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Enhanced Th2 Cell Differentiation and Allergen-Induced Airway Inflammation in Zfp35-Deficient Mice¹

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Studies of human asthma and of animal models of allergic airway inflammation revealed a crucial role for Th2 cells in the pathogenesis of allergic asthma. Kruppel-type zinc finger proteins are the largest family of a regulatory transcription factor for cellular development and function. Zinc finger protein (Zfp) 35 is an 18-zinc finger motif-containing Kruppel-type zinc finger protein, while its function remains largely unknown. The aim of this study was to clarify the role of Zfp35 in the pathogenesis of Th2-dependent allergic inflammation, such as allergic asthma. We examined airway eosinophilic inflammation and hyperresponsiveness in two mouse models, which use our newly generated Zfp35-deficient (Zfp35−/−) mice and adoptive transfer of cells. In Zfp35−/− mice, Th2 cell differentiation, Th2 cytokine production, eosinophilic inflammation, and airway hyperresponsiveness were substantially enhanced. Furthermore, adoptive transfer of Ag-sensitized Zfp35−/− CD4 T cells into the asthmatic mice resulted in enhanced airway inflammation and airway hyperresponsiveness. These results indicate that Zfp35 controls Th2 cell differentiation, allergic airway inflammation, and airway hyperresponsiveness in a negative manner. Thus, Zfp35 may control Th2-dependent diseases, such as allergic asthma. The Journal of Immunology, 2009, 183: 0000–0000.

Asthma is a chronic inflammatory disease of the lower airways that causes airway hyperresponsiveness (AHR)¹ to a wide variety of specific and nonspecific stimuli (1, 2). Acute allergic asthma represents airway inflammation predominated by eosinophils, hypersecretion of mucus, and AHR. The involvement of Th2 cells in the pathogenesis of allergic asthma has been well recognized through the studies of human asthma as well as of animal models of allergic airway inflammation (3–9).

It is well recognized that CD4⁺ effector Th cells are categorized into three subsets, Th1, Th2, and Th17 cell subsets. Th1 cells produce IFN-γ and direct cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and are involved in humoral immunity and allergic reactions. Th17 cells, a recently identified population (10), produce IL-17 and play a critical role in the pathogenesis of autoimmune diseases (11–13). Several transcription factors that control the differentiation of these Th subsets have been identified. Among them, a zinc finger transcription factor GATA3 appears to be a key transcription factor for Th2 cell differentiation (14, 15), T-bet for Th1 (16), and Retinoid-related orphan receptor γt for Th17 cell differentiation (17).

Transcription factors play critical roles in a variety of cellular processes, such as differentiation, proliferation, cell death, and survival through specific induction/repression of the target genes (18–20). The Cys²His² (C₄H₂) type zinc finger proteins (Zfps) are one of the well-defined eukaryotic DNA-binding proteins, and ~700 genes existed in the human genome (21–23). The C₄H₂ type zinc finger motif is composed of 21 aa with a consensus sequence of CX₂CXₓFX₅LX₂HX₃H and contains two conserved cysteine and histamine residues that coordinate a zinc ion and fold the domain into a finger-like structure (24, 25). The investigation of C₄H₂ type Zfps suggested their unique and specific roles in the regulation of lymphocyte development. For example, Egr-3, Zfp608, Th-POK, and Gfi-1 are shown to be involved in the differentiation of T cells (26–29). We have recently reported that Schnurri-2 plays a crucial role in the control of Th2 cell differentiation by regulating NF-kB functions (30, 31). In particular, Zfps that possess many zinc finger motifs, such as PLZF, SKAT-2, ZEB, and ZNF304 (these have 9, 14, 7, and 13 zinc finger motifs, respectively), control the IL-4 production from either CD4 T or NKT cells (32–35).

We selected Zfp35 (also known as znf271) in a subtraction analysis where a relatively increased expression of Zfp35 was detected in IL-4-producing cells in comparison to IL-4⁺⁺⁺, producing cells in vitro-generated Th2 cells. Zfp35 belongs

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3 Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; Zfp, zinc finger protein; Tg, transgenic; RL, lung resistance.

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to C2H2-type Zfp family and contains 18 continuous typical C2H2 zinc fingers in its C-terminal and contains one acidic region with a numbers of glutamic and aspartic acid domain in its N terminus (36). A human homolog of Zfp35, ZNF239, was identified in EBV-positive B cells and shows 79% homology at the amino acid level compared with mice (37). A human homolog of Zfp35, ZNF239, was identified in the cDNA library of the spleen by the Mammalian Gene Collection Program and shows 79% homology at the amino acid level compared with mice (37). The rat homolog Zfp239 was identified in EBV-positive B cells and shows 79% homology with a numbers of glutamic and aspartic acid domain in its N terminus (36). A human homolog of Zfp35, ZNF EB, was identified in splenic CD4 T cells of wild-type (+/+), Zfp35<sup>−/−</sup> (+/−), and Zfp35<sup>−/−</sup> (−/−) mice, and then expression levels of Zfp35 were determined by the RT-PCR method. Three independent experiments were performed with similar results. n.d., Not detected.

**Materials and Methods**

**Mice**

We have newly established Zfp35<sup>−/−</sup> mice and were backcrossed to either BALB/c or C57BL/6<sup>−/−</sup> mice. Zfp35<sup>−/−</sup> × DO11.10-transgenic (Tg) mice (39) were used at 6–8 wk old. BALB/c and C57BL/6 mice were purchased from CLEA Japan. All mice used in this study were maintained under specific pathogen-free conditions. Animal care was conducted in accordance with the guidelines of Chiba University.

**Cell purification**

Splenic CD4<sup>+</sup> T cells were purified by using magnetic beads and an AutoMACS Sorter (Miltenyi Biotec) yielding a purity of >98%. Where indicated, splenic CD4<sup>+</sup> T cells with naive phenotype (CD44<sup>low</sup>) and eosinophils in the bronchoalveolar lavage (BAL) fluid were isolated on a FACSAria cell sorter (BD Biosciences), yielding a purity of >98% as previously described (40). The lung mononuclear cells were isolated as described previously (41).

**In vitro Th1/Th2 cell differentiation cultures**

Naive splenic CD4<sup>+</sup> T cells were stimulated with 3 μg/ml immobilized anti-TCR-β mAb (H57-597) in the presence of 25 U/ml IL-2 and 1–100 U/ml IL-4 as described previously (42). For Th1 cell differentiation, naive splenic CD4<sup>+</sup> T cells were stimulated with 3 μg/ml immobilized anti-TCR-β mAb (H57-597) in the presence of 25 U/ml IL-2, IL-12, and anti-IL-4 mAb. In brief, 1.5 × 10<sup>5</sup> sorted DO11.10 Tg CD4<sup>+</sup> CD4<sup>+</sup> CD4<sup>+</sup> T cells were stimulated with antigenic OVA peptide (Loh15, OVA<sub>323–339</sub>) 0.1 μM) and 10<sup>5</sup> irradiated (3500 rad) normal BALB/c splenocytes. Appropriate cytokines and anti-cytokine Abs were added in Th1/Th2 cell differentiation cultures as described previously (40). In typical DO11.10 Tg T cell cultures, Th2 cell-skewed (IL-12 with anti-IFN-γ mAb) and Th1 cell-skewed (IL-12 with anti-IL-4 mAb) conditions were used.

**ELISA for the measurement of cytokine concentration**

The production of IL-2, IL-4, IL-5, IL-10, and IFN-γ was measured by ELISA as described elsewhere (43). The production of IL-13 was measured by a mouse IL-13 ELISA kit (R&D Systems) according to the manufacturer’s protocol.

**Immunofluorescent staining and flow cytometry analysis**

In general, one million cells were stained with Abs as indicated according to a standard method (44, 45).

**Quantitative RT-PCR**

Quantitative RT-PCR was performed as described previously using an Applied Biosystems PRISM 7500 Sequence Detection System under standard conditions (46, 47). The primers for TaqMan probes for the detection of Zfp35 (exons 2 and 3), muc5ac, gob5, IL-4, IL-5, IL-13, eosinophil peroxidase (EPO), and HPRT were purchased from Applied Biosystems.

**Collection and analysis of BAL fluid**

One day after the last challenge with OVA, BAL was prepared as previously described (48). Total BAL fluid was collected and cells in 100-μl aliquots were counted. One hundred thousand viable BAL cells were cytocentrifuged onto slides by a Cytospin 3 (Shandon) and stained with May-Grünwald-Giemsa solution (Merck) as described elsewhere (49). Five hundred leukocytes were counted on each slide. Cell types were identified...
using morphological criteria. The percentages of each cell type were calculated. Cytokines levels in the BAL fluid were measured 6 h after the last OVA challenges.

Immunization, challenge, and CD4 T cell transfer

Mice were immunized with 1 mg of OVA in alum on day 0. They were challenged on days 7 and 9 intranasally with 100 \( \mu \)g of OVA in 30 \( \mu \)l of saline under anesthesia as previously described (50). Splenic CD4 T cells from OVA-immunized wild-type or zfp35-deficient BALB/c mice on day 10 were isolated by the panning method as previously described (40). Prepared CD4 T cells (1.5 \( \times \) 10^7 cells) were i.v. transferred into wild-type BALB/c mice. Transferred mice were challenged on days 11 and 13 intranasally as above.

OVA inhalation and analysis of airway inflammation

AHR responses were assessed by methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics) as described previously (50). Airway function was also assessed by measuring the changes in lung resistance (RL) and dynamic compliance in response to increasing doses of inhaled methacholine, as described previously (51, 52).

Analysis of lung histology

The lung samples taken on day 11 were sectioned, stained with H&E reagents, periodic acid-Schiff reagents, and Luna reagents as described previously (50).

Statistical analysis

The significance between two groups was determined by two-tailed Student’s \( t \) test. Mann-Whitney \( U \) tests were used to determine the level of difference in the degree of AHR.

Results

Establishment of knockout mice for Zfp35 gene

To functional analyze the Zfp35, the targeting strategy to inactivate Zfp35 is described in Fig. 1. Germline chimeras and mice heterozygous for the mutated Zfp35 gene were generated as described in Materials and Methods. Southern blot analysis with the external probe confirmed the correct recombination (Fig. 1B).

We also assessed the mRNA expression of Zfp35 in freshly prepared splenic CD4 T cells from BALB/c and Zfp35-deficient mice. Real-time RT-PCR analysis demonstrated that there was no detectable Zfp35 transcript in Zfp35-deficient splenic CD4 T cells (Fig. 1C).

Phenotypic and function characterization of CD4 T cells in Zfp35-deficient (Zfp35^−/−) mice

We found that moderate numbers of CD4 and CD8 T cells were present in Zfp35-deficient mice of a BALB/c and C57BL/6 background (Fig. 1D and data not shown). The cell surface expression of TCR-\( \beta \), CD3\v, CD25, CD69, CD44, and CD62L on thymic and splenic CD4 and CD8 T cells was found to be comparable to those of controls (data not shown). In addition, anti-TCR-\( \beta \) mAb-, IL-2-, or peptide-induced proliferative responses were indistinguishable between wild-type and Zfp35^−/− splenic CD4 T cells (data not shown). Thus, no obvious defect in the phenotype or the activation of Zfp35^−/− splenic CD4 T cells was noted.
Enhanced OVA- and anti-TCR Ab-induced Th2 cell differentiation in Zfp35−/− mice

To examine the influence of Th cell differentiation in the naive Zfp35−/− CD4 T cells, we used the in vitro Th1/Th2 cell differentiation method and examined the response by intracellular cytokine staining (40, 53). Naive splenic CD4 T cells (CD4−CD44low) were purified by cell sorting (purity >98%). An assessment of the capability of Zfp35−/− CD4 T cells to differentiate into Th1/Th2 cells in vitro indicated a moderate enhancement of Th2 cell differentiation under Th2 conditions (18.8% vs 26.5% and 29.3% vs 42.3%; Fig. 2B), while Th1 cell differentiation was equivalent at the same IL-12 dose (Fig. 2A). The ability to produce Th2 cytokines was then assessed by ELISA. As shown in Fig. 2E, the production of IL-4, IL-5, and IL-13 increased about 2- to 4-fold in zfp35-deficient Th2 cells. IFN-γ production was not detectable in the cultured Th2 cells.

Next, to further examine the efficiency in Th2 cell differentiation of Zfp35−/− T cells, purified splenic CD4 T cells were stimulated with immobilized anti-TCRβ mAb in graded doses of exogenous cytokines. Th2 cell differentiation was enhanced in Zfp35−/− T cells at any doses of exogenous IL-4, particularly the enhancement was more prominent under relatively lower IL-4 conditions, whereas Th1 cell differentiation was unaffected (Fig. 2, C and D). In addition, proliferative responses induced with antigenic peptide, IL-2, or IL-4 were indistinguishable between wild-type and Zfp35−/− effector Th2 cells (data not shown). These results thus far suggest that Zfp35 regulates Th2 cell differentiation in a negative manner.

Enhanced OVA-induced eosinophilic inflammation and AHR in Zfp35−/− mice

To assess the role of Zfp35 in allergic airway inflammation, the BAL fluid of OVA-immunized and OVA-inhaled wild-type and Zfp35−/− (BALB/c background) mice was collected 48 h after the last OVA challenge (Fig. 3A). The number of total infiltrating cells and the absolute number of eosinophils and the percentages of eosinophils per
Zfp35

CD4 T cells are given in parentheses. Three independent experiments were done with similar results.

Stained with anti-CD4 and one of anti-CD25, anti-CD44, and anti-CD69 mAbs. The percentages of each quadrant are shown. The percentages among the regions in Zfp35−/− mice (data not shown).

The expression of surface markers on CD4 T cells of OVA-immunized and -challenged Zfp35−/− mice. A. g-D-induced goblet cell hyperplasia was evaluated by periodic acid-Schiff staining. Representative photographic views of wild-type and Zfp35−/− mice (BALB/c background) are shown (a–d). B. Total RNA was prepared from the lung of asthmatic wild-type or Zfp35−/− mice and mRNA levels of muc5ac and gob5 were examined. The data represent the mean values of muc5ac and gob5 mRNA expression normalized with hprt expression. Three independent experiments were done with similar results (∗∗, p < 0.01 and ∗∗∗, p < 0.005). n.d., Not detected; Cont., control.

Specific staining of mucus-containing epithelial cells was not detected in wild-type or Zfp35−/− mice when OVA was not challenged (Fig. 4A, control (Cont.), a and b). After the OVA challenge, Zfp35−/− mice showed a mildly increased mucus production in the airway epithelium in comparison to wild-type mice (Fig. 4A, c and d). Subsequently, we examined the expression of muc5ac and gob5 in the asthmatic lung of Zfp35−/− mice. The expression of these genes in the lung of Zfp35−/− mice significantly increased as compared wild-type mice (Fig. 4B). Taken together, these results indicated that allergic airway inflammation, mucus production, and AHR were all enhanced in Zfp35−/− mice. Thus, Zfp35 appears to be one of the key transcription factors in mediating Th2-dependent airway inflammation.

Increased Th2 cytokine production from CD4 T cells of BAL fluid in immunized Zfp35−/− mice

Th2 cells predominantly express CCR3 and CCR4 (54–56). Consequently, we examined the cell surface expression of CCR3 and CCR4 on CD4 T cells in OVA-immunized and -challenged Zfp35−/− mice. The expression of these chemokine receptors was very low either on CD44high or CD44low splenic CD4 T cells, and no difference was detected between wild-type and Zfp35−/− mice (Fig. 5A), thus indicating that CD4 T cells in the spleen of Zfp35−/− mice were not spontaneously polarized.

We also examined the expression of CD25, CD44, and CD69 on the splenic CD4 T cells and lung CD4 T cells in OVA-immunized and -challenged Zfp35−/− mice (Fig. 5B). The number of CD25+ and CD69+ CD4 T cells in the lung slightly increased in the
OVA-immunized and -challenged mice in comparison to nonimmunized mice, but the numbers of CD25⁺, CD44⁺, and CD69⁺ cells in the Zfp35⁻/⁻ groups were comparable to those of the wild type. These results indicate that CD4 T cells in the spleen and the asthmatic lung of Zfp35⁻/⁻ mice were not spontaneously activated even after OVA immunization and challenge.

Increased Th2 cytokine production from CD4 T cells and eosinophils in the BAL fluid in Zfp35⁻/⁻ mice

We isolated the BAL fluid from OVA-immunized and -challenged Zfp35⁻/⁻ mice, and CD4 T cells and I-A^d-negative, Gr-1, and CCR3-positive eosinophils were isolated by a cell sorter. The mRNA expression of IL-4, IL-5, and IL-13 in the BAL fluid CD4 T cells was assessed, and the expression of IL-4 and IL-13 was increased in Zfp35⁻/⁻ mice (Fig. 6A). The percentages of I-A^d-negative, Gr-1, and CCR3-positive eosinophils were slightly increased (Fig. 6B). The levels of IL-4, IL-5, IL-13, and EPO mRNA expression in the sorted eosinophils were examined. The levels of IL-4, IL-13, and EPO expression were increased substantially in the Zfp35⁻/⁻ groups in comparison to the wild type (Fig. 6C). The expression of IL-5 was not detected in this experimental system.

Modulation of airway inflammation by adoptively transferred Zfp35⁻/⁻ CD4 T cells

Finally, we addressed whether OVA-primed T cells with altered Zfp35 expression are able to modulate OVA-induced allergic inflammation. Following immunization with OVA, splenic CD4 T cells from BALB/c and Zfp35⁻/⁻ mice (BALB/c background) were prepared and transferred into BALB/c mice that had been also immunized with OVA once (Fig. 7A). Two and 4 days after cell transfer, mice were challenged with intranasal administration of OVA and the inflammatory infiltrates in the BAL fluid were analyzed. As shown in Fig. 7, B and C, the adoptive transfer of CD4 T cells from Zfp35⁻/⁻ mice resulted in increased levels of eosinophilic infiltrates. As shown in Fig. 7, D and E, the hyperresponsiveness to methacholine in Zfp35⁻/⁻ mice was enhanced compared with that in control mice. These results indicate that the expression levels of Zfp35 in CD4 T cells appear to affect the OVA-induced airway inflammation and AHR.

Discussion

In this report, we demonstrated that Zfp35⁻/⁻ CD4 T cells differentiate into Th2 cells more efficiently (Fig. 2). The development of eosinophilic airway inflammation, mucus hyperproduction, and AHR was shown to be enhanced in Zfp35⁻/⁻ mice (Figs. 3 and 4). Th2 cytokine expression of CD4 T cells and eosinophils of BAL fluid in the challenged Zfp35⁻/⁻ mice increased (Fig. 6). Moreover, the transfer of OVA-sensitized Zfp35⁻/⁻ CD4 T cells modulated the airway inflammation (Fig. 7), thus indicating that Zfp35 plays an important role in the pathogenesis of allergic airway inflammation and hyperresponsiveness through the control of Th2 cell generation and Th2 cytokine expression.

The absolute number of eosinophils in the BAL fluid is enhanced after OVA challenge (Figs. 3C and 7C). An increase in the percentage of eosinophils in the Zfp35⁻/⁻ mice was also observed in the experiments shown in Figs. 3 and 7, and no remarkable change was observed in the cell transfer experiments (Fig. 7B). In addition, an increased IL-4 and IL-13 production in the eosinophils in the BAL fluid was detected (Fig. 6C). Thus, Zfp35 in Th2 cells and also in eosinophils appears to play an important role in the regulation of OVA-induced allergic airway inflammation.

No preferential expression of Zfp35 was detected between Th1 and Th2 cells (M. Kitajima and T. Nakayama, unpublished observation), while the enhancement of Th2 cell differentiation but not Th1 or Th17 cell differentiation was detected (Fig. 2 and our unpublished observation). GATA3 is one of crucial factors in Th2 differentiation and airway inflammation (57–59). Therefore, Zfp35 may regulate Th2-specific processes, such as those involving GATA3. However, the GATA3-dependent promoter activity on IL-5 promoter was not affected by Zfp35 (M. Kitajima and T. Nakayama, unpublished observation). Thus, Zfp35 appears to control a process that is required for Th2 cell differentiation but not GATA3-mediated transcriptional processes. Increasing numbers of transcription factors implicated in the development of allergic airway inflammation are reported; STAT factors, NF-κB, NFAT, AP-1 family protein, and Th2 cell-related transcription factors including GATA3, JunB, and c-Maf (60). Zinc finger transcription factors including Schnurri-2 (41) and repressor of GATA (ROG) (61) control the allergic airway inflammation.

The Zfp35 gene was clustered with znf191 and znf397 in mouse chromosome 18A12 (62, 63). ZNF191 has been identified in a T cell line, contains four C2H2 zinc finger domains, and znf191-deficient mice died at ~7.5 days after fertilization (64). ZNF397 possesses nine C2H2 zinc finger domains and is expressed in the spleen and thymus at low levels and acts as a transcriptional enhancer of Th2 differentiation in Zfp35-deficient mice.
The precise immunological function of these molecules was not investigated at this time. We examined the levels of mRNA expression of znf191 and znf397 in splenic \( \text{Zfp35}^-/- \) CD4 T cells by a RT-PCR method, and the expression was found to be normal (M. Kitajima and T. Nakayama, unpublished observation). This indicates that the observed effects on Th2 cell differentiation and airway inflammation in \( \text{Zfp35}^-/- \) deficient mice are not the result of an altered expression of the neighboring genes znf191 and znf397 in the same cluster.

The evolitional relationship among amino acid sequences of different Zfps with phylogenetic tree analysis was reported (65). ZNF322 and ZNF328 are closely related to \( \text{Zfp35} \) in the phylogenetic tree (65, 66). ZNF322 contains nine zinc finger domains and regulates in a positive manner in MAPK signaling pathways. Although no apparent effect of \( \text{Zfp35} \) on the activation of the ERK MAPK cascade was detected in Th2 cells (M. Kitajima and T. Nakayama, unpublished observation), \( \text{Zfp35} \) may also have a regulatory function on the MAPK signaling cascade in other cells.

\( \text{Znf328} \) contains 15 zinc finger domains and 1 KRAB domain and is known to be a hot spot for structural cytogenetic changes in numerous different malignancies (37). The mRNA expression of \( \text{Znf328} \), a human homolog of \( \text{Zfp35} \), is transiently induced upon activation of human B cells by anti-IgM F(ab')\(_2\) or a phorbol ester plus calcium ionophore (37). These observations suggest that \( \text{Znf328} \) is a candidate key molecule involved in the pathogenesis of hematopoietic malignancies and immune disorders. Thus, \( \text{Zfp35}^-/- \) deficient mice may provide a good experimental system for the analysis of the role of this interesting molecule.

Zinc deficiency was reported to be correlated with the Th2 inflammatory cytokine profile (67). Case-control studies in Scotland (68) and Spain (69) found that the combined risk of atopy, bronchial reactivity, and allergic-type symptoms were 4- to 5-fold higher in individuals with low zinc intake compared with those with higher zinc intakes. Significant decreases in serum, plasma, or hair zinc levels have also been reported in some asthmatic individuals (70). Intracellular Zn exists in two discreet pools, the first being nonexchangeable and tightly bound to metalloenzymes, and the second being a more labile and dynamic pool that is rapidly exchangeable and able to be altered by Zn deprivation or supplementation (71). It is possible that \( \text{Zfp35} \) containing 18 continuous zinc (72) finger motifs partly maintain intracellular zinc homeostasis, thereby controlling allergic asthma.

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Disclosures

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