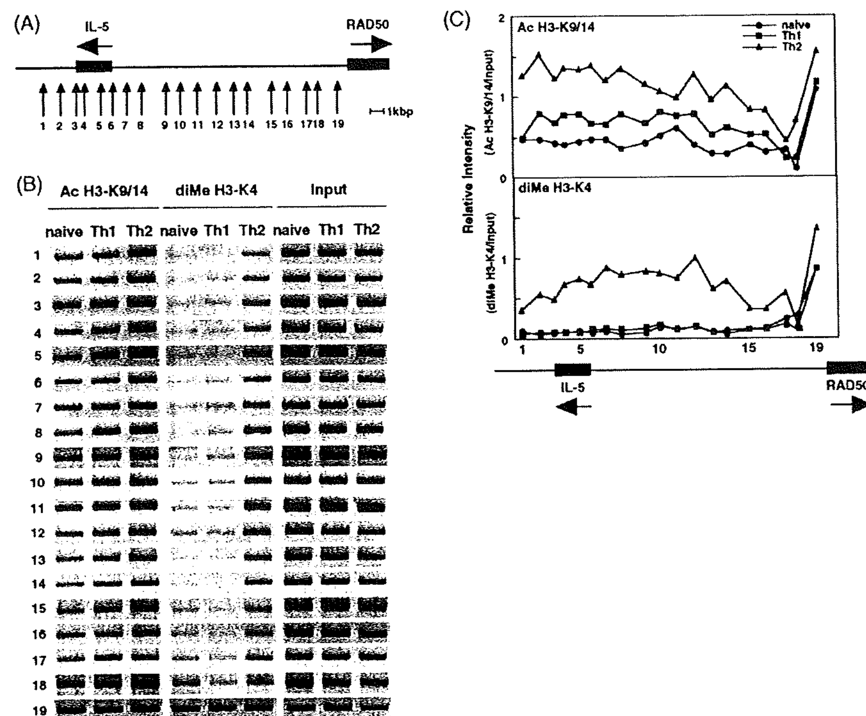

Fig. 2. Hyper-acetylation of histone H3-K9 and hyper-methylation of histone H3-K4 around the IL-5 and RAD50 loci in developing Th2 cells. (A) Schematic representation of the human IL-5 and RAD50 loci and the locations of PCR primer pairs used in the ChIP assay. (B) Naïve CD4 T, developing Th1 and Th2 cells were prepared and subjected to ChIP assays with the primer pairs indicated in (A). A representative result of a HV is shown. (C) The relative PCR band intensities (immunoprecipitates with anti-acetyl H3-K9 or anti-dimethyl H3-K4/input DNA) in each primer pair shown in panel B are shown.

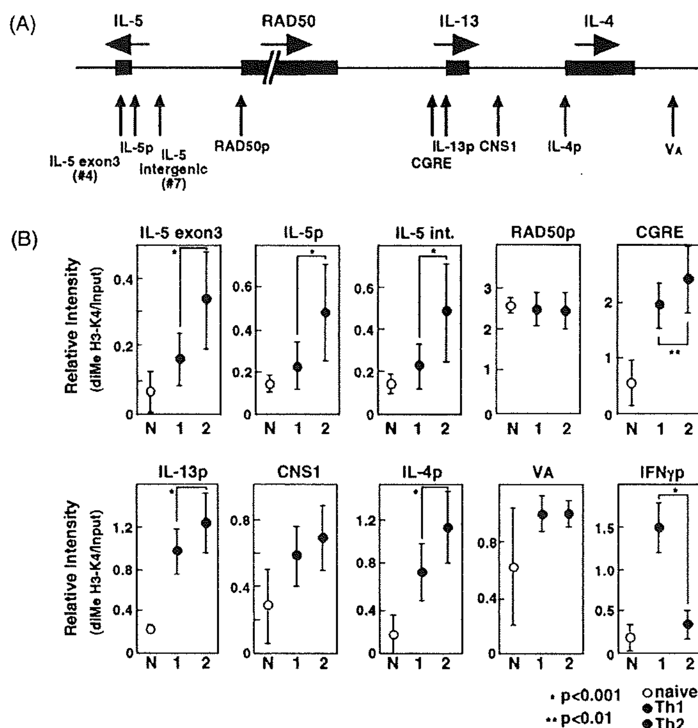

Fig. 3. Hyper-methylation of histone H3-K4 at the Th2 cytokine gene loci in *in vitro* differentiated Th2 cells. (A) A schematic representation of the Th2 cytokine gene loci and the locations of PCR primer pairs used in the ChIP assay. The primer named IL-5 exon 3 is the same as #4 primer used in Fig. 2. The primer named IL-5 intergenic is the same as #7 primer used in Fig. 2. (B) The methylation status of histone H3-K4 at the indicated region in naïve CD4T and *in vitro* differentiated Th1 and Th2 cells from 20 HV. ChIP assays with an anti-dimethyH3-K4 antibody, and the measurement of the relative intensities of the PCR bands were performed as described in Fig. 1. The mean values with standard deviation are shown. The student *p* values are shown in each panel.

3.4. mRNA expression levels of transcription factors that are preferentially expressed in Th1/Th2 cells

In a mouse system, the mRNA expression levels of various transcription factors (GATA3, T-bet, Eomesodermin, HLX, and ROG) are well documented to be involved in Th1/Th2 cell differentiation. Consequently, we wanted to examine the expression levels of these factors in our *in vitro* differentiated human Th1/Th2 cells. mRNA prepared from naïve and the *in vitro* cultured Th1 and Th2 cells from 20 HV were subjected to real time PCR analysis, and the relative expression (/18S) of the above transcription factors are shown in Fig. 4A. The mean values with standard deviations are shown in Fig. 4B. The expression levels of GATA3 mRNA were significantly higher in the developing Th2 cells than those of the developing Th1 cells. Whereas the mRNA expression levels of T-bet, HLX, and ROG were higher in the developing Th1 cells than in the Th2 cells. The levels of Eomesodermin were not statistically different between Th1 and Th2 cells. Moreover, the cytokine production data are shown in Fig. 4C. The results indicate that basically no IL-4, IL-5 or IFNγ was produced in naïve T cells, while there was a preferential induction of IL-4 and IL-5 in Th2 cells, and that of IFNγ in Th1 cells.

4. Discussion

In the present study, we established *in vitro* Th1/Th2 cell differentiation culture system using human peripheral blood naïve CD4 T cells. Using these *in vitro* developing Th1 and Th2 cells, we provide the first evidence indicating that in the human system, histone H3-K9/14 hyper-acetylation and H3-K4 hyper-methylation occurred throughout the Th2 cytokine gene loci in developing Th2 cells. Similar to mouse Th2 cells, chromatin remodeling of the intergenic region of the IL-5 and RAD50 gene loci was observed preferentially in Th2 cells.

The Th2 cytokine genes, IL-4, IL-5 and IL-13 are localized within a 125-kb region in the mouse and human (Mohrs et al., 2001). These cytokines are coordinately expressed in Th2 cells. We recently demonstrated that histone H3-K9 hyper-acetylation of the Th2 cytokine gene loci occurs in mouse Th2 cells in a Th2-specific and STAT6/GATA3-dependent manner (Yamashita et al., 2002). In addition, the long-range histone hyper-acetylation regions within the IL-13/IL-4 gene loci (Yamashita et al., 2002) were revealed in developing Th2 cells. The methylation of histone H3-K4 is also known to be an epigenetic marker typically associated with transcriptionally active chromatin (Milne et al., 2002; Nakamura et al., 2002). However, the methylation status of histone H3-K4 at the Th2 cytokine gene loci in human Th2 cells were not formally investigated. In the present study, we extensively investigated the histone H3-K4 methylation within the Th2 cytokine loci using 20 healthy volunteers' Th1/Th2 cell samples, and found that hyper-methylation occurs preferentially in the human developing Th2 cells (Figs. 1 and 3). Histone H3-K9 hyper-acetylation at the enhancer regions for Th2 cytokines (CNS1 and V_A enhancer) was preferentially detected in mouse Th2 cells (Yamashita et al., 2002). Interestingly, however, the

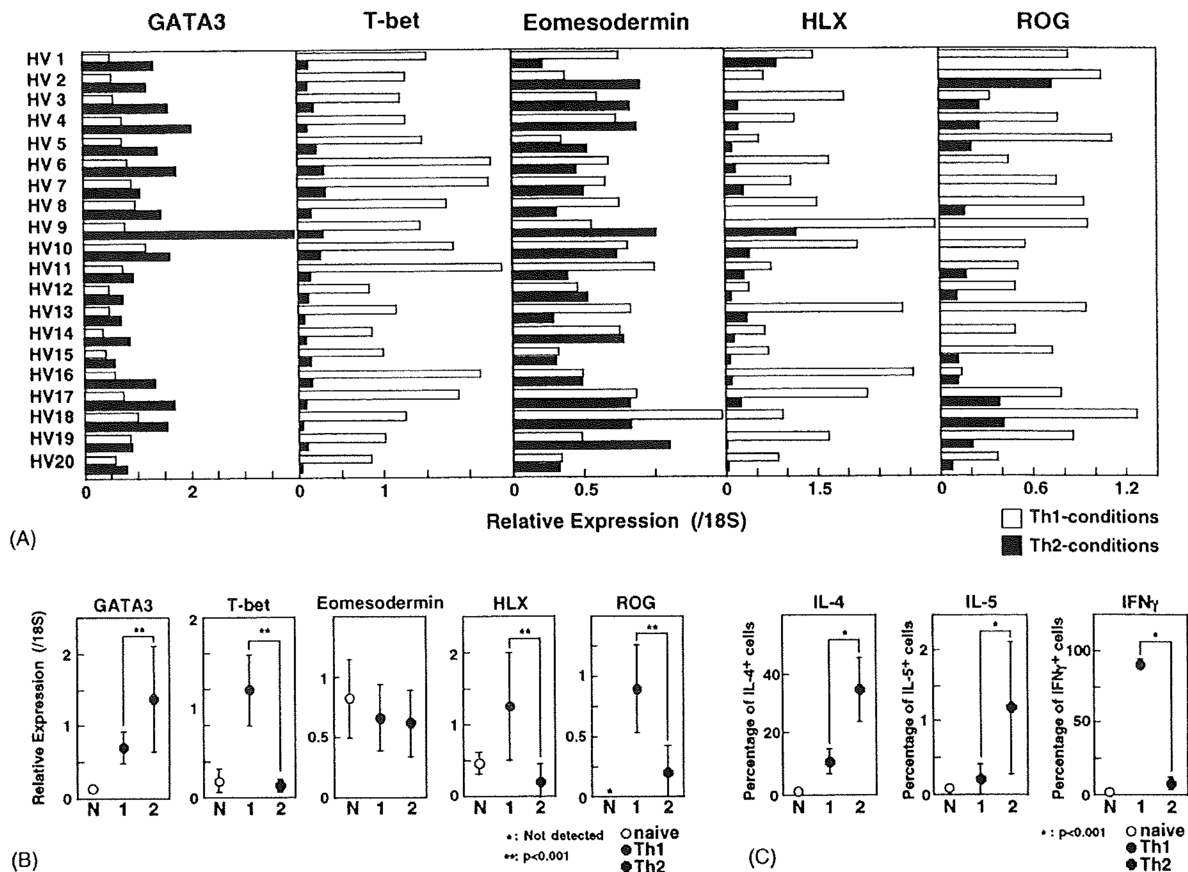

Fig. 4. mRNA expression levels of GATA3, T-bet, Eomesodermin, HLX and ROG in Th1 and Th2 cells. mRNA was prepared from *in vitro* cultured Th1 and Th2 cells from 20 HV, and real time quantitative PCR analysis was performed. (A) The relative expression (/18S) of the GATA3, T-bet, Eomesodermin, HLX, and ROG in Th1 and Th2 cells from 20 healthy volunteers are shown. (B) The mean values and standard deviations are shown. The Student *p* values are shown in each panel. (C) We performed cytoplasmic staining (IL-4, IL-5 and IFNγ) of naïve, Th1 and Th2 cells (20 HV), and the mean values and standard deviations are shown. The Student *p* values are shown in each panel.

levels of the methylation at CNS1 and V_A enhancer regions were not statistically higher in Th2 cells (Fig. 3). Although it is not clear at this time which molecules are responsible for this difference, one cause may be the difference in the increase in the expression of GATA3 between mouse and human Th2 cells. We detected more than a five-fold increase in GATA3 expression in mouse developing Th2 cells as compared with naïve CD4 T or Th1 cells (Omori et al., 2003), whereas the GATA3 expression in the developing human Th2 cells increased at most by about two-fold (Fig. 4). Since the binding affinity of GATA3 to the CNS1 and V_A enhancer regions were low in comparison to CGRE, another GATA3-binding motif critical for chromatin remodeling of the IL-4/IL-13 gene loci (Yamashita et al., 2002), chromatin remodeling of these regions (CNS1 and V_A enhancer) may not be efficiently induced.

More recently, we demonstrated a long-range histone hyper-acetylation with transcriptions within the IL-5 and the intergenic regions between the IL-5 and RAD50 genes (Inami et al., 2004). Similar to mouse Th2 cells, the present analysis revealed that the long-range histone hyper-acetylation of H3-K9/14 accompanied with hyper-methylation of histone H3-K4 occurred in human Th2 cells (Fig. 2). In addition, a relatively unmodified (hypo-acetylation) region was detected at 1600–1200 bp upstream of the RAD50 gene in Th2 cells (Fig. 2). Similar unmodified regions were observed in the mouse system (Inami et al., 2004). Therefore, similar molecular events may operate during the chromatin remodeling of the IL-5 gene locus in mouse and human developing Th2 cells.

As we expected, the expression levels of GATA3 were significantly higher in Th2 cells, while those of T-bet were higher in Th1 cells (Fig. 4). HLX is a Th1-restricted homeobox transcription factor, which appears to interact with T-bet to achieve the induction of an optimal expression of IFNγ in mouse Th1 cells (Mullen et al., 2002). ROG is a POZ (BTB) domain-containing zinc finger repressor, and the expression levels of ROG mRNA were significantly higher in the mouse developing Th1 cells than in Th2 cells (Omori et al., 2003). Similar to the mouse system, we observed an increased expression of HLX and ROG in human Th1 cells using 20 healthy volunteers' Th1/Th2 cells (Fig. 4). Eomesodermin is induced in mouse effector CD8 T cells, and it is necessary for the full effector differentiation of CD8 T cells, including the acquisition of a sufficient IFNγ expression. The expression of Eomesodermin is restricted to CD8 T cells (Pearce et al., 2003). We detected a low level expression of Eomesoder-

min, but no preferential difference was observed between human Th1 and Th2 cells (Fig. 4).

In a mouse system, histone modifications associated with the silencing of the IL-4 and IL-13 genes in Th1 cells were reported (Koyanagi et al., 2005). We thus need to await further analyses addressing the problem of whether similar modifications may also exist in human Th1 cells. There were some differences among individuals in the methylation status of histone H3-K4 at the Th2 cytokine gene loci in 20 HV (Fig. 3 and Supplemental Figure 4). It would therefore be interesting to examine whether the levels of the histone H3-K4 methylation status are associated with the relative risk for certain Th2 immune diseases, including allergic asthma.

In summary, we herein analyzed the chromatin remodeling of the Th2 cytokine gene loci in human developing Th2 cells, particularly in regard to the methylation status of H3-K4, and thus found chromatin remodeling to occur throughout the Th2 cytokine gene loci including the intergenic regions accompanied with an increased GATA3 expression.

Acknowledgements

We thank Dr. Yoichi Suzuki for valuable suggestions and Ms. Hikari Asou, Satoko Norikane and Kaoru Sugaya and Mr. Toshihiro Ito for their excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid for: Scientific Research in Priority Areas #17016010 and #17047007; Scientific Research B #17390139, Scientific Research C #18590466; Grant-in-Aid for Young Scientists #17790318; Special Coordination Funds for Promoting Science and Technology), the Ministry of Health, Labor and Welfare (Japan), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (Japan), The Japan Health Science Foundation, Kanae Foundation and Uehara Memorial Foundation and Mochida Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2006.11.004.

References

Ansel, K.M., Djuretic, I., Tanasa, B., Rao, A., 2006. Regulation of Th2 differentiation and IL4 locus accessibility. Annu. Rev. Immunol. 24, 607–656.

Avni, O., Lee, D., Macian, F., Szabo, S.J., Glimcher, L.H., Rao, A., 2002. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. Nat. Immunol. 3, 643–651.

Barnes, P.J., Karin, M., 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. 336, 1066–1071.

Constant, S.L., Bottomly, K., 1997. Induction of Th1 and Th2 CD4+T cell responses: the alternative approaches. Annu. Rev. Immunol. 15, 297–322.

Das, J., Chen, C.H., Yang, L., Cohn, L., Ray, P., Ray, A., 2001. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat. Immunol. 2, 45–50.

Dong, C., Yang, D.D., Wysk, M., Whitmarsh, A.J., Davis, R.J., Flavell, R.A., 1998. Defective T cell differentiation in the absence of JNK1. Science 282, 2092–2095.

Gately, M.K., Renzetti, L.M., Magram, J., Stern, A.S., Adorini, L., Gubler, U., Presky, D.H., 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. 16, 495–521.

Inami, M., Yamashita, M., Tenda, Y., Hasegawa, A., Kimura, M., Hashimoto, K., Seki, N., Taniguchi, M., Nakayama, T., 2004. CD28 costimulation controls histone hyperacetylation of the interleukin 5 gene locus in developing th2 cells. J. Biol. Chem. 279, 23123–23133.

Ishikawa, E., Motohashi, S., Ishikawa, A., Ito, T., Uchida, T., Kaneko, T., Tanaka, Y., Horiguchi, S., Okamoto, Y., Fujisawa, T., Tsuboi, K., Taniguchi, M., Matsumura, A., Nakayama, T., 2005. Dendritic cell maturation by CD11c-T cells and Valpha24+ natural killer T-cell activation by alpha-galactosylceramide. Int. J. Cancer 117, 265–273.

Kimura, M.Y., Hosokawa, H., Yamashita, M., Hasegawa, A., Iwamura, C., Watarai, H., Taniguchi, M., Takagi, T., Ishii, S., Nakayama, T., 2005. Regulation of T helper type 2 cell differentiation by murine Schnurri-2. J. Exp. Med. 201, 397–408.

Koyanagi, M., Baguet, A., Martens, J., Margueron, R., Jenuwein, T., Bix, M., 2005. EZH2 and histone 3 trimethyl lysine 27 associated with IL4 and IL13 gene silencing in Th1 cells. J. Biol. Chem. 280, 31470–31477.

Lee, G.R., Kim, S.T., Spilianakis, C.G., Fields, P.E., Flavell, R.A., 2006. T helper cell differentiation: regulation by cis elements and epigenetics. Immunity 24, 369–379.

Lee, H.J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., Arai, N., 2000. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J. Exp. Med. 192, 105–115.

Lohning, M., Richter, A., Radbruch, A., 2002. Cytokine memory of T helper lymphocytes. Adv. Immunol. 80, 115–181.

Messi, M., Giacchetto, I., Nagata, K., Lanzavecchia, A., Natoli, G., Sallusto, F., 2003. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. Nat. Immunol. 4, 78–86.

Milne, T.A., Briggs, S.D., Brock, H.W., Martin, M.E., Gibbs, D., Allis, C.D., Hess, J.L., 2002. MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol. Cell 10, 1107–1117.

Mohrs, M., Blankespoor, C.M., Wang, Z.E., Loots, G.G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E.M., Locksley, R.M., 2001. Deletion of a coordinate regulator of type 2 cytokine expression in mice. Nat. Immunol. 2, 842–847.

Morinobu, A., Kanno, Y., O'Shea, J.J., 2004. Discrete roles for histone acetylation in human T helper 1 cell-specific gene expression. J. Biol. Chem. 279, 40640–40646.

Mosmann, T.R., Coffman, R.L., 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv. Immunol. 46, 111–147.

Mullen, A.C., Hutchins, A.S., High, F.A., Lee, H.W., Sykes, K.J., Chodosh, L.A., Reiner, S.L., 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. Nat. Immunol. 3, 652–658.

Murphy, K.M., Ouyang, W., Farrar, J.D., Yang, J., Ranganath, S., Asnagli, H., Afkarian, M., Murphy, T.L., 2000. Signaling and transcription in T helper development. Annu. Rev. Immunol. 18, 451–494.

Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C.M., Canaani, E., 2002. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol. Cell 10, 1119–1128.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., Paul, W.E., 1999. The IL-4 receptor: signaling mechanisms and biologic functions. Annu. Rev. Immunol. 17, 701–738.

O'Garra, A., 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 8, 275–283.

Omori, M., Yamashita, M., Inami, M., Ukai-Tadenuma, M., Kimura, M., Nigo, Y., Hosokawa, H., Hasegawa, A., Taniguchi, M., Nakayama, T., 2003. CD8 T cell-specific downregulation of histone hyperacetylation and gene activation of the IL-4 gene locus by ROG, repressor of GATA. Immunity 19, 281–294.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., Murphy, K.M., 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 9, 745–755.

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.A., Shen, H., Cereb, N., Yang, S.Y., Lindsten, T., Rossant, J., Hunter, C.A., Reiner,

S.L., 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science 302, 1041–1043.

Reiner, S.L., Locksley, R.M., 1995. The regulation of immunity to *Leishmania major*. Annu. Rev. Immunol. 13, 151–177.

Rincon, M., Enslen, H., Raingeaud, J., Recht, M., Zapton, T., Su, M.S., Penix, L.A., Davis, R.J., Flavell, R.A., 1998. Interferon-gamma expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. EMBO J. 17, 2817–2829.

Santangelo, S., Cousins, D.J., Winkelmann, N.E., Staynov, D.Z., 2002. DNA methylation changes at human Th2 cytokine genes coincide with DNase I hypersensitive site formation during CD4(+) T cell differentiation. J. Immunol. 169, 1893–1903.

Seder, R.A., Paul, W.E., 1994. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12, 635–673.

Shibata, Y., Kamata, T., Kimura, M., Yamashita, M., Wang, C.R., Murata, K., Miyazaki, M., Taniguchi, M., Watanabe, N., Nakayama, T., 2002. Ras activation in T cells determines the development of antigen-induced airway hyperresponsiveness and eosinophilic inflammation. J. Immunol. 169, 2134–2140.

Shirai, T., Magara, K.K., Motohashi, S., Yamashita, M., Kimura, M., Suwazomo, Y., Nogawa, K., Kuriyama, T., Taniguchi, M., Nakayama, T., 2003. TH1-biased immunity induced by exposure to Antarctic winter. J. Allergy Clin. Immunol. 111, 1353–1360.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100, 655–669.

Turner, B.M., 2002. Cellular memory and the histone code. Cell 111, 285–291.

Yamashita, M., Hirahara, K., Shinnakasu, R., Hosokawa, H., Norikane, S., Kimura, M.Y., Hasegawa, A., Nakayama, T., 2006. Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. Immunity 24, 611–622.

Yamashita, M., Kimura, M., Kubo, M., Shimizu, C., Tada, T., Perlmutter, R.M., Nakayama, T., 1999. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc. Natl. Acad. Sci. USA 96, 1024–1029.

Yamashita, M., Shinnakasu, R., Asou, H., Kimura, M., Hasegawa, A., Hashimoto, K., Hatano, N., Ogata, M., Nakayama, T., 2005. Ras-ERK MAPK cascade regulates GATA3 stability and Th2 differentiation through ubiquitin-proteasome pathway. J. Biol. Chem. 280, 29409–29419.

Yamashita, M., Ukai-Tadenuma, M., Kimura, M., Omori, M., Inami, M., Taniguchi, M., Nakayama, T., 2002. Identification of a conserved GATA3 response element upstream proximal from the interleukin-13 gene locus. J. Biol. Chem. 277, 42399–42408.

Yang, D.D., Conze, D., Whitmarsh, A.J., Barrett, T., Davis, R.J., Rincon, M., Flavell, R.A., 1998. Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity 9, 575–585.

Yano, S., Ghosh, P., Kusaba, H., Buchholz, M., Longo, D.L., 2003. Effect of promoter methylation on the regulation of IFN-gamma gene during in vitro differentiation of human peripheral blood T cells into a Th2 population. J. Immunol. 171, 2510–2516.

Zhang, D.H., Cohn, L., Ray, P., Bottomly, K., Ray, A., 1997. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J. Biol. Chem. 272, 21597–21603.

Zheng, W., Flavell, R.A., 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89, 587–596.

International Immunology, Vol. 19, No. 6, pp. 755–762
doi:10.1093/intimm/dxm042

© The Japanese Society for Immunology. 2007. All rights reserved.
For permissions, please e-mail: journals.permissions@oxfordjournals.org

Schnurri-2 regulates T_h2-dependent airway inflammation and airway hyperresponsiveness

Chiaki Iwamura, Motoko Y. Kimura, Kenta Shinoda, Yusuke Endo, Akihiro Hasegawa, Masakatsu Yamashita and **Toshinori Nakayama**

Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Keywords: asthma, NF-κB, Schnurri-2-deficient mice

Abstract

Schnurri (Shn)-2 is a large zinc finger-containing protein, which plays a critical role in cell growth, signal transduction and lymphocyte development. In *Shn-2*-deficient (*Shn-2*$^{-/-}$) CD4 T cells, the activation of nuclear factor-κB is up-regulated and their ability to differentiate into T_h2 is enhanced. Here, we extend our investigation and demonstrate that Shn-2 regulates T_h2 responses *in vivo* using an ovalbumin-induced allergic asthma model. Eosinophilic inflammation, mucus hyperproduction and airway hyperresponsiveness (AHR) were all enhanced in *Shn-2*$^{-/-}$ mice. Moreover, eosinophilic infiltration and AHR were enhanced in mice given a transfer of *Shn-2*$^{-/-}$ effector T_h2. Shn-2 in T_h2 is thus considered to play an important role as a negative regulator in allergic airway inflammation.

Introduction

Drosophila Schnurri (Shn) is a large zinc-finger containing protein with a molecular weight of ~270 kDa. *Drosophila* Mad–Medea and Shn interact with each other and act as nuclear targets in the *Drosophila* decapentaplegic-signaling pathway (1–3). In vertebrates, this signaling pathway may equate to the bone morphogenetic protein/transforming growth factor-β/activin-signaling pathways which play diverse roles in the developmental processes. Vertebrates have at least three orthologs of Shn, namely Shn-1, Shn-2 and Shn-3 (4). mRNA expression of Shn-2 was detected primarily in the brain, heart and spleen. The vertebrate homologs of Shn were originally identified as proteins that bind to the nuclear factor-κB (NF-κB) site of various genes (5). Recently, the role of Shn-2 in the positive selection of thymocytes has been reported (6), and Shn-3-deficient $CD4^+CD8^+$ thymocytes were shown to exhibit a defect in cell survival (7). We recently demonstrated that Shn-2 binds to the NF-κB motif directly, thus resulting in the repression of the transcriptional activity of NF-κB through the competition of NF-κB binding in T cells (8). Shn-2-deficient (*Shn-2*$^{-/-}$) CD4 T cells showed an increased capability to differentiate into T_h2, due to the constitutive activation of NF-κB and the subsequent up-regulation of GATA3 expression (8). However, the precise physiological roles of these Shn family member proteins in *in vivo* immune responses still remain largely unknown.

T_h2 play an important role in allergic asthma by inducing allergen-specific IgE production, airway inflammation, airway hyperresponsiveness (AHR) and mucus hyperproduction (9–13). The administration of allergens adsorbed with alum induces reproducible allergen-specific acquired immune responses that are dependent on T_h2 producing IL-4, IL-5 and IL-13. A subsequent allergen challenge via the airway causes the rapid activation of T_h2, mast cells and B cells. This activation results in increased vascular permeability, cellular infiltration into the lung tissue, smooth muscle contraction and mucus secretion.

In this study, we investigated the role of Shn-2 in allergic inflammation using *Shn-2*$^{-/-}$ mice. Our results suggest that Shn-2 plays a crucial role in the regulation of allergic airway inflammation and AHR.

Methods

Mice

Shn-2$^{-/-}$ mice have been described previously (6, 8). The animals used in this study were backcrossed to BALB/c >12 times and were 7–9 weeks old. Anti-ovalbumin (OVA)-specific TCRαβ (DO11.10) transgenic (Tg) mice were provided by Dennis Loh (Washington University School of Medicine, St Louis, MO, USA) (14). BALB/c mice were purchased from Clea Inc., Tokyo, Japan. Mice used in this study were at 7–9 weeks of age. Three independent experiments were performed for each experiment. All mice used in this study were maintained under specific pathogen-free conditions. All animal care was conducted in accordance with the guidelines of Chiba University.

Correspondence to: T. Nakayama; E-mail: tnakayama@faculty.chiba-u.jp

Transmitting editor: A. Singer

Received 23 November 2006, *accepted* 16 March 2007
Advance Access publication 9 May 2007

Sensitization and airway challenge with OVA

The mice were sensitized by an intra-peritoneal injection of 100 μg OVA (Sigma-Aldrich, St Louis, MO, USA) adsorbed to 1 mg alum (LSL, Tokyo, Japan) on day 0. OVA solution in PBS (100 μg per 30 μl) was administered intra-nasally to each mouse on days 7 and 9.

Measurement of AHR

The degree of AHR was assessed by methacholine-induced airflow obstruction 24 h after the last antigen challenge. The respiratory parameters were obtained by exposure of mice to 0.9% saline mist, followed by incremental doses of aerosolized methacholine (0, 3, 6, 12, 24 and 48 mg ml^{-1} in saline). Airflow obstruction was monitored and analyzed by whole-body plethysmograph (Buxco Electronics, Wilmington, NC, USA) as described previously (15). The results are expressed as the average in percentages of baseline enhanced paused values. The degree of AHR was also assessed by a computer-controlled small animal ventilator (SCIREQ, Montreal, Canada) (16). In brief, the mice were anesthetized with 100 μl per 10 g body weight of 50 mg ml^{-1} pentobarbital sodium given intra-peritoneally. After performing a tracheotomy, the trachea was cannulated with a blunted 18-gage needle. These mice were ventilated with a tidal volume of 10 ml kg^{-1} at a frequency of 180 breath min^{-1}. Each mouse was challenged with increasing doses of methacholine aerosol. After each challenge, lung resistance (RL) was recorded during tidal breathing every 10 s. The maximum values of RL were determined and expressed as the percent changes from baseline after saline exposure.

Collection of bronchioalveolar lavage fluid

Bronchioalveolar lavage (BAL) was performed 48 h after the last OVA challenge as described previously (17). All BAL fluid was collected and the cells were counted in 100-μl aliquots. One hundred thousand viable BAL cells were cytocentrifuged onto slides by a Cytospin 4 (Thermo Electron, Waltham, MA, USA) and stained with May-Grunwald–Giemza solution (MERCK, Darmstadt, Germany). Two hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The percentages of each cell type were calculated. Cytokine levels in the BAL fluid were measured 6 h after the last OVA challenges. IL-5, IL-13 and eotaxin-2 levels in BAL fluid were measured by ELISA as previously described (18).

Lung histology

The mice were sacrificed by asphyxiation at 48 h after the last OVA challenge, and the lungs were infused with 10% (v/v) formalin in PBS for fixation. The lung samples were sectioned, stained with hematoxylin and eosin (H&E) reagents or periodic acid–Schiff (PAS) reagent and examined for pathological changes under a light microscope at ×200. The number of infiltrated mononuclear cells in the peribronchiolar regions was calculated by direct counting in four different fields per slide.

Lung mononuclear cell preparation and a flow cytometry analysis

The lungs were sliced into small cubes and then incubated for 30 min in 5 ml RPMI 1640 solution containing collagenase (20 U ml^{-1}) (Worthington, Lakewood, NJ, USA) and trypsin inhibitor (0.3 mg ml^{-1}) (Sigma-Aldrich). Lung mononuclear cells were separated by centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK). For staining, one million cells were incubated on ice for 30 min with the appropriate staining reagents, according to a standard method (19). The reagents used in this study were anti-CD8α-PE (53-6.7) and anti-CD4-APC[K2] (RM4-5) purchased from PharMingen (San Diego, CA, USA). A flow cytometry analysis was performed on FACScalibur™[K3] (Becton Dickinson, Franklin lakes, NJ, USA) and the results were analyzed using the CELLQUEST™ software program (Becton Dickinson).

Adoptive cell transfer of T_h2 for the development of airway inflammation and AHR

Effector T_h2 were generated as previously described (20). In brief, splenic CD4 T cells purified from DO11.10 OVA-specific TCR Tg or *Shn-2*$^{-/-}$ DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 3 μM) plus antigen presenting cells under T_h2 culture conditions for 6 days *in vitro*. These effector T_h2 (5×10^{-6}) were transferred intravenously into BALB/c recipient mice on day 0. These recipient mice were not irradiated. On day 1 and 3, OVA solution (100 μg per 30 μl) was administrated intra-nasally to each mouse. The degree of AHR was measured on day 4. BAL fluid was collected on day 5.

Quantitative PCR analysis

Total RNA was isolated from the lung (three mice in each group) using the TRIzol reagent (Sigma-Aldrich). Reverse transcription (RT) was carried out with Superscript II RT (Invitrogen, Carlsbad, CA, USA). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under standard conditions. The primers and TaqMan probes for the detection of Muc5ac, thymus activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC) and hypoxanthine-guanine phosphoribosyltransferase (hprt) were purchased from Applied Biosystems. The expression of mRNA was normalized using the hprt signal.

Data analysis

The statistical analysis was performed using the two-tailed Student's *t*-test. Mann–Whitney *U*-tests were used to determine the level of difference in the degree of AHR. The values are the mean ± SD.

Results

Enhanced eosinophilic infiltration in BAL fluid and AHR in Shn-2$^{-/-}$ *mice*

We recently reported that the ability to differentiate into T_h2 *in vitro* was enhanced in *Shn-2*$^{-/-}$ naive CD4 T cells (8). The aim of this study was to clarify the role of Shn-2 in T_h2-dependent *in vivo* immune responses, such as OVA-induced allergic airway inflammation. Wild-type and *Shn-2*$^{-/-}$ mice were immunized with OVA–alum on day 0 and challenged with OVA intra-nasally on day 7 and 9. On day 11, BAL fluid

was harvested and examined. The absolute numbers of eosinophils, lymphocytes, neutrophils and macrophages were determined by cell counts based on morphological criteria. As shown in Fig. 1(A), total cell numbers of infiltrating leukocyte significantly increased in the Shn-2$^{-/-}$ allergy-induced mice. A significant increase in the absolute number of eosinophils was also observed. In *Shn-2*$^{-/-}$ mice, both OVA immunization and OVA challenge were required for the induction of allergic inflammation. The levels of IL-5, IL-13 and eotaxin-2 were increased in allergy-induced *Shn-2*$^{-/-}$ mice in comparison to the levels in wild-type mice (Fig. 1B). No IL-4 was detected in the BAL fluid (data not shown).

We examined the degree of AHR in the allergy-induced *Shn-2*$^{-/-}$ mice by measuring methacholine-induced airflow obstruction with a whole-body plethysmograph (Fig. 1C) and a mechanical ventilator (Fig. 1D). The degree of AHR in *Shn-2*$^{-/-}$ mice was enhanced in comparison to that of wild-type mice. These data indicate that OVA-induced airway inflammation and AHR are therefore enhanced in allergy-induced *Shn-2*$^{-/-}$ mice.

Enhanced lung inflammation and mucus production in the lung of Shn-2$^{-/-}$ *mice*

We examined the histological changes in the lungs of allergy-induced *Shn-2*$^{-/-}$ mice by H&E staining (Fig. 2A, left panels). No massive inflammatory cell infiltration was noted in the lungs of wild-type and *Shn-2*$^{-/-}$ mice that did not receive the OVA challenge (Fig. 2A, panels a and c). Substantial numbers of mononuclear cells were infiltrated in the peribronchiolar regions in wild-type mice after the OVA challenge (Fig. 2A, panel b), and the infiltration extended to the surrounding area in *Shn-2*$^{-/-}$ mice (Fig. 2A, panel d). The number of infiltrated cells also increased in *Shn-2*$^{-/-}$ mice (Fig. 2A, right panels).

We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in allergy-induced *Shn-2*$^{-/-}$ mice are shown (Fig. 2B). No specific staining was detected in wild-type and *Shn-2*$^{-/-}$ mice without the OVA challenge (Fig. 2B, panels a and c). Moderate staining was noted in wild-type bronchioles, whereas the staining levels increased in the

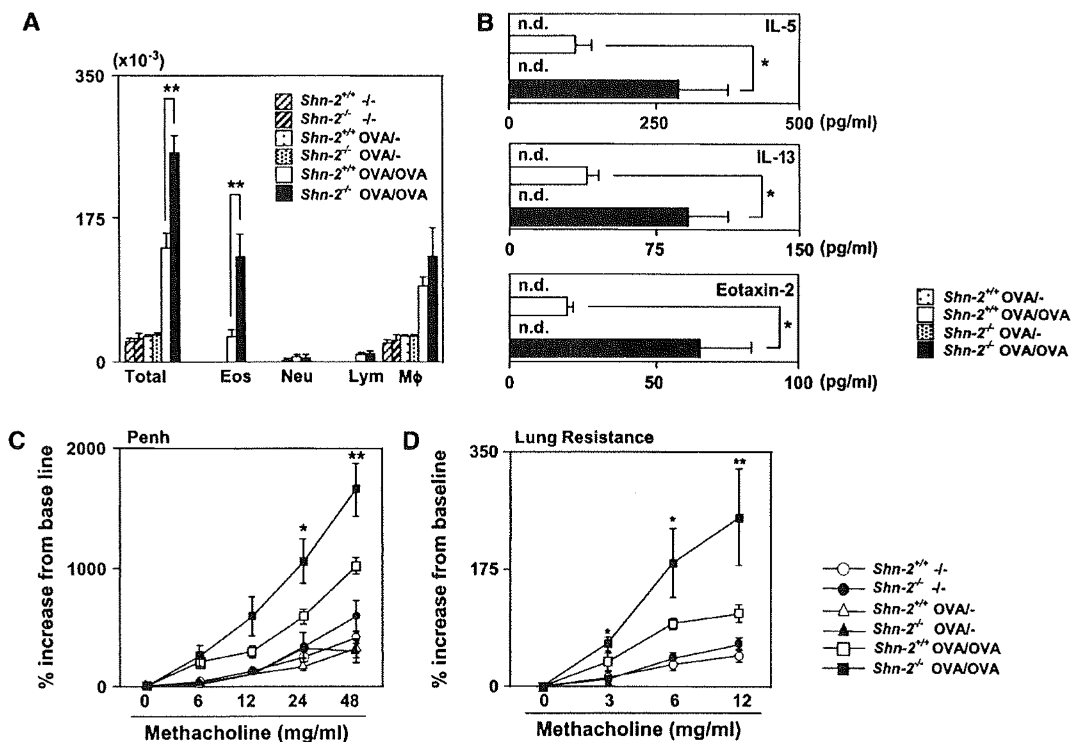

Fig. 1. Enhanced airway inflammation and AHR in *Shn-2*$^{-/-}$ mice. Airway inflammation and AHR were induced with OVA sensitization and challenges. (A) The absolute numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mϕ) in the BAL fluid are shown. The results were obtained using the values from cell counting, the percentages of the cells, total cell number per milliliter and the volume of BAL fluid recovered. Samples ware collected 48 h after the last OVA challenge. Mean values with SDs (n = 5) are shown. Four independent experiments were done with similar results. –/–: without OVA priming or OVA challenge, OVA/–: with OVA priming but not OVA challenge and OVA/OVA: with OVA priming and OVA challenge. (B) The levels of IL-5, IL-13 and eotaxin-2 in BAL fluid were determined by ELISA. Samples were collected 6 h after the last OVA challenge. Mean values with SDs (n = 5) are shown. (C and D) One day after the last OVA challenge, AHR in response to increasing doses of methacholine was assessed by measuring enhanced pause (C) and RL (D). Five animals from each group were individually examined, and the mean values and SDs are indicated. Four independent experiments were done with similar results. The differences were statistically significant between wild-type and *Shn-2*$^{-/-}$ mice with OVA sensitization and OVA challenge (*P < 0.05 and **P < 0.01).

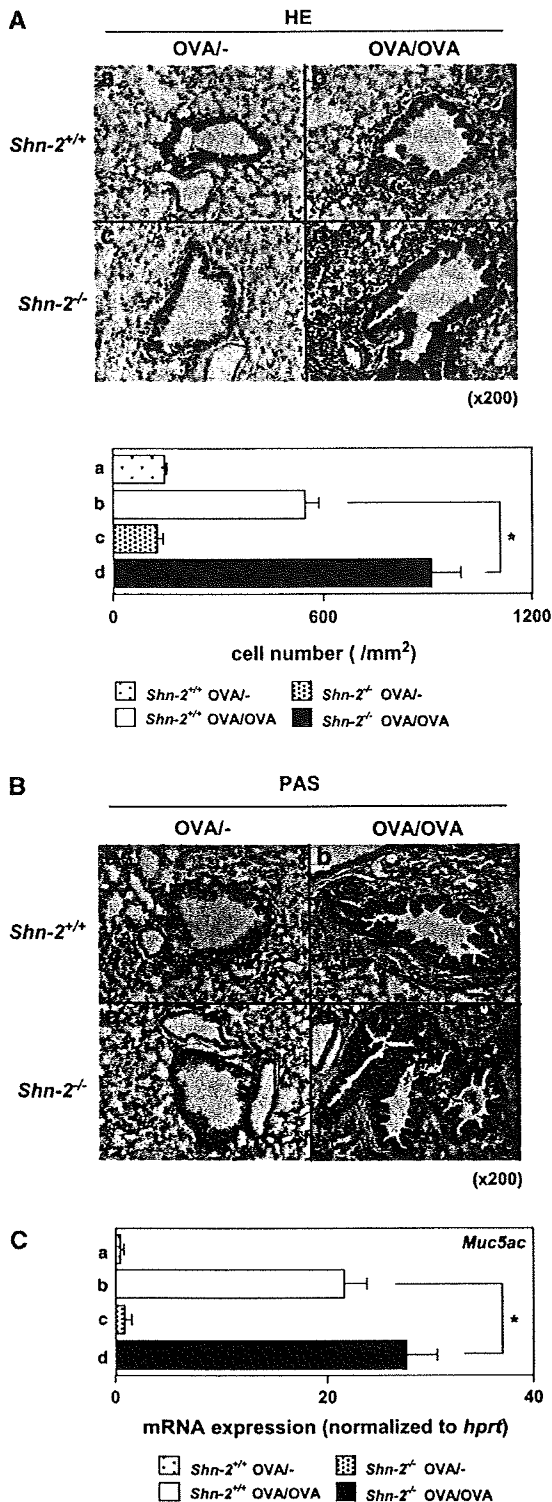

Fig. 2. Enhanced leukocyte infiltration into the lung and mucus production in allergy-induced $Shn\text{-}2^{-/-}$ mice. The levels of OVA-induced airway inflammation and mucus production in $Shn\text{-}2^{-/-}$ mice were examined by a histological analysis and a quantitative real-time RT-PCR. (A) Antigen-induced leukocyte infiltration into the lung was evaluated using H&E staining (left panels). The numbers of infiltrated mononuclear cells in the perivascular and peribronchiolar regions were calculated by direct counting from four different fields per slide (right panels). The mean values with SDs (n = 5) are shown (*P < 0.05). (B) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photographic views of wild-type and $Shn\text{-}2^{-/-}$ mice are shown. (C) Total mRNA was prepared from the lung of allergy-induced wild-type or $Shn\text{-}2^{-/-}$ mice, and mRNA levels of Muc5ac were examined. The data represent the mean values of Muc5ac mRNA expression normalized with *hprt* expression. Three independent experiments were done with similar results (*P < 0.05).

$Shn\text{-}2^{-/-}$ bronchioles (Fig. 2B, panels b and d). Consequently, we examined the expression of Muc5ac in the lungs of $Shn\text{-}2^{-/-}$ mice, and a slight, but significant increase in the expression was noted in the $Shn\text{-}2^{-/-}$ mouse lungs (Fig. 2C). These results indicate that the levels of mucus hyperproduction were moderately enhanced in the lungs of the allergy-induced $Shn\text{-}2^{-/-}$ mice in comparison to those of the wild-type mice.

The increased number of lung CD4 T cells in $Shn\text{-}2^{-/-}$ mice is accompanied with an enhanced production of TARC and MDC

Previous studies have reported a reduced number of CD4 T cells in the spleen of $Shn\text{-}2^{-/-}$ mice (8). We therefore examined whether the number of CD4 T cells were reduced in OVA-sensitized $Shn\text{-}2^{-/-}$ mice. Lung leukocytes were stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. The percentages of $CD4^+$ and $CD8^+$ cells in $Shn\text{-}2^{-/-}$ mice significantly decreased in comparison to those in wild-type mice (Fig. 3A). After the OVA challenge, the percentage of CD4 T cells increased substantially in the $Shn\text{-}2^{-/-}$ mice (12.1 versus 23.4%). The absolute numbers of total leukocytes and CD4 T cells in the lung decreased significantly in $Shn\text{-}2^{-/-}$ mice (Fig. 3B, upper panels). However, a dramatic increase in the total numbers of lung leukocytes and CD4 T cells was observed in the $Shn\text{-}2^{-/-}$ mice after the OVA challenge (Fig. 3B, lower panels).

In our previous study, the proliferative ability of CD4 T cells in the $Shn\text{-}2^{-/-}$ mice was comparable to that in the wild-type mice (8). To investigate the reason why the CD4 T cell levels increased in the allergy-induced $Shn\text{-}2^{-/-}$ mice, we examined the mRNA expression of TARC and MDC. These chemokines are known to be selective attractants for T_h2 migration (21). As shown in Fig. 3(C), the mRNA expression levels of TARC and MDC in the lung from the $Shn\text{-}2^{-/-}$ mice were significantly higher than those in the wild-type mice. The increased expression of TARC and MDC may thus explain the dramatic increase observed in the number of CD4 T cells in the lungs of $Shn\text{-}2^{-/-}$ mice.

$Shn\text{-}2^{-/-}$ effector T_h2 enhanced AHR and eosinophilic infiltration into the lungs in recipient mice

We performed adoptive transfer experiments to determine whether the enhancement of airway inflammation and AHR observed in $Shn\text{-}2^{-/-}$ mice is mediated via $Shn\text{-}2^{-/-}$ T_h2. Effector T_h2 from $Shn\text{-}2^{-/-}$ DO11.10 OVA-specific TCR Tg mice were prepared as described in the Methods. On days

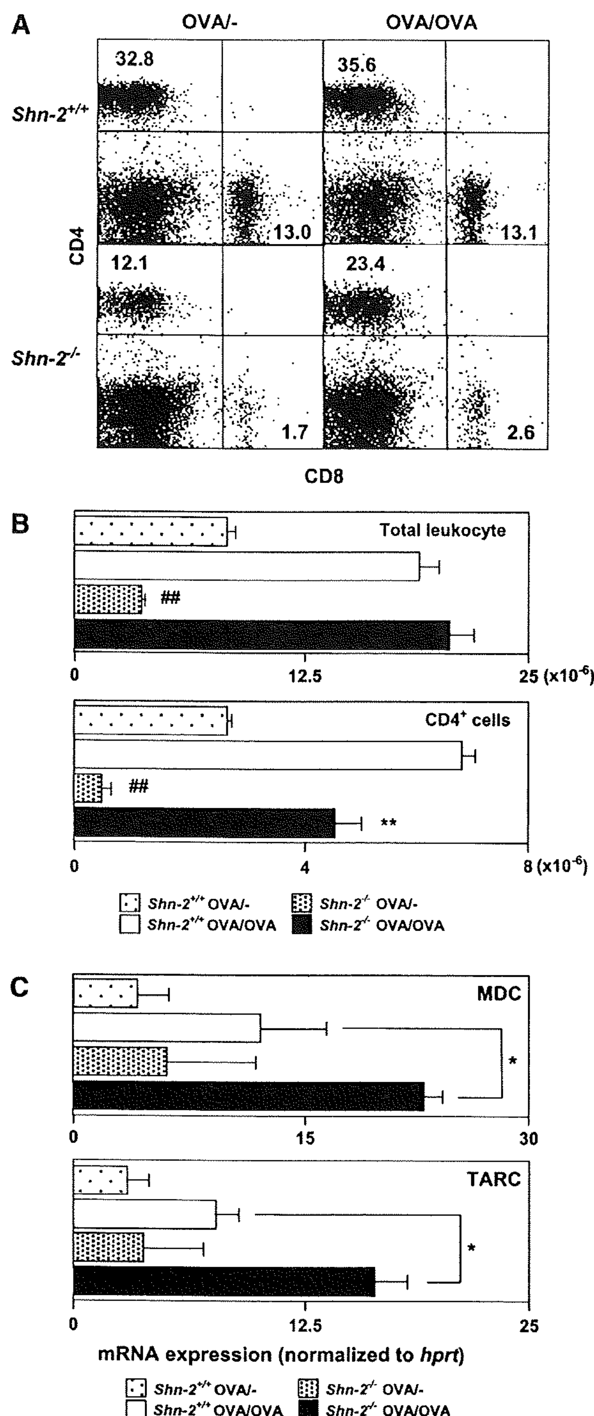

Fig. 3. Increased CD4 T cells in the lung tissues after OVA challenge in *Shn-2*$^{-/-}$ mice. Lung mononuclear cells from *Shn-2*$^{-/-}$ mice were prepared as described in the Methods. (A) Representative CD4/CD8 profiles of the lung leukocytes from wild-type or *Shn-2*$^{-/-}$ mice before (OVA/−) and after OVA challenge (OVA/OVA). The percentages of cells in each quadrant are shown. (B) Total numbers of leukocytes harvested in the lung (upper panels) and the number of CD4 T cells (lower panels) are shown. Differences in the number of leukocytes and CD4 T cells were statistically significant between the wild-type and the *Shn-2*$^{-/-}$ mice without OVA challenge (##P < 0.01). The differences in the number of CD4 T cells were statistically significant between the wild-type mice and the *Shn-2*$^{-/-}$ mice with OVA sensitization and OVA challenge (**P < 0.01). (C) Total mRNA was prepared from the lung of allergy-induced wild-type or *Shn-2*$^{-/-}$ mice. A real-time RT-PCR analysis for TARC and MDC as well as *hprt* (as a control) was performed. Representative data of three individual animals from three independent experiments are shown (*P < 0.05).

1 and 3 after effector T_h2 transfer into syngeneic BALB/c mice, airway inflammation was induced by the OVA intranasal administration. A significant increase in the absolute number of eosinophilic infiltration in BAL fluid was observed in the mice that received *Shn-2*$^{-/-}$ T_h2 (Fig. 4A). The levels of IL-5 and eotaxin-2 in the BAL fluid from the mice receiving *Shn-2*$^{-/-}$ T_h2 increased markedly more than that from the mice receiving wild-type T_h2 (Fig. 4B). The levels of IL-4 were not increased but moderately decreased. The levels of IL-13 were comparable. The degree of AHR in the mice receiving *Shn-2*$^{-/-}$ T_h2 also increased more than that in the mice receiving wild-type T_h2 (Fig. 4C and D). These results suggest that the hyperactivation of effector T_h2 in *Shn-2*$^{-/-}$ mice exacerbates the development of allergic airway inflammation.

We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in the mice that received wild-type or *Shn-2*$^{-/-}$ T_h2 are shown (Fig. 4E). Moderate staining was noted in both bronchioles (Fig. 4E, panels a and b). The mRNA expression of Muc5ac in the lung of mice that received *Shn-2*$^{-/-}$ T_h2 was examined, and it was found to be comparable to that in the mice receiving wild-type T_h2 (Fig. 4F). These results indicate that the levels of mucus hyperproduction in the lungs of the mice that received *Shn-2*$^{-/-}$ T_h2 are therefore comparable to those of the mice that received wild-type T_h2.

Discussion

We previously reported that the activation of NF-κB to be up-regulated in *Shn-2*$^{-/-}$ CD4 T cells, and their ability to differentiate into T_h2 was enhanced (8). In this study, we demonstrated that OVA-induced allergic inflammation and AHR are enhanced in the *Shn-2*$^{-/-}$ mice as well as in the wild-type mice transferred with *Shn-2*$^{-/-}$ effector T_h2. These results indicate that Shn-2 regulates OVA-induced airway inflammation and AHR through the control of CD4 T cell activation.

We observed an increased IL-5, IL-13 and eotaxin-2 level in the BAL fluid in OVA-sensitized and OVA-challenged *Shn-2*$^{-/-}$ mice (Fig. 1B). IL-13 is known to induce AHR in the absence of inflammatory cells (13). IL-5 and eotaxin-2 are known to attract eosinophils (22). Since eosinophils release granule proteins that are cytotoxic to the airway epithelium such as major basic proteins, eosinophilia may exacerbate the airway obstruction and AHR (23). Therefore, it is likely that the overproduction of these factors (IL-5, IL-13 and eotaxin-2) resulted in the enhanced eosinophilic infiltration and AHR in the airways of the *Shn-2*$^{-/-}$ mice. The hyperproduction of mucus also plays an important role in the pathogenesis of various asthmatic features and is linked with

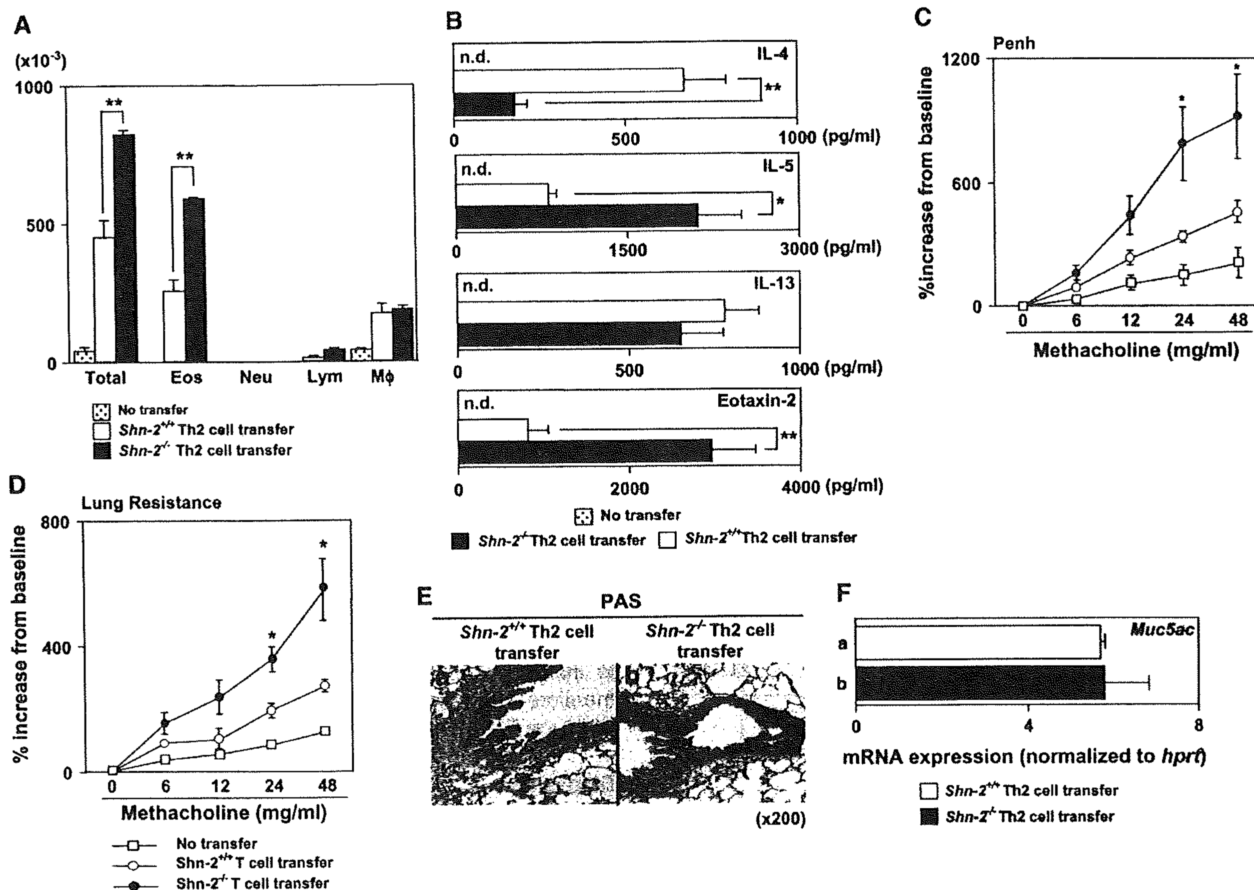

Fig. 4. Enhanced airway inflammation and AHR in mice receiving *Shn-2*$^{-/-}$ effector T_h2. Naive CD4 T cells from DO11.10 OVA-specific TCR Tg or *Shn-2*$^{-/-}$ DO11.10 OVA-specific TCR Tg mice were cultured under T_h2 conditions for 6 days. The effector T_h2 (5×10^6) were transferred into BALB/c mice 1 day before the first OVA challenge. (A) The absolute number of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mϕ) in the BAL fluid are shown. The mean values with SDs ($n = 5$) are shown. Three independent experiments were done with similar results (**$P < 0.01$). (B) The levels of IL-4, IL-5, IL-13 and eotaxin-2 in the BAL fluid were determined by ELISA. Samples ware collected 6 h after the last OVA challenge. The mean values with SDs ($n = 5$) are shown. Three independent experiments were done with similar results (*$P <$ 0.05 and **$P < 0.01$). n.d. not detectable. (C and D) AHR was monitored by measuring enhanced pause (left panel) and RL (right panel) as described in the Methods. The mean values with SDs ($n = 5$) are shown. (E) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photographic views of the lung in the mice receiving wild-type or *Shn-2*$^{-/-}$ T_h2 are shown. (F) The data represent the mean values of Muc5ac mRNA expression in the lung of mice receiving wild-type or *Shn-2*$^{-/-}$ T_h2. It was normalized with *hprt* expression. Three independent experiments were done with similar results. Differences in AHR were statistically significant between wild-type and *Shn-2*$^{-/-}$ T cell transfer groups (*$P < 0.05$).

asthma fatality (24, 25). IL-13 has been shown to induce mucus hypersecretion *in vivo* and *in vitro* (26). Therefore, an overproduction of IL-13 may induce severe mucus secretion in the *Shn-2*$^{-/-}$ lung. IL-13 was also shown to induce TARC and MDC production from keratinocytes and bronchial epithelial cells (27, 28). The increased number of CD4 T cells in the lungs of the *Shn-2*$^{-/-}$ mice may be due to the increased amount of IL-13, which thus resulted in the overproduction of TARC and MDC.

To investigate whether the exacerbation of airway inflammation and AHR was due to the deficiency of Shn-2 in T_h2, we performed a set of experiments with adoptive transfer of T_h2 (Fig. 4). As a result, an enhanced degree of eosinophilic infiltration and increased levels of IL-5 and eotaxin-2 in BAL fluid were observed in wild-type recipient mice transferred with *Shn-2*$^{-/-}$ T_h2. The degree of AHR was also amplified. Therefore, the exacerbation of airway inflammation and AHR appears to be at least in part due to the enhanced T_h2 activities of *Shn-2*$^{-/-}$ T_h2.

However, the levels of IL-13 in the BAL fluid from the mice that received *Shn-2*$^{-/-}$ T_h2 were comparable to those from the mice that received wild-type T_h2. The levels of mucus hypersecretion did not increase in the mice that received *Shn-2*$^{-/-}$ T_h2 (Fig. 4E and F). These results may indicate that the overproduction of IL-13 in the BAL fluid of *Shn-2*$^{-/-}$ mice was not only from CD4 T cells but also from other IL-13-producing cells such as mast cells, basophils and eosinophils. We observed the Shn-2 expression in naive CD4 T cells as well as bone marrow-derived mast cells (BMMCs) (Supplementary Figure 1A, available at *International Immunology*

Online). The phenotypic features (expression profiles of c-kit and FcεRI) were indistinguishable between wild-type and *Shn-2$^{-/-}$* BMMCs (Supplementary Figure 1B, available at *International Immunology* Online.). In addition, the levels of IL-5, IL-6 and IL-13 production after cross-linking of FcεRI using anti-DNP IgE and DNP–BSA were comparable (Supplementary Figure 1C, available at *International Immunology* Online.). The IL-4 production was not detected by ELISA in either group (C. Iwamura and T. Nakayama, unpublished observation). These results indicate that the function of *Shn-2$^{-/-}$* mast cells is thus within the normal range.

We observed that the production of T_h2-dependent antibodies (IgG1 and IgE) induced by OVA–alum immunization was decreased in *Shn-2$^{-/-}$* mice as compared with that seen in wild-type mice (M. Y. Kimura and T. Nakayama, unpublished observation). The level of IL-4 in the BAL fluid in the mice that received *Shn-2$^{-/-}$* T_h2 decreased (Fig. 4B). This could be the reason why the IgG1 and IgE levels are decreased in the *Shn-2$^{-/-}$*mice. It is unclear at this time why the production of IL-4 in the BAL fluid of mice that received *Shn-2$^{-/-}$* T_h2 decreased. One possible explanation is the fact that GATA3 may play a more important role in the regulation of IL-5 production than IL-4.

In addition to T_h2, other lymphocytes substantially regulate allergic diseases. It has been reported that CD8 T cells and NKT cells are the source of IL-13 and can induce the airway inflammation and AHR independently from conventional CD4 T cells (29–31). Regulatory T cells are also known to control allergic diseases (12, 32). In *Shn-2$^{-/-}$* mice, the absolute number of $CD4^+CD25^+$ T cells decreased in comparison to that of wild-type mice (C. Iwamura and T. Nakayama, unpublished observation). Furthermore, some *Shn-2$^{-/-}$* mice after 16 weeks of age died from severe whole-body inflammation of an enlarged spleen and draining lymph nodes (C. Iwamura and T. Nakayama, unpublished observation). Therefore the down-regulation of regulatory T cells may exacerbate the airway inflammation in *Shn-2$^{-/-}$* mice. Not only for T cells but also for non-T cell populations, such as airway smooth muscle cells, eosinophils and epithelial cells, which have all been reported to play important roles in the development of asthma (9, 22, 33). Although we need to await a more comprehensive study, it is possible that some of these cells may express Shn-2, and thereby contribute to the exacerbation of airway inflammation and AHR in *Shn-2$^{-/-}$* mice.

Recently, the role of NF-κB in the pathogenesis of allergic diseases was investigated in experimental allergic murine models (34–36). Since *Shn-2$^{-/-}$* CD4 T cells showed the constitutive activation of NF-κB (8), we used an adoptive transfer system to examine whether or not the effector T_h2 with an increased activation level of NF-κB exacerbate airway inflammation and AHR. Our preliminary results showed significant increases in the absolute number of eosinophils and the degree of AHR was observed in the mice that received T_h2 over-expressing NF-κB (p65) (C. Iwamura and T. Nakayama, unpublished observation). These data suggest that the increased levels of NF-κB activation in *Shn-2$^{-/-}$* T_h2 may thus enhance both airway inflammation and AHR.

In summary, OVA-induced eosinophilic airway inflammation, AHR and mucus hyperproduction were all found to be enhanced in *Shn-2$^{-/-}$* mice. Therefore, Shn-2 appears to play a key role as an *in vivo* negative regulator of the T_h2-dependent allergic airway responses.

Supplementary data

Supplementary Figure 1 is available at *International Immunology* Online.

Acknowledgements

We are grateful to Erwin Gelfand for the introduction of the measurement of RL and to Yoichi Suzuki for his valuable advice on the statistical analysis. We also thank Kaoru Sugaya, Hikari Asou, Satoko Norikane and Toshihiro Ito for their excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid for Scientific Research in Priority Areas #17016010 and #17047007, Scientific Research B #17390139, Scientific Research C #18590466; Grant-in-Aid for Young Scientists #17790318; Special Coordination Funds for Promoting Science and Technology), the Ministry of Health, Labor and Welfare (Japan), The Japan Health Science Foundation, Kanae Foundation, Uehara Memorial Foundation, Mochida Foundation and Sagawa Foundation.

Abbreviations

AHR	airway hyperresponsiveness
BAL	bronchioalveolar lavage
BMMC	bone marrow-derived mast cell
H&E	hematoxylin and eosin
hprt	hypoxanthine guanine phosphoribosyl transferase
MDC	macrophage-derived chemokine
NF-κB	nuclear factor-κB
OVA	ovalbumin
PAS	periodic acid–Schiff
RL	lung resistance
RT	reverse transcription
Shn	Schnurri
Shn-2$^{-/-}$	Shn-2 deficient
TARC	thymus activation-regulated chemokine
Tg	transgenic

References

1 Affolter, M., Marty, T., Vigano, M. A. and Jazwinska, A. 2001. Nuclear interpretation of Dpp signaling in *Drosophila. EMBO J.* 20:3298.
2 Arora, K., Dai, H., Kazuko, S. G. *et al.* 1995. The *Drosophila* schnurri gene acts in the Dpp/TGF beta signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell* 81:781.
3 Staehling-Hampton, K., Laughon, A. S. and Hoffmann, F. M. 1995. A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for dpp signaling. *Development* 121:3393.
4 Wu, L. C. 2002. ZAS: C2H2 zinc finger proteins involved in growth and development. *Gene Expr.* 10:137.
5 Maekawa, T., Sakura, H., Sudo, T. and Ishii, S. 1989. Putative metal finger structure of the human immunodeficiency virus type 1 enhancer binding protein HIV-EP1. *J. Biol. Chem.* 264: 14591.
6 Takagi, T., Harada, J. and Ishii, S. 2001. Murine Schnurri-2 is required for positive selection of thymocytes. *Nat. Immunol.* 2:1048.
7 Allen, C. E., Muthusamy, N., Weisbrode, S. E., Hong, J. W. and Wu, L. C. 2002. Developmental anomalies and neoplasia in animals and cells deficient in the large zinc finger protein KRC. *Genes Chromosomes Cancer* 35:287.
8 Kimura, M. Y., Hosokawa, H., Yamashita, M. *et al.* 2005. Regulation of T helper type 2 cell differentiation by murine Schnurri-2. *J. Exp. Med.* 201:397.

9 Busse, W. W. and Lemanske, R. F. Jr 2001. Asthma. *N. Engl. J. Med.* 344:350.
10 Hamelmann, E. and Gelfand, E. W. 2001. IL-5-induced airway eosinophilia—the key to asthma? *Immunol. Rev.* 179:182.
11 Lloyd, C. M., Gonzalo, J. A., Coyle, A. J. and Gutierrez-Ramos, J. C. 2001. Mouse models of allergic airway disease. *Adv. Immunol.* 77:263.
12 Umetsu, D. T. and DeKruyff, R. H. 2006. The regulation of allergy and asthma. *Immunol. Rev.* 212:238.
13 Wills-Karp, M. 2004. Interleukin-13 in asthma pathogenesis. *Immunol. Rev.* 202:175.
14 Murphy, K. M., Heimberger, A. B. and Loh, D. Y. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes *in vivo*. *Science* 250:1720.
15 Kamata, T., Yamashita, M., Kimura, M. *et al.* 2003. src homology 2 domain-containing tyrosine phosphatase SHP-1 controls the development of allergic airway inflammation. *J. Clin. Invest.* 111:109.
16 Hamelmann, E., Schwarze, J., Takeda, K. *et al.* 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766.
17 Hansen, G., Berry, G., DeKruyff, R. H. and Umetsu, D. T. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103:175.
18 Kimura, M., Koseki, Y., Yamashita, M. *et al.* 2001. Regulation of Th2 cell differentiation by mel-18, a mammalian polycomb group gene. *Immunity* 15:275.
19 Nakayama, T., June, C. H., Munitz, T. I. *et al.* 1990. Inhibition of T cell receptor expression and function in immature CD4+CD8+ cells by CD4. *Science* 249:1558.
20 Yamashita, M., Kimura, M., Kubo, M. *et al.* 1999. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. *Proc. Natl Acad. Sci. USA* 96:1024.
21 Lukacs, N. W. 2001. Role of chemokines in the pathogenesis of asthma. *Nat. Rev. Immunol.* 1:108.
22 Rothenberg, M. E. and Hogan, S. P. 2006. The eosinophil. *Annu. Rev. Immunol.* 24:147.
23 Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17:255.
24 Sheehan, J. K., Richardson, P. S., Fung, D. C., Howard, M. and Thornton, D. J. 1995. Analysis of respiratory mucus glycoproteins in asthma: a detailed study from a patient who died in status asthmaticus. *Am. J. Respir. Cell Mol. Biol.* 13:748.
25 Takeyama, K., Dabbagh, K., Lee, H. M. *et al.* 1999. Epidermal growth factor system regulates mucin production in airways. *Proc. Natl Acad. Sci. USA* 96:3081.
26 Fahy, J. V. 2002. Goblet cell and mucin gene abnormalities in asthma. *Chest* 122:320S.
27 Hartl, D., Latzin, P., Zissel, G., Krane, M., Krauss-Etschmann, S. and Griese, M. 2006. Chemokines indicate allergic bronchopulmonary aspergillosis in patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 173:1370.
28 Sekiya, T., Miyamasu, M., Imanishi, M. *et al.* 2000. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J. Immunol.* 165:2205.
29 Akbari, O., Stock, P., Meyer, E. *et al.* 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9:582.
30 Meyer, E. H., Goya, S., Akbari, O. *et al.* 2006. Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells. *Proc. Natl Acad. Sci. USA* 103:2782.
31 Miyahara, N., Swanson, B. J., Takeda, K. *et al.* 2004. Effector CD8+ T cells mediate inflammation and airway hyper-responsiveness. *Nat. Med.* 10:865.
32 Hawrylowicz, C. M. 2005. Regulatory T cells and IL-10 in allergic inflammation. *J. Exp. Med.* 202:1459.
33 Cookson, W. 2004. The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nat. Rev. Immunol.* 4:978.
34 Das, J., Chen, C. H., Yang, L., Cohn, L., Ray, P. and Ray, A. 2001. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat. Immunol.* 2:45.
35 Poynter, M. E., Irvin, C. G. and Janssen-Heininger, Y. M. 2002. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am. J. Pathol.* 160:1325.
36 Tenda, Y., Yamashita, M., Kimura, M. Y. *et al.* 2006. Hyperresponsive T(H)2 cells with enhanced nuclear factor-kappaB activation induce atopic dermatitis-like skin lesions in Nishiki-nezumi Cinnamon/Nagoya mice. *J. Allergy Clin. Immunol.* 118:725.

Critical Reviews™ in Immunology, 27(6):539–546 (2007)

Immune Mechanisms of Allergic Airway Disease: Regulation by Transcription Factors

Masakatsu Yamashita, Atsushi Onodera, & Toshinori Nakayama*
Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan

* Address all correspondence to Toshinori Nakayama, Department of Immunology (H3), Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, 260-8670 Japan; Tel.: 81-43-226-2200; Fax: 81-43-227-1498; tnakayama@faculty.chiba-u.jp

Referee: Steven F. Ziegler, Ph.D., Director, Immunology Program, Benaroya Research Institute, 1201 9th Avenue, Seattle WA 98101

ABSTRACT: Allergic asthma is an airway inflammatory disease characterized by chronically increased expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, enzymes, and receptors. Several transcription factors are known to play a crucial role in the pathogenesis of chronic inflammatory diseases such as asthma, including signal transducer and activator of transcription factors, nuclear factor-kappaB, nuclear factor of activated T cells, activator protein-1 family proteins, and Th2 cell–related transcription factors including GATA3, JunB, and c-Maf. Modulation of the activity of certain transcription factors has been shown to result in the inhibition of inflammation during asthma. Therefore, both agonists and inhibitors of transcription factors may be potential tools for the treatment of asthma. In this review, we summarize the current knowledge of the role of well-established transcription factors in asthma.

KEY WORDS: STAT6, NFκB, NFAT, AP-1, GATA3, JunB

I. INTRODUCTION

Asthma is a chronic inflammatory disease of the lower airways that causes airway hyperresponsiveness to a wide variety of specific and nonspecific stimuli.[1,2] In addition to inflammatory cells, several elements in the airway wall contribute to increased thickness and structural changes referred to as airway remodeling. Mucus gland hypertrophy and metaplasia into mucus-secreting cells occurs, and results in excessive mucus levels in the airway lumen. There, mucus mixes with infiltrated macrophages, lymphocytes, and eosinophils and shed epithelial cells. Airway remodeling and inflammation result in airway hyperresponsiveness (AHR). AHR is defined as an increased bronchoconstrictor response to a nonspecific stimulus. The strength of AHR correlates with the level of airway inflammation.

Hallmarks of asthma include airway inflammation predominated by eosinophils, mucus hyperproduction, Th2 cytokines (IL-4, -5, and -13), and allergen-specific IgE.[3] Most previously studied animal models suggest a Th2 paradigm for allergic diseases, with increased activation of Th2 cells that produce Th2 cytokines resulting in IgE production and the recruitment and activation of eosinophils. This notion has been supported by clinical studies showing that Th2-like cytokines were released from the lymphocytes of asthmatic patients.[4–6] Asthma can also be characterized at the molecular level by upregulated expression of genes encoding multiple inflammatory proteins, including Th2 cytokines and chemokines, adhesion molecules, pro-inflammatory receptors, and mediator-synthesizing enzymes. The expression of these genes is tightly regulated by transcription factors. Several transcription factors have been

Received: 2/2/07; Accepted: 4/26/07
1040-8401/07/$35.00
© 2007 by Begell House, Inc.

implicated in the inflammatory process in allergic airway inflammation, such as signal transducer and activator of transcription (STAT) factors, nuclear factor-kappaB (NFκB), nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1) family proteins, and Th2 cell-related transcription factors including GATA3, JunB, and c-Maf. The number of transcription factors involved in the regulation of inflammatory processes in allergic airway inflammation is increasing and has recently been reported to include CCAAT/enhancer binding protein (C/EBP), peroxisome proliferator-activated receptor (PPAR), and Nrf2. This review will summarize the transcription factors that regulate the pathogenesis of allergic airway inflammation, while also discussing their possible use for therapy.

II. TRANSCRIPTION FACTORS INVOLVED IN THE REGULATION OF ALLERGIC AIRWAY DISEASES

A. The Signal Transducer and Activator of Transcription 6

Ligation of either the IL-4 or IL-13 receptor by their respective cytokines leads to phosphorylation and activation of activator of transcription 6 (STAT6). STAT6 signaling is essential for class switching to IgE in B-lymphocytes and for Th2 cell differentiation.[7] In an experimental mouse asthma model, airway hyperresponsiveness (AHR), mucus production, eosinophilic infiltration into BALF (bronchioalveolar lavage fluid) and lung parenchyma, and serum IgE level were significantly reduced in STAT6-deficient mice.[8,9] The expression of chemokines such as eotaxin, MDC (CCL22), and TARC (CCL17) were also reduced in the asthmatic lung of STAT6-deficient mice.[10] Furthermore, adoptive transfer of STAT6-deficient CD4 T cells from OVA-sensitized mice into STAT6-deficient donors failed to induce AHR, whereas the adoptive transfer of wild-type OVA-sensitized CD4 T cells was able to induce AHR,[11] suggesting that STAT6 expressed in CD4 T cells plays a crucial role in the induction of AHR. Having said this, STAT6 expressed in airway epithelial cells also appears to play an important role in the pathogenesis of asthma.[12] Blockade of IL-13 inhibited allergen-induced AHR and mucus hyperproduction.[13,14] Also, delivery of IL-13 alone to the airway could cause all of these effects. Significantly, epithelial cell-specific reconstitution of STAT6 in STAT6-deficient mice with a STAT6 transgene under the control of a Clara cell secretory protein promoter was sufficient to restore IL-13–induced AHR and mucus hyperproduction.[12] This piece of evidence demonstrates the importance of STAT6 expressed in epithelial cells in asthmatic symptoms. Therefore, the IL-4/IL-13–STAT6 signaling pathways may be potential therapeutic targets in asthma. In this regard, a STAT6 inhibitor peptide, derived from the STAT6-binding region of the IL-4 receptor alpha chain, inhibited IL-4 dependent phosphorylation of STAT6 in different human and murine cell lines.[15]

B. GATA3

GATA3 was identified as a lineage-specific transcription factor selectively expressed in Th2-lineage cells.[16–19] Preferential expression of GATA3 in Th2 cells is linked to signals mediated through STAT6.[20] GATA3 was reported to be upregulated in the asthmatic lung.[21] GATA3 induces the expression of Th2 cytokines (IL-4, IL-5, and IL-13) through the induction of chromatin remodeling of Th2 cytokine gene loci.[16,22–24] Furthermore, conditional deletion of GATA3 in developed Th2 cells strongly inhibited the expression of IL-5 and IL-13, but only moderately blocked IL-4 production.[22,25,26]

Several studies have revealed a direct link between GATA3 expression and allergic airway inflammation. In a murine model, Finotto et al. showed that inhaled GATA3 antisense oligonucleotides suppressed Th2 cytokine production and AHR.[27] In addition, an analysis of transgenic mice expressing an inducible dominant-negative GATA3 in a T cell–specific manner provided evidence that GATA3 is involved in antigen-mediated Th2 cytokine production and airway inflammation.[28] In human studies, the analysis of GATA3 expression by immuno-histochemistry techniques revealed increased frequencies of GATA3-expressing cells in airway biopsies from asthmatic patients in comparison to those from normal subjects, and the number of GATA3-positive cells dramatically increased following antigen challenge.[21,29]

C. Leucine Zipper–Containing Transcription Factor Family

Activation protein-1 (AP-1) is a member of the c-Fos and c-Jun families of transcription factors, which dimerize with each other via their leucine zipper domains. AP-1 interacts with nuclear factor of activated T cells (NFAT) and regulates IL-4 and IL-5 production from T-lymphocytes.[30] Among the AP-1 family of proteins, JunB is preferentially expressed in Th2 cells and is involved in the production of IL-4.[31] JunB-null mice were embryonic lethal but transgenic mice expressing JunB under the control of a ubiquitin promoter rescued the development of fetuses.[32] Analysis of T-lymphocytes from these mice revealed a very low level of JunB expression and significantly reduced production of IL-4. In addition, OVA-induced airway inflammation in these mice was also severely impaired.[32]

c-Maf is a Th2-specific leucine zipper–containing transcription factor that is important for transcriptional activation of IL-4.[33] c-Maf is thought to directly activate expression of the IL-4 gene, but not the IL-13 gene, in concert with other factors such as NIP-45, NFAT, and JunB.[31,34,35] Although the physiological roles of c-Maf in asthma remain unclear, the expression of c-Maf has been reported to increase in bronchial biopsies of atopic asthmatic patients.[36]

D. NFAT

NFAT consists of a family a calcium-sensitive transcription factors that is critically important in the expression of many T cell–related cytokines, including IL-2 and IL-4.[30,37] NFAT proteins are constitutively expressed in the cytoplasm, but they translocate into the nucleus after dephosphorylation by the calcium-activated phosphatase calcineurin. This process is inhibited by cyclosporine A and FK506, which both inhibit calcineurin activation. In addition, both of these calcineurin inhibitors have been shown to have anti-inflammatory effects in asthma.[38]

There are five currently known NFAT family members that differ in their tissue distribution and regulation. NFAT1 (NFATp or NFATc2), NFAT2 (NFATc or NFATc1), and NFAT4 (NFATx or NFATc3) are all predominantly expressed in immune cells.[30] The expression of NFAT is not restricted to T cells; it is also detected in B cells, mast cells, basophils, eosinophils, and muscle cells.[39,40] Experiments using gene-targeted and chimeric mice have revealed an important role for NFAT2, but not NFAT1 and NFAT4, in Th2 polarization.[41,42] The deletion of NFAT1 (especially in combination with NFAT4) has been demonstrated to result in a phenotype of hyperactive immunity, characterized by heightened Th2 cytokine production and eosinophil recruitment to the lung.[34,41,43] Both NFAT1 and NFAT2 can activate the IL-4 or IL-13 promoters in transient transfection assays,[44] and enhanced IL-4 gene expression in NFAT1-null mice probably does not reflect a direct repressive effect of NFAT1 on Th2 cytokine gene expression.

E. T-bet

Mice lacking the Th1-specific transcription factor T-bet have profound defects in the development of Th1 cells and production of IFNγ.[45] Spontaneous development of asthmalike symptoms, such as airway hyperreactivity, inflammation, and remodeling, was observed in T-bet–deficient mice.[46] Ca^{2+}-homeostasis of airway smooth muscle cells is also altered in T-bet–deficient mice and contributes to increased bronchial hyperresponsiveness.[47] Furthermore, reduced expression of T-bet has been reported in both T-lymphocytes and the lung of asthmatic patients.[46] However, the molecular mechanisms underlying the reduction of T-bet expression in asthmatic subjects remains to be elucidated.

F. Nuclear Factor κB (NFκB)

NFκB is a ubiquitously expressed transcription factor that regulates the expression of multiple cytokines, chemokines, inducible nitric oxide synthase (iNOS), the inducible form of cyclooxygenase (COX-2), and cell adhesion molecules that are involved in asthma pathogenesis. The activation of NFκB by cytokines, oxidants, immune stimuli, and viruses has been demonstrated in many inflammatory cells.[48–50] NFκB is present in the cytoplasm in an inactive form, complexed with an inhibitory protein, IκB. On activation,

IκB kinase phosphorylates IκB, and after subsequent ubiquitination, the proteasome rapidly degrades IκB, thus resulting in its dissociation form NFκB. Five mammalian members have been identified, namely, p50 (NFκB1), p52 (NFκB2), p65 (RelA), c-Rel, and RelB. Distinct expression profiles for each of the five NFκB members have been observed in developing tissues and organs. One of the first in vivo studies showed p50, p65, and p52 expression to be found in virtually all cell types by in situ staining of embryonic tissues, with highest expression observed in the thymus.[51] However, only c-Rel and RelB were detected in lymphoid tissues. These tissue expression patterns are consistent with the observation that p50/p65 heterodimers are readily activated in most cell types, while c-Rel complexes are predominantly found in hematopoietic lineages and RelB subunits are preferentially complexed in the spleen and thymus.[51,52]

In a murine model of allergen-induced asthma, it was shown that c-Rel-deficient mice are less susceptible to pulmonary inflammation and asthmatic airway hyperresponsiveness.[53] Furthermore, c-Rel–deficient mice also do not develop eosinophilia and they have virtually no increase in total serum IgE. These data suggest that c-Rel is a potent mediator of immune responses in allergic inflammation, and it is therefore suggested to be a potential therapeutic target for asthma. However, in the case of parasitic infection caused by helminth, c-Rel–deficient mice were demonstrated to induce a Th2 response sufficient to expel the infection, whereas p50 knockout and p52 knockout mice were unable to do so.[54] In addition, p50 knockout mice have also been found to be deficient in both the production of Th2 cytokines (IL-4, IL-5, and IL-13) and in the expression of GATA3.[55]

G. Peroxisome Proliferator-Activated Receptor (PPAR)

To date, three different PPAR subtypes have been identified, namely, PPARα, PPARγ, and PPARβ (also known as PPARδ). The name PPAR is derived from the fact that activation of PPARα induced peroxisome proliferation in rodent hepatocytes. However, the activation of PPARβ or PPARγ fails to induce this response. Among the known PPARs, PPARγ has been suggested to be an immunomodulator and to have potential as a novel anti-inflammatory target for asthma.[56] PPARγ has been found in cells of the immune system such as monocytes/macrophages, B and T cells, and dendritic cells. PPARγ agonists suppressed the production of IL-8 in airway epithelial cells,[57] suggesting the possibility of reducing lymphocyte recruitment and therefore airway inflammation. PPARγ agonists also inhibited other inflammatory cytokines such as IL-1β, IL-6, and TNF-α in stimulated human peripheral blood monocytes.[58] PPARγ regulates gene expression by binding as a heterodimer with the retinoid X receptor (RXR), a member of the nuclear hormone receptor superfamily activated by 9-cis-retinoic acids. PPARγ/RXR heterodimer reduces the availability of coactivators that are required for induction of gene expression by other transcriptional factors.[59] Therefore, transcription factors such as AP-1, NFkB, NFAT, or STAT6 cannot induce the expression of pro-inflammatory genes. In addition, PPARγ/RXR complexes directly bind to transcription factors such as NFκB family proteins (p50 and p65) and NFAT, thereby preventing them from inducing gene transcription in macrophages and T cells.[60,61] Last but not least, eosinophils play a crucial role in the development of asthma, and IL-5 and eotaxin are pivotal cytokines/chemokines for eosinophil activation. Recent studies demonstrated that a PPARγ agonist reduced both IL-5–stimulated eosinophil survival and eotaxin-induced eosinophil chemotaxis.[62] Therefore, the development of PPARγ agonists may be useful as therapeutics for chronic inflammatory airway diseases.

H. CCAAT/Enhancer Binding Proteins (C/EBPs)

The C/EBPs constitute a family of transcription factors involved in the regulation of cellular differentiation, cell cycle regulation, and cytokine gene expression.[63] Six members of this family have so far been identified. They are designated C/EBPα to C/EBPγ and they share strong sequence homology.

C/EBPβ and C/EBPδ are readily induced by pro-inflammatory stimuli such as LPS, glucagons, and cytokines.[63] IL-4 expression and reduction