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SUMF2 Interacts With Interleukin-13 and Inhibits Interleukin-13 Secretion in Bronchial Smooth Muscle Cells

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ABSTRACT

IL-13 is a central mediator of allergic inflammation and secreted by Th2 and bronchial smooth muscle cells (BSMC). However, little is known about the regulation of IL-13 secretion. To address it, a cDNA library of BSMC was screened for the proteins interacted with IL-13 by yeast two-hybridization. Besides IL-13 receptors, human sulfatase modifying factor 2 (SUMF2) was interacted with IL-13. Furthermore, SUMF2 and IL-13 were co-immunoprecipitated from BSMC, which was independent of IL-13 glycosylation. Interestingly, high levels of SUMF2 were expressed by BSMC, accompanied by significantly higher levels of intracellular IL-13, but lower levels of IL-13 secretion from BSMC. In contrast, little of SUMF2 was detected in lymphocytes, accompanied by lower levels of intracellular IL-13, but significantly higher levels of 12 kDa form of IL-13 secretion. Moreover, knockdown of SUMF2 expression by transfection with SUMF2-specific siRNA did not alter IL-13 mRNA transcription, but significantly reduced intracellular IL-13 levels, associated with increased levels of IL-13 secretion from BSMC. While induction of transient SUMF2 expression in lymphocytes failed to modulate IL-13 mRNA transcription. It significantly increased the contents of 12 kDa form of intracellular IL-13, accompanied by significantly reduced levels of IL-13 in their supernatants. In addition, blockage of *N*-glycosylation by treatment with tunicamycin eliminated 17 kDa form of intracellular IL-13, but failed to promote IL-13 secretion in BSMC. Collectively, our novel data clearly indicated that SUMF2 interacted with IL-13 and inhibited IL-13 secretion in BSMC and lymphocytes, which was independent of IL-13 glycosylation. J. Cell. Biochem. 108: 1076–1083, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: SULFATASE MODIFYING FACTOR 2; INTERLEUKIN 13; SECRETION; GLYCOSYLATION; BRONCHIAL SMOOTH MUSCLE CELLS; LYMPHOCYTES

Interleukin (IL)-4, IL-5, and IL-13 can be secreted by antigen-activated Th2 cells and high levels of these cytokines are strongly associated with the development of allergic inflammation. IL-13 is also secreted by mast cells, basophils, bronchial smooth muscle cells (BSMC), and airway epithelial cells, and has been thought to be a crucial mediator of bronchial asthma [Wills-Karp, 2004; Chan et al., 2006; Allahverdian et al., 2008; Tliba and Panettieri, 2009]. IL-13 can promote allergen-induced airway hyper-reactivity [Farghaly et al., 2008; Moynihan et al., 2008; Chiba et al., 2009], epithelial cell damage [Booth et al., 2007], mucous cell metaplasia [Tesfaigzi, 2008; Xiang et al., 2008], and eosinophilia aggregation [Venkayya et al., 2002; Fulkerson et al., 2006; Hunninghake et al., 2007]. Indeed, high levels of IL-13 are usually detected in bronchial tissue, bronchoalveolar lavage fluids, and sputum from asthma patients [Saha et al., 2008]. Apparently, IL-13 is a molecular therapeutic target for the development of new medicines for intervention of asthma [Izuhara et al., 2006; Blease, 2008; Kasaian and Miller, 2008]. The expression of IL-13 is mainly regulated by the GATA3 and other factors [Zhu et al., 2006]. However, the regulation of IL-13 secretion is poorly understood.

Sulfatase modifying factor (SUMF) is one member of the formylglycine-generating enzyme (FGE) family and can catalyze the oxidation of cysteine in the active site of sulfatases into C-alpha-formylglycine (FGly), which is necessary for catalytic activities of the sulfatases [Landgrebe et al., 2003; Zito et al., 2005]. SUMF1 and SUMF2 are co-located in the luminal space of the endoplasmic

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reticulum (ER), where the newly synthesized sulfatases are post-translationally modified by the formation of FGly. Although both SUMF1 and SUMF2 share similar expression pattern, unlike SUMF1, SUMF2 lacks the enzymatic activity for the generation of FGly because the SUMF2 lacks two catalytic cysteins, Cys336 and Cys341, in the active site of SUMF1 [Mariappan et al., 2005]. SUMF2 can bind to SUMF1 and sulfatase, regulating their activities and the process of FGly formation [Zito et al., 2005]. Notably, human IL-13 is usually secreted as non-glycosylated form with a molecular mass of 12 kDa although it can be a glycosylated protein with a mass of 17 kDa in the cytoplasma [McKenzie et al., 1993]. Therefore, IL-13 is subjected to post-translational modification, which usually occurs in the ER. Given that SUMF2 is predominately resident in the ER, we hypothesized that SUMF2 may regulate the post-translational modification and secretion of IL-13 in the ER.

To test this hypothesis, a cDNA library of BSMC was screened for the proteins interacted with IL-13 and found that SUMF2 was interacted with IL-13. Importantly, we found that SUMF2 inhibited the secretion of IL-13 in both BMSC and lymphocytes. We discussed the implication of our findings.

MATERIALS AND METHODS

CELL CULTURE

Human BSMC and SMCM (smooth muscle cell medium) were purchased from SicenCell Research Laboratories (Carlsbad). The BSMC were isolated from normal human bronchi and bronchioles. The cells at 10^5/ml were cultured in SMCM supplemented with 10% fetal calf serum (FBS), 10^3 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mg/ml amphotericin B, 12 mM NaOH, 1.7 μM $CaCl_2$, 2 mM L-glutamine, and 25 mM HEPES (Sciencell) and exposed to fresh SMCM every other day. The cells were cultured in the presence and absence of tunicamycin (5 μg/ml; Sigma) for determination of the impact of glycosylation inhibition on IL-13 secretion.

CONSTRUCTION OF A cDNA LIBRARY AND PLASMID FOR BAIT IL-13 EXPRESSION

Total RNA was extracted from BSMC and reversely transcribed into cDNA, followed by cloning into the pMyr vector using the CytoTrap XR Library Construction kit (Stratagene, USA), according to the manufacturer's instructions. The DNA fragment for the coding region of the IL-13 gene (GenBank NM_002188) from the BSMC cDNA library was amplified by RT-PCR with the specific primers 5′-TGC*GGATCC*AGGCCCTGTGCCTCCCTC-3′ (forward) and 5′-GC*CCATGG*AGTTTCAGTTGAACTGTCCCTCG-3′ (reverse). Subsequently, the amplified DNA fragment was inserted into the pSos plasmid at the sites of BamHI and NcoI (Stratagene) to generate the plasmid of pSos-IL-13. Its accuracy was demonstrated by DNA sequencing, and the expression of IL-13 was characterized by Western blot analysis.

YEAST TWO-HYBRIDIZATION SCREENING

The proteins interacted with IL-13 in BSMC were screened by the yeast two-hybridization assay using the expression of IL-13 as the bait protein and the CytoTrap two-hybrid system (Stratagene), according to the manufacturer's instructions. Briefly, the BSMC cDNA library constructed in the pMyr vector was co-transformed with the bait plasmid into yeast cells with transformation efficiency at 1.5 × 103 cfu/μg DNA. The colonies containing proteins interacted with IL-13 were judged if the colonies grew in synthetic glucose minimal medium (SD/glu(-UL)) medium at 25°C and synthetic galactose minimal medium (SD/gal(-UL)) medium at 37°C, but not in SD/glu(-UL) medium at 37°C. The potential positive colonies were further characterized by PCR using the specific primers of 5′-ACTACTAGCAGCTGTAATAC-3′ and 5′-CGTGAATGTAAGCGTGACAT-3′, followed by DNA sequencing.

LYMPHOCYTE ISOLATION

Human blood samples were collected from volunteers and their mononuclear cells were isolated by gradient centrifugation on Histopaque-1077 (Sigma), according to manufacturer's instructions. The mononuclear cell layer was carefully collected and stimulated with 10 μg/ml Con A (Sigma) in 10% FBS RPMI 1640 medium for 3 h. The unadhered lymphocytes were harvested and stained with fluorescent antibodies against CD3 and CD19 (BD PharMigen), followed by examining under a fluorescent microscope. The isolated lymphocytes with a purity of >92% were used for the following experiments.

CO-IMMUNOPRECIPITATION

Human BSMC were cultured in SMCM and when at confluence, they were harvested. After washing with PBS, the cells (5×10^6) were treated with 3 ml ice-cold RIPA lysis buffer (10 mM Tris, 1% NP-40, 0.1% Deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, pH 7.4) at 4°C for 10 min, followed by centrifuging at 10,000*g* at 4°C for 10 min. Their supernatants (1 ml) were mixed with 2 μg anti-SUMF2, anti-IL-13 antibody, or control anti-β-actin (BOSTER or Abcam Biotechnology), and incubated at 4°C for 1 h under gentle agitation, respectively. Subsequently, the mixture was reacted with 20 μl Protein G PLUS-Agarose (Santa Cruz Biotechnology) and incubated at 4°C under gentle agitation for 1 h, followed by centrifuging at 500*g* at 4°C for 5 min. The generated pellets were washed extensively with PBS and the precipitated proteins were dissolved in 40 μl crack buffer. After boiling, 20 μl aliquot containing about 20 μg proteins were analyzed by Western blot assay with both anti-IL-13 and anti-SUMF2 antibody, respectively. The cell lysates without immunoprecipitation were simultaneously analyzed and used as controls.

WESTERN BLOT ASSAY

BSMC or lymphocytes (5×10^6) were lysed in lysis buffer [15 mM Tris; 2 mM EDTA; 50 mM β-mercaptoethanol; 20% glycerol; 0.1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 1 mM sodium fluoride; 1 mM sodium orthovanadate; and 1 μg/ml each aprotinin, leupeptin, and pepstatin (pH 7.5)]. After quantifying the protein concentrations using a spectrophotometer (Bio-Rad Laboratories), equal amount of protein lysate (20 μg/lane) from each sample was separated by 12% SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore), followed by blocking with 5% skimmed milk. The specific antigens were probed with monoclonal antibody against human IL-13 (mouse IgG1, 1:50, BOSTER) and SUMF2 (mouse IgG1, 1:500, Abcam Biotechnology),

respectively, at 4°C overnight. The mouse IgG1 isotype was used as negative controls and anti-β-actin was used for detecting the internal control of β-actin. After washing, the bound antibodies were visualized using alkaline phosphatase-conjugated goat anti-mouse IgG1 and NBT/BCIP substrates (Zhongshan). To analyze the secreted IL-13, the total proteins in the harvested supernatants were first precipitated using absolute ethanol and the form of secreted IL-13 was then analyzed by Western blot assays.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from 10^6 cultured human lymphocytes or BSMC by TRizol, according to the manufacturer's instruction (Invitrogen), respectively and then reversely transcribed into cDNA using Strata Script™ reverse transcriptase (Stratagene). The levels of SUMF2, IL-13, IL-2, IL-4, and GAPDH mRNA transcripts were characterized by quantitative RT-PCR using the SYBR Green PCR Core reagents kit (Applied Biosystems) and the specific primers on a DA7600 PCR amplifier (DAAN). The primers were designed according to the reported gene sequences of human SUMF2 (GenBank accession number: NM_015411), homo sapiens IL-13 (NM_002188), homo sapiens IL-4 (NM_000589), homo sapiens IL-2 (NM_000586), and GAPDH (NM_002046). The sequences of specific primers were presented in Table I. The PCR reactions (50 μl/tube, in duplicate) were denatured at 94°C for 2 min and subjected to 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The relative levels of mRNA transcripts were analyzed by normalizing the values of individual samples to GAPDH.

INDUCTION OF SUMF2 EXPRESSION IN LYMPHOCYTES

To induce human SUMF2 expression in lymphocytes, the gene for human SUMF2 was amplified from the BSMC cDNA library by PCR using the specific primers of 5′-*CCGG ATC* CGTGCATGA-GGGCGCATG-3′, and 5′-CC*GAATTC*ACCACCCGGCTGCTTAC-3′. After digestion with *Bam*HI and *Eco*RI, the PCR products were cloned into the pcDNA6/HisA plasmid (Invitrogen) to generate the recombinant plasmid of pcDNA6/HisA-SUMF2. Following transforming into *E. coli* DH5α competent cells, the positive colonies were identified by DNA sequencing.

Freshly isolated human lymphocytes (5×10^5/ml) were transfected in duplicate with the plasmid of pcDNA6/HisA-SUMF2 or control pcDNA6/HisA by Lipofectamine 2000 (Invitrogen) in RPMI 1640 medium for 2 h and then cultured in 10% FBS RPMI 1640 containing 2 μg/ml blasticidin for 3 days. The cells were stimulated

TABLE I. The Sequences of Specific Primers

Gene		Primer sequence	Length
SUMF2	Forward	5′-CAGAACAACTACGGGCTCTATGAC-3′	494 bp
	Reverse	5′-ACTTGGCCTCTAACAGAAACACC-3′	
IL-13	Forward	5′-GTCAACATCACCCAGAACCAGA-3′	400 bp
	Reverse	5′-TAACCCTCCTTCCCGCCTAC-3′	
IL-4	Forward	5′-TCTCACCTCCCAACTGCTTCC-3′	429 bp
	Reverse	5′-CTCATGATCGTCTTTAGCCTTTCC-3′	
IL-2	Forward	5′-CAACTGGAGCATTTACTGCTGG-3′	235 bp
	Reverse	5′-CCCTGAATTAGTCGTTATAGTTGC-3′	
GAPDH	Forward	5′-GGTGAAGGTCGGAGTCAACGG-3′	227 bp
	Reverse	5′-CCTGGAAGATGGTGATGGGATT-3′	

with 10 μg/ml Con A in 10% FBS RPMI 1640 for 40 h and their supernatants were harvested for analysis of IL-13, IL-2, and IL-4 by enzyme-linked immunosorbent assay (ELISA). The cells were collected for analysis of IL-13 and SUMF2 expression by RT-PCR and Western blot assays.

RNA INTERFERENCE

BSMC at 10^5/ml were transfected in duplicate with 80 nM siRNA pools (2 siRNA duplexes) targeting the SUMF2 gene (Abcam Biotechnology) using Lipofectamine 2000 in Opti-MEM I media (Invitrogen), according to the manufacturer's instructions. Briefly, Lipofectamine 2000 and siRNA (20 μm stock) were diluted in Opti-MEM I at a ratio of 1:25 at room temperature for 5 min, respectively. Equal volume of the siRNA and Lipofectamine 2000 solutions were then mixed and incubated at room temperature for 15 min with gentle agitation, followed by adding the mixture into the cells. After overnight incubation, the cells were exposed to complete medium for additional 2 days.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The concentrations of IL-13, IL-2, and IL-4 in the supernatants collected from cultured BSMC and lymphocytes were determined by ELISA using IL-13, IL-2, and IL-4 specific ELISA kits (Diaclone Research, France), respectively, according to the manufacturer's instructions. The minimum level of IL-13, IL-2, or IL-4 for detection was about 1.5, 10, or 0.5 pg/ml, respectively.

STATISTICAL ANALYSIS

Data were expressed as means ± SEM. The difference between two groups was analyzed by Student *t*-test using SPSS 10.0 for Windows. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

SCREENING PROTEINS INTERACTED WITH IL-13

To screen proteins interacted with IL-13, a cDNA library was generated from BSMC and cloned into the pMyr vector. Furthermore, the gene fragment for encoding IL-13 was amplified by PCR from the cDNA library of BSMC and generated the bait plasmid of pSos-IL-13. After sequencing, the bait plasmid, together with the cDNA library in the pMyr vector, was co-transfected into yeast cells for screening proteins interacted with IL-13. A total 52 positive colonies were obtained and 16 out of 52 positive colonies were randomly selected and further analyzed by PCR (Fig. 1). Apparently, three different genes were detected with varying sizes. Analysis of their DNA sequences revealed that they had 100% sequences matched with the human IL-13 receptor α1 (IL-13Rα1, NM_001560, Supplementary Fig. 1); the IL-13 receptor α2 (IL-13Rα2, NM_001560, Supplementary Fig. 2); and the SUMF2 gene (NM_015411, Supplementary Fig. 3), determined by NCBI/BLAST analysis. Although IL-13Rα1 and α2 were expected the SUMF2 was surprisingly detected, suggesting that SUMF2 interacted with IL-13 in yeast cells.

Fig. 1. Analysis of positive yeast colonies by PCR. After screening the proteins interacted with IL-13, 16 out of 52 yeast colonies were analyzed by PCR using specific primers, followed by agarose gel electrophoresis. Data are a representative image from five repeated experiments. M: DNA L2000 marker. Lanes 1, 3, and 7–11: fragment of the human IL-13Rα1 gene; Lane 5: fragment of the human IL-13Rα2 gene; Lanes 12–16: fragment of the human SUMF2 gene; Lanes 2, 4, and 6: unknown DNA sequences, determined by DNA sequencing and BLAST analysis (their DNA sequences were presented in Supplementary Figs. 1–3).

SUMF2 INTERACTS WITH IL-13, INDEPENDENT OF IL-13 GLYCOSYLATION

Human BSMC can produce IL-13 and provide an in vitro experimental model for the examination of whether SUMF2 could interact with IL-13 by co-immunoprecipitation assay. BSMC lysate was incubated with anti-IL-13 or anti-SUMF2 and the antigen/antibody complex was precipitated by protein-G agarose beads, followed by Western blot analysis. Firstly, SUMF2 and both forms of 12 and 17 kDa IL-13 were detected in BSMC lysates, suggesting that those proteins were expressed by BSMC (Fig. 2A,B, Normal Tun$^-$ lane). Following co-immunoprecipitation with anti-SUMF2, both forms of 12 and 17 kDa IL-13 were observed in BSMC (Fig. 2A, Co-IP Tun$^-$ lane), suggesting that both forms of IL-13 interacted with SUMF2. Furthermore, treatment of BSMC lysate with anti-IL-13 successfully precipitated SUMF2 (Fig. 2B), further demonstrating that IL-13 interacted with SUMF2 in BSMC. However, treatment with anti-β-actin only precipitated β-actin alone in BSMC.

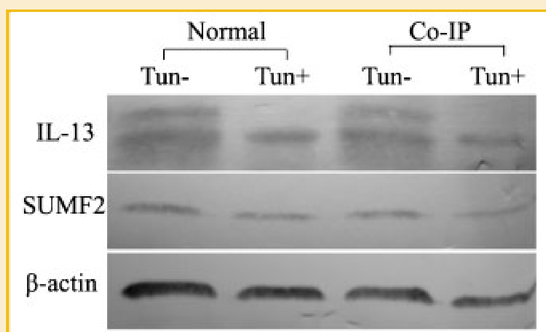

Fig. 2. SUMF2 interacts with IL-13 in BSMC. The interaction of SUMF2 with IL-13 in BSMC was characterized by immunoprecipitation assay. BSMC lysates were incubated with anti-SUMF2 or anti-IL-13 and the resulting antigen/antibody complex was precipitated by protein-G agarose beads, followed by Western blot analysis. BSMC lysates without any antibody treatment were used as controls (normal) and treated and probed with anti-β-actin as positive controls. To examine the impact of IL-13 *N*-glycosylation on the interaction of SUMF2 with IL-13, BSMC were pre-treated with, or without, tunicamycin and their lysates were further analyzed by immunoprecipitation. Data shown are representative images from five repeated experiments. A: Analysis of IL-13 (probed with anti-IL-13) after immunoprecipitation with anti-SUMF2. B: Analysis of SUMF2 (probed with anti-SUMF2) after immunoprecipitation with anti-IL-13. Tun$^-$: Lysate from tunicamycin-untreated BSMC; Tun$^+$: Lysate from tunicamycin-treated BSMC.

To determine the impact of IL-13 glycosylation on the interaction with SUMF2, BSMC were treated with tunicamycin (5 μg/ml) for 24 h and the interaction of SUMF2 with IL-13 was characterized by co-immunoprecipitation. Obviously, tunicamycin treatment almost eliminated the form of 17 kDa IL-13, indicating the inhibition of IL-13 glycosylation. After treatment with anti-SUMF2, a strong band of 12 kDa IL-13 were observed (Fig. 2A, Co-IP Tun$^+$ lane) while SUMF2 was also precipitated by anti-IL-13 (Fig. 2B, Co-IP Tun$^+$ lane). Given that IL-13 at 12 kDa is non-glycosylated form our data indicate that SUMF2 naturally interacts with IL-13 in BSMC and their interaction can be independent of IL-13 glycosylation.

DIFFERENTIAL EXPRESSION OF SUMF2 AND IL-13 IN LYMPHOCYTES AND BSMC

IL-13 is expressed in both human lymphocytes and BSMC, and the expression of SUMF2 and IL-13 in those cells was further characterized by RT-PCR, Western blot assay, and ELISA. First, while a strong band of the SUMF2 gene was detected in BSMC only a very weak band was observed in lymphocytes by RT-PCR, suggesting that very low levels of SUMF2 mRNA were transcribed in lymphocytes (Fig. 3A). Second, the relative levels of IL-13 mRNA transcripts in BSMC were significantly higher than that in lymphocytes ($P < 0.05$, Fig. 3B). Furthermore, analysis of their proteins revealed that while higher levels of IL-13 and SUMF2 were detected in BSMC, predominately with the 12 kDa non-glycosylated form of IL-13, moderated levels of IL-13, mainly with the 17 kDa glycosylated form, were observed in lymphocytes (Fig. 3C). Interestingly, only very little SUMF2 protein was detected in lymphocytes. Moreover, quantification of IL-13 in the supernatants of cultured cells indicated that significantly higher levels of IL-13 were secreted by lymphocytes, as compared with that by BSMC ($P < 0.05$, Fig. 3D). Finally, characterization of intracellular and secreted IL-13 by lymphocytes demonstrated that high levels of 17 kDa form of IL-13 displayed in intracellular lymphocytes while almost only 12 kDa form of IL-13 was detected in the supernatants of cultured lymphocytes (Fig. 3E). Together, these data demonstrated that high level of SUMF2 was expressed by BSMC, but little by lymphocytes and IL-13 was differentially expressed and secreted between BSMC and lymphocytes.

SUMF2 INHIBITS IL-13 SECRETION

The interaction of SUMF2 with IL-13 and differential expression of SUMF2 between BSMC and lymphocytes raised the question about the function of SUMF2 in the expression and secretion of IL-13 in those cells. To address this issue, we employed two strategies by knockdown of SUMF2 expression in BSMC with siRNA technology and induction of transient SUMF2 expression in lymphocytes. Transfection of BSMC with SUMF2-specific siRNA almost completely abrogated the expression of SUMF2 in BSMC, evidenced by little detection of SUMF2 mRNA transcript in BSMC (Fig. 4A).

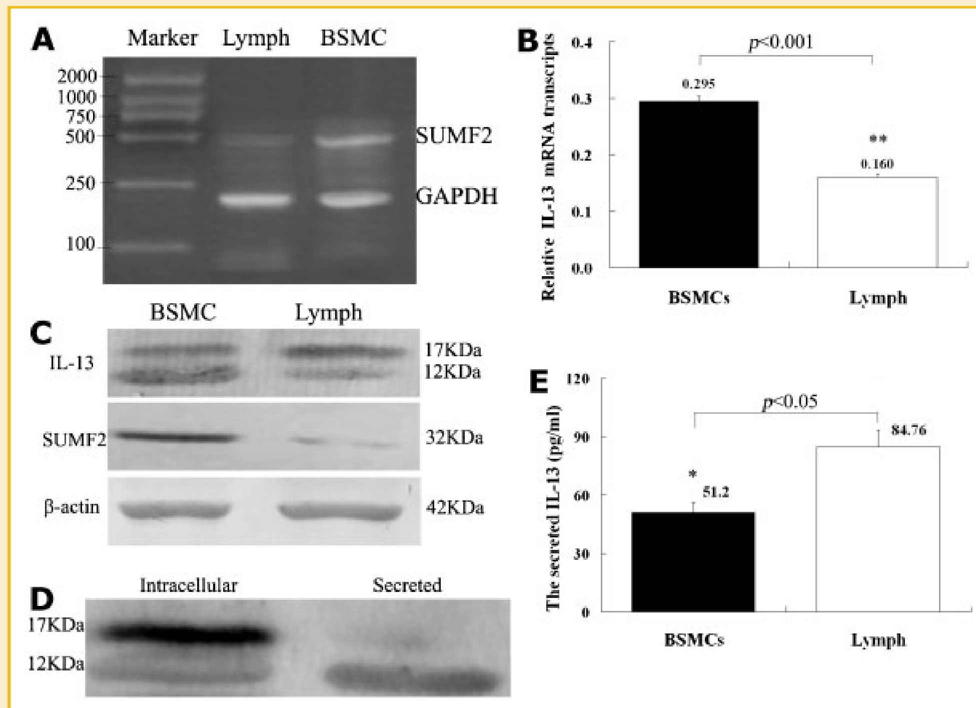

Fig. 3. Analysis of SUMF2 and IL-13 expression in BSMC and lymphocytes. The SUMF2 and IL-13 expression and IL-13 secretion were characterized by RT-PCR, Western blot, and ELISA, respectively. Data are representative images or expressed as mean ± SEM of each group (n = 10 per group) from five independent experiments. A: Analysis of SUMF2 expression by RT-PCR. The SUMF2 and control GAPDH were simultaneously analyzed in the same tube. B: The relative levels of IL-13 mRNA transcripts. The values of each group to control GAPDH were determined by quantitative RT-PCR. C: Western blot analysis of SUMF2 and IL-13 expression. D: Western blot analysis of secreted IL-13. E: The levels of secreted IL-13. The concentrations of IL-13 in the supernatants of BSMC and lymphocytes were determined by ELISA. Lymph: Lymphocytes.

Similarly, transfection of lymphocytes with the pcDNA6/HisA-SUMF2, but not control pcDNA6/hisA, induced high levels of SUMF2 mRNA transcripts in lymphocytes. Furthermore, knockdown of SUMF2 in BSMC and induction of SUMF2 expression in lymphocytes did not modulated the expression of IL-13 because the levels of IL-13 mRNA transcripts were similar between siRNA-untransfected and transfected BSMC or between SUMF2-untransfected and transfected lymphocytes (Fig. 4B). Interestingly, transfection of BSMC with SUMF2-specific siRNA reduced the levels of intracellular IL-13 while transfection of lymphocytes with the SUMF2 gene increased intracellular IL-13 contents (Fig. 4C). Consistently, the levels of IL-13 were significantly elevated in the supernatants of BSMC while reduced in the supernatants of lymphocytes. However, modulation of SUMF2 expression failed to alter in the expression and secretion of IL-2 and IL-4 mRNA transcripts in both types of cells. Apparently, SUMF2 did not modulate the expression of IL-13, but specifically inhibited the secretion of IL-13 in both types of cells.

SUMF2 INHIBITS THE SECRETION OF IL-13, INDEPENDENT OF IL-13 GLYCOSYLATION

Finally, we examined whether IL-13 *N*-glycosylation could affect inhibitory effect of SUMF2 on IL-13 secretion. BSMC were treated with, or without, tunicamycin (5 μg/ml) for 24 h and the levels of intracellular and secreted IL-13 were characterized by Western blot assay and ELISA, respectively. Treatment with tunicamycin did not significantly change the expression of SUMF2 because similar levels of SUMF2 were detected in tunicamycin-untreated and treated BSMC. However, this treatment significantly increased the intracellular levels of 12 kDa form of IL-13 and almost eliminated 17 kDa form of IL-13, demonstrating that tunicamycin efficiently blocked the process of IL-13 *N*-glycosylation in BSMC. Importantly, similar levels of IL-13 were detected in the supernatants of tunicamycin-untreated and treated BSMC. These data suggest that 12 kDa form of IL-13 is likely to be secreted by BSMC and the inhibitory effect of SUMF2 on IL-13 secretion is independent of IL-13 glycosylation (Fig. 5).

DISCUSSION

IL-13 is a crucial mediator of allergic inflammation and produced by Th2, BSMC, and other cells. While GATA-3 acts as a transcription factor, regulating Th2 differentiation and IL-13 expression [Zhu et al., 2006; Yao et al., 2008], little is known whether other factors could modulate IL-13 secretion. In this study, we attempted to identify the proteins interacted with IL-13 and their regulatory roles in the expression and secretion of IL-13 in both human BSMC and lymphocytes. After the establishment of a cDNA library with BSMC cells, the proteins, which interacted with IL-13, were screened by two-hybridization. As expected, human IL-13Rα1 and IL-13Rα2 were identified. Surprisingly, SUMF2 and other unknown proteins

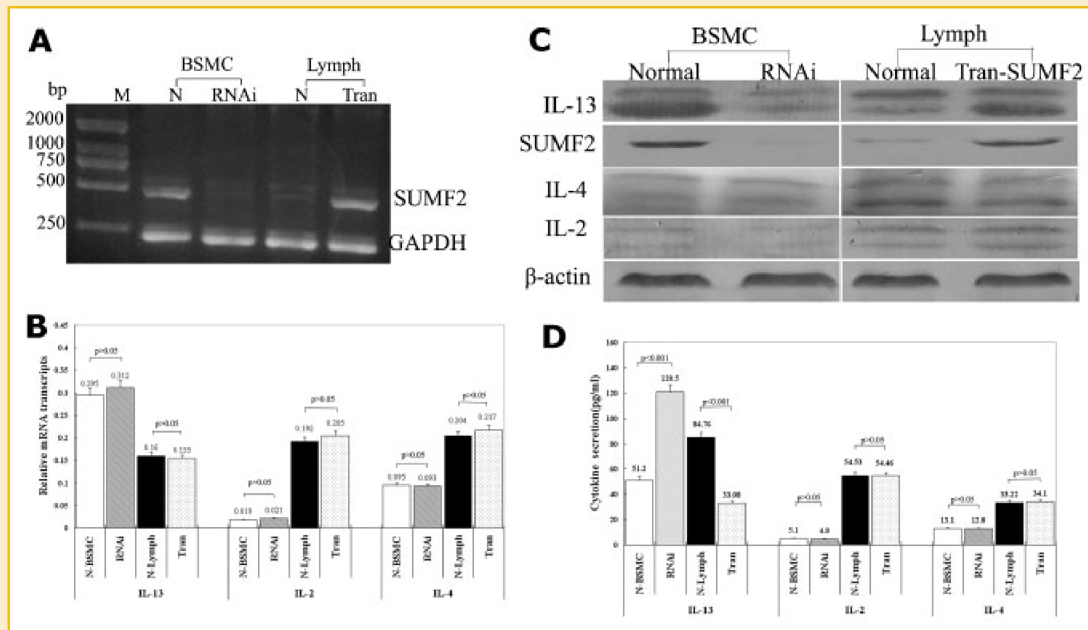

Fig. 4. The modulatory effect of SUMF2 expression on IL-13 expression and secretion. BSMC were transfected with, or without, SUMF2-specific siRNA to knockdown SUMF2 expression and lymphocytes were transfected with, or without, the SUMF2 gene for inducing transient SUMF2 expression. Their SUMF2, IL-13, IL-2, and IL-4 expression and IL-13, IL-2, and IL-4 secretion were characterized by RT-PCR, quantitative RT-PCR, Western blot, and ELISA, respectively. Data are representative images or expressed as mean ± SEM of each group (n = 10 per group) from five independent experiments. A: RT-PCR analysis of SUMF2 mRNA transcripts. M: DNA L2000 marker; N: Normal (unmanipulated) BSMC or lymphocytes; RNAi: BSMC that had been transfected with SUMF2-psecific siRNA; Tran: Lymphocytes that had been transfected with the SUMF2 gene. The SUMF2 and control GAPDH were simultaneously analyzed in the same tube. B: The relative levels of IL-13, IL-2, and IL-4 mRNA transcripts, determined by quantitative RT-PCR. C: Western blot analysis of SUMF2, IL-13, IL-2, and IL-4 expression. D: The levels of secreted IL-13, IL-2, and IL-4. The concentrations of IL-13, IL-2, and IL-4 in the supernatants of BSMC and lymphocytes were analyzed by ELISA. The levels of IL-2 mRNA transcripts and secreted proteins in BSMC were near the experimental background, indicating no expression of IL-2 in BSMC.

were interacted with IL-13 in yeast cells (Fig. 1). The interaction of SUMF2 with IL-13 was further demonstrated by immunoprecipitation because incubation of BSMC lysates with anti-SUMF2 precipitated both 12 and 17 kDa forms of IL-13 and treatment with anti-IL-13 captured SUMF2 proteins, even after blockage of IL-13 *N*-glycosylation (Fig. 2). These data clearly indicate that SUMF2 is expressed by BSMC and SUMF2 interacts with both forms of IL-13, independent of IL-13 glycosylation.

Analysis of SUMF2 and IL-13 expression and IL-13 secretion suggested that the expression of SUMF2 was associated with low levels of IL-13 secretion in BSMC and lymphocytes. Firstly, high levels of SUMF2 were expressed in BSMC, but little of it in lymphocytes. Secondly, higher levels of IL-13 mRNA transcripts and proteins were detected in SUMF2-expressing BSMC, but significantly lower concentrations of IL-13 were measured in their supernatants. In contrast, high levels of IL-13 were secreted by lymphocytes, which expressed a little SUMF2. Furthermore, transfection of BSMC with SUMF2-specific siRNA not only abrogated SUMF2 expression, but also reduced the contents of intracellular IL-13. The reduced levels of IL-13 were not caused by SUMF2 inhibition on IL-13 expression, rather than by promoting IL-13 secretion. Evidently, similar levels of IL-13 mRNA transcripts in unmanipulated and siRNA-transfected BSMC cells, however, significantly higher levels of IL-13 in the supernatants of BSMC. Conversely, induction of SUMF2 expression in lymphocytes did not significantly modulate the expression of IL-13, but it significantly inhibited the secretion of IL-13, which was supported by higher levels of intracellular IL-13 and lower levels of secreted IL-13 in the supernatants of cultured lymphocytes. Finally, blockage of IL-13 glycosylation did not affect the inhibition of SUMF2 on the IL-13 secretion. Collectively, these novel data provide first evidence to demonstrate that SUMF2 interacts with IL-13 and inhibits IL-13 secretion, independent of IL-13 glycosylation. Notably, modulation of SUMF2 expression did not affect the expression and secretion of IL-2 and IL-4 in both types of cells. Apparently, SUMF2 specifically regulates the secretion of IL-13. However, the specificity of SUMF2 remains to be further demonstrated.

SUMF2 is one of the FGE family members and binds to SUMF1 and sulfatase. However, SUMF2 has no FGE activity [Mariappan et al., 2005; Zito et al., 2005]. Recent data show that human SUMF2 is retained in ER through its C-terminal tetrapeptide PGEL, a non-canonical variant of the classic KDELER-retention signal [Gande et al., 2008]. Notably, IL-13 is a secreted cytokine and after translation, it is translocated into the lumen of the ER through its N-terminal signal peptides. It is possible that SUMF2 binds to both unglycosylated and glycosylated IL-13, perhaps in the ER in an unknown manner, which inhibits the intracellular IL-13 transportation and secretion. However, the precise mechanisms underlying the regulation of SUMF2 expression and interaction of SUMF2 with IL-13 remain to be further investigated. Notably, SUMF2 was

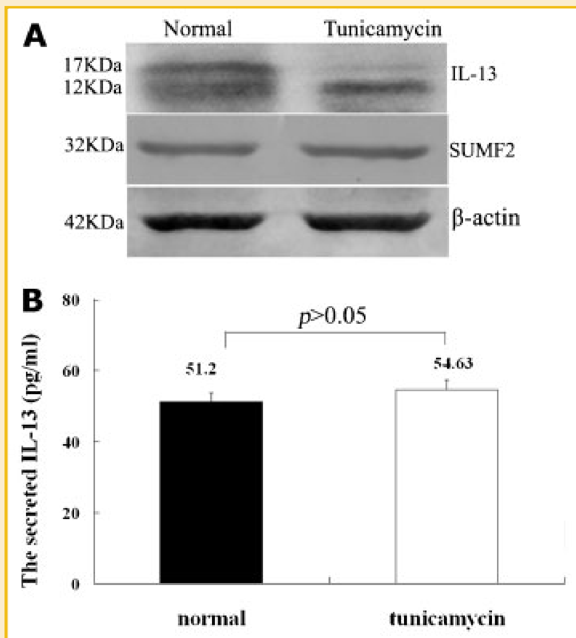

Fig. 5. The inhibitory effect of SUMF2 on IL-13 secretion is independent of IL-13 glycosylation. BSMC were treated with, or without, 5 μg/ml tunicamycin. Their intracellular and secreted IL-13 was characterized by Western blot assay and ELISA, respectively. Data are representative images or expressed as mean ± SEM of each group (n = 10) from five independent experiments. A: Western blot analysis of SUMF2 and IL-13 expression in BSMC. Normal: Unmanipulated BSMC; Tunicymycin: Tunicamycin-treated BMSC. B: The levels of secreted IL-13. The concentrations of secreted IL-13 in the supernatants of BSMC were determined by ELISA.

differentially expressed and secreted by lymphocytes and BSMC. Conceivably, some pathogenic factors may down-regulate the expression of SUMF2 and promote high levels of IL-13 secretion, particularly in BSMC, leading to airway inflammatory. Therefore, our findings may provide new insights into the regulation of IL-13 secretion and the pathogenic process of airway inflammation. If the inhibition of SUMF2 on the secretion of IL-13 is demonstrated in vivo, therapeutic modulation of SUMF2 expression to reduce the secretion of IL-13 may be valuable for intervention of airway inflammatory.

Previous studies have shown that mature IL-13 contains 112 amino acids with a molecular mass of about 12 kDa, which is a non-glycosylated form and secreted in the supernatants [McKenzie et al., 1993]. Other studies to characterize IL-13 are usually based on its antigenicity by ELISA [Burd et al., 1995]. There is little information about its intracellular molecular base. Interestingly, we found that two forms of intracellular IL-13 with molecular weight of 12 and 17 kDa were detected in both BSMC and lymphocytes. However, only 12 kDa form of IL-13 was detected in the supernatants. The relative levels of both forms of intracellular IL-13 appeared not to be associated with the secretion as BSMC had higher ratio of 12–17 kDa intracellular IL-13; however, they secreted lower levels of IL-13, as compared with that of lymphocytes. Strikingly, lower levels of 17 kDa form of IL-13 were detected in intracellular BSMC while higher levels of it in lymphocytes, which was negatively associated with the expression of SUMF2. Given that there are four N-linked glycosylation sites in IL-13 it is possible that the 17 kDa form of IL-13 was a glycosylated form while the one with smaller mass was unglycosylated. Indeed, treatment of BSMC with tunicamycin to block endogenous glycosylation increased the contents of intracellular IL-13 with a molecular mass of 12 kDa. Possibly, SUMF2 may negatively regulate the glycosylation of IL-13 in BSMC. Therefore, during the post-translational process, IL-13 is firstly subjected to glycosylation for intracellular transportation and then decarbohydrate before secretion, which is different from other cytokines. Alternatively, the 17 kDa form of intracellular IL-13 may be resulted from different splices of IL-13 gene [McKenzie et al., 1993]. We are interested in further investigating the post-translational process of IL-13 and whether the 17 kDa form of IL-13 is bioactive.

In summary, our data showed that SUMF2 interacted with IL-13 and inhibited IL-13 secretion in both BSMC and lymphocytes, which was independent of IL-13 glycosylation. Furthermore, higher levels of SUMF2 were expressed by BSMC, but little by lymphocytes. In addition, two forms of intracellular IL-13 were detected in both types of cells, but the 12 kDa form of IL-13 was predominately secreted by both BSMC and lymphocytes. Given that IL-13 is a crucial mediator for the development of airway inflammation, our findings that SUMF2 inhibited IL-13 secretion may provide new insights into the regulation of IL-13 secretion and understanding the pathogenesis of airway inflammation.

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