

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)³ 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 γ t 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 (C₂H₂)-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 C₂H₂-type zinc finger motif is composed of 21 aa with a consensus sequence of CX₂CX₃FX₅LX₂HX₃H 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 C₂H₂-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- κ B 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^{low}-producing cells in comparison to IL-4^{high}-producing cells in 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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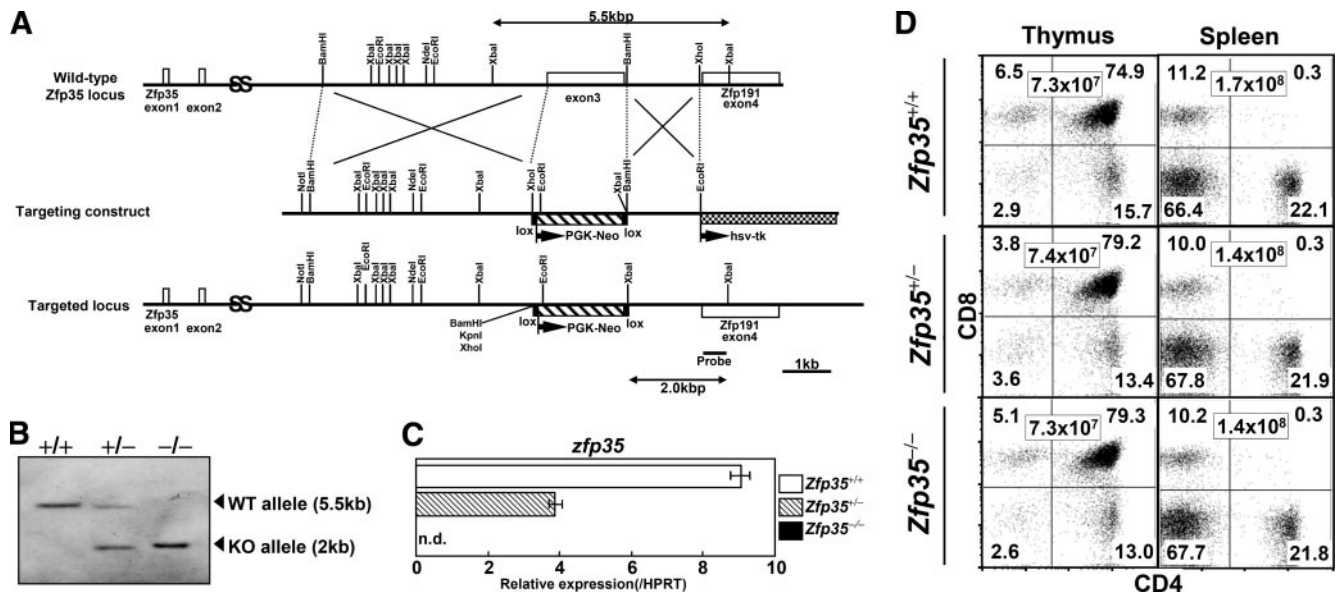


FIGURE 1. Targeted disruption of mouse *Zfp35* gene. **A**, Schematic representation of the *Zfp35* gene, the targeting vector, and the predicted disrupted gene. The probes used for Southern blot analyses are shown along with the predicted sizes of the hybridizing fragments. The open boxes represent translated exons, respectively. **B**, Southern blot analysis of genomic DNA extracted from the tails of wild-type (+/+), *Zfp35*^{+/-} (+/-), and *Zfp35*^{-/-} (-/-) mice. Genomic DNA digested with *Xba*I was hybridized with the probe to yield 5.5- and 2.0-kb bands; these represented the wild-type (WT) and knockout (KO) alleles, respectively. **C**, RNAs were prepared from fresh splenic CD4⁺ T cells of wild-type (+/+), *Zfp35*^{+/-} (+/-), and *Zfp35*^{-/-} (-/-) 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. **D**, Representative CD4/CD8 profiles of thymocytes and splenocytes of *Zfp35*^{-/-} mice with a BALB/c background. Representative yields of thymocytes and splenocytes are shown in the box.

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*, ZNF^{EB}, was identified in EBV-positive B cells and shows ~79% homology at the amino acid level compared with mice (37). The rat homolog *Zfp239* was identified in the cDNA library of the spleen by the Mammalian Gene Collection Program and shows ~84% homology at the amino acid level compared with mice (38). However, the physiological function of *Zfp35* and homologs in the immune system has not been investigated.

In this study, we have established *Zfp35*-deficient (*Zfp35*^{-/-}) mice with either a BALB/c or a C57BL/6 background and investigated the role of *Zfp35* in Th1/Th2 cell differentiation and also in the pathogenesis of Th2-dependent allergic airway inflammation. Our results indicate that *Zfp35* negatively regulates Th2 cell differentiation and Th2-driven airway allergic inflammation.

Materials and Methods

Mice

We have newly established *Zfp35*^{-/-} mice and were backcrossed to either BALB/c or C57BL/6 10 times. *Zfp35*^{-/-} × DO11.10-transgenic (Tg) (an anti-OVA-specific TCR-αβ 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 T cells were purified by using magnetic beads and an AutoMACS Sorter (Miltenyi Biotec) yielding a purity of >98%. Where indicated, splenic CD4 T cells with naive phenotype (CD44^{low}) 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 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 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 × 10⁴ sorted DO11.10 Tg CD44^{low} CD4 T cells were stimulated with antigenic OVA peptide (Loh15, OVA_{323–339}, 0.1 μM) and 10⁵ 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-4 with anti-IL-12 mAb and 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 were 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

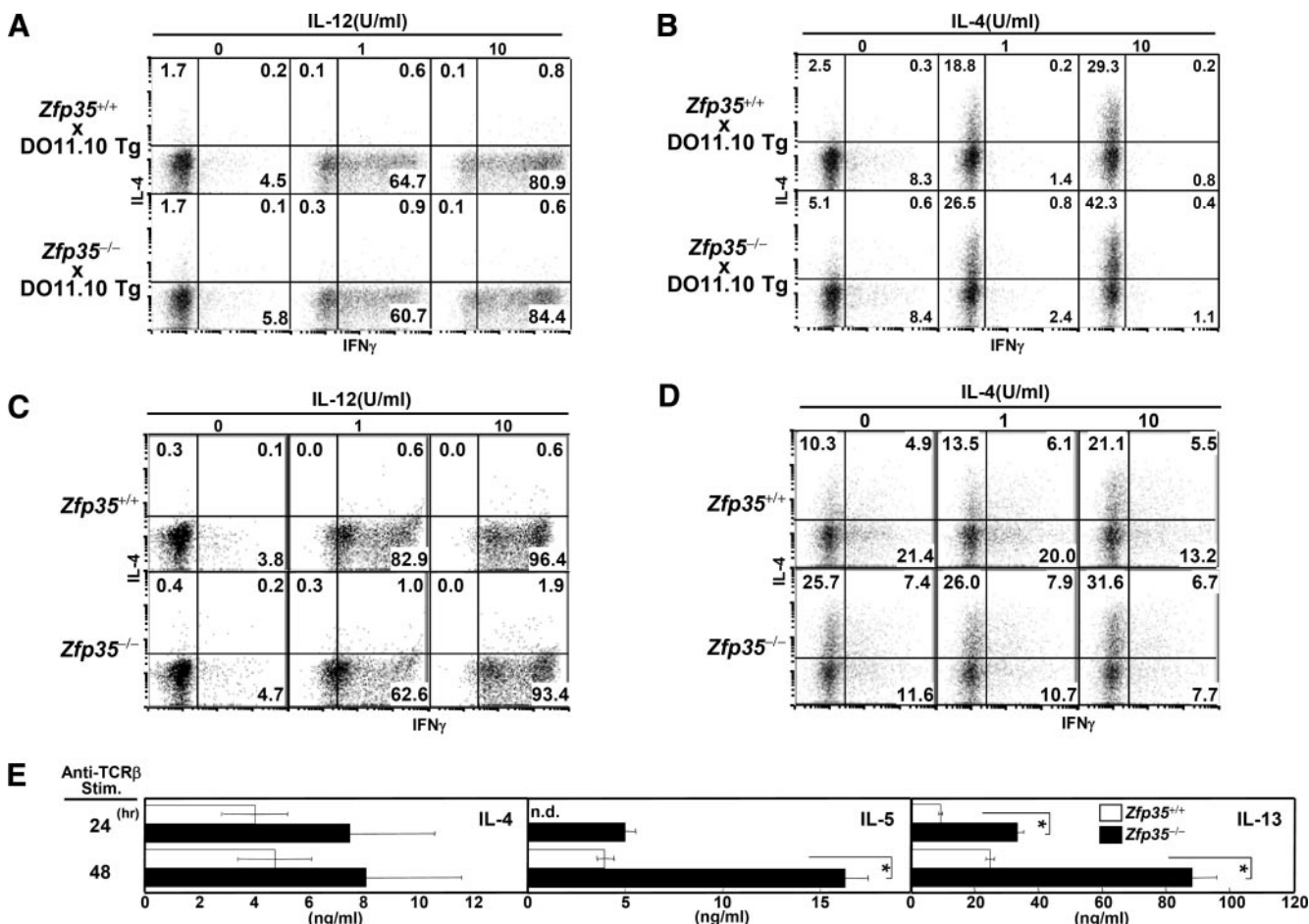


FIGURE 2. Enhanced Th2 cell differentiation in $Zfp35^{-/-}$ CD4 T cells. *A* and *B*, The ability to differentiation into Th1/Th2 cells from $Zfp35^{-/-}$ $\times$ DO 11.10 Tg mice was assessed. The results are representative of five experiments. *C* and *D*, Naive (CD44^{low}) CD4 T cells from $Zfp35^{-/-}$ mice were purified by cell sorting and stimulated with immobilized anti-TCR- β mAb (3 μ g/ml) under Th1/2 conditions for 5 days. The results are representative of five experiments. *E*, A fraction of the same differentiated cell cultures used in *B* were restimulated with anti-TCR- β mAb for the indicated times. The mean cytokine concentration is shown with SDs. Three independent experiments were performed with similar results. n.d., Not detected.

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 μ g of OVA in 30 μ 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 (1.5×10^7 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 3' external probe confirmed the correct recombination (Fig. 1*B*). 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. 1*C*).

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. 1*D* and data not shown). The cell surface expression of TCR- β , CD3 ϵ , 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- β 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.

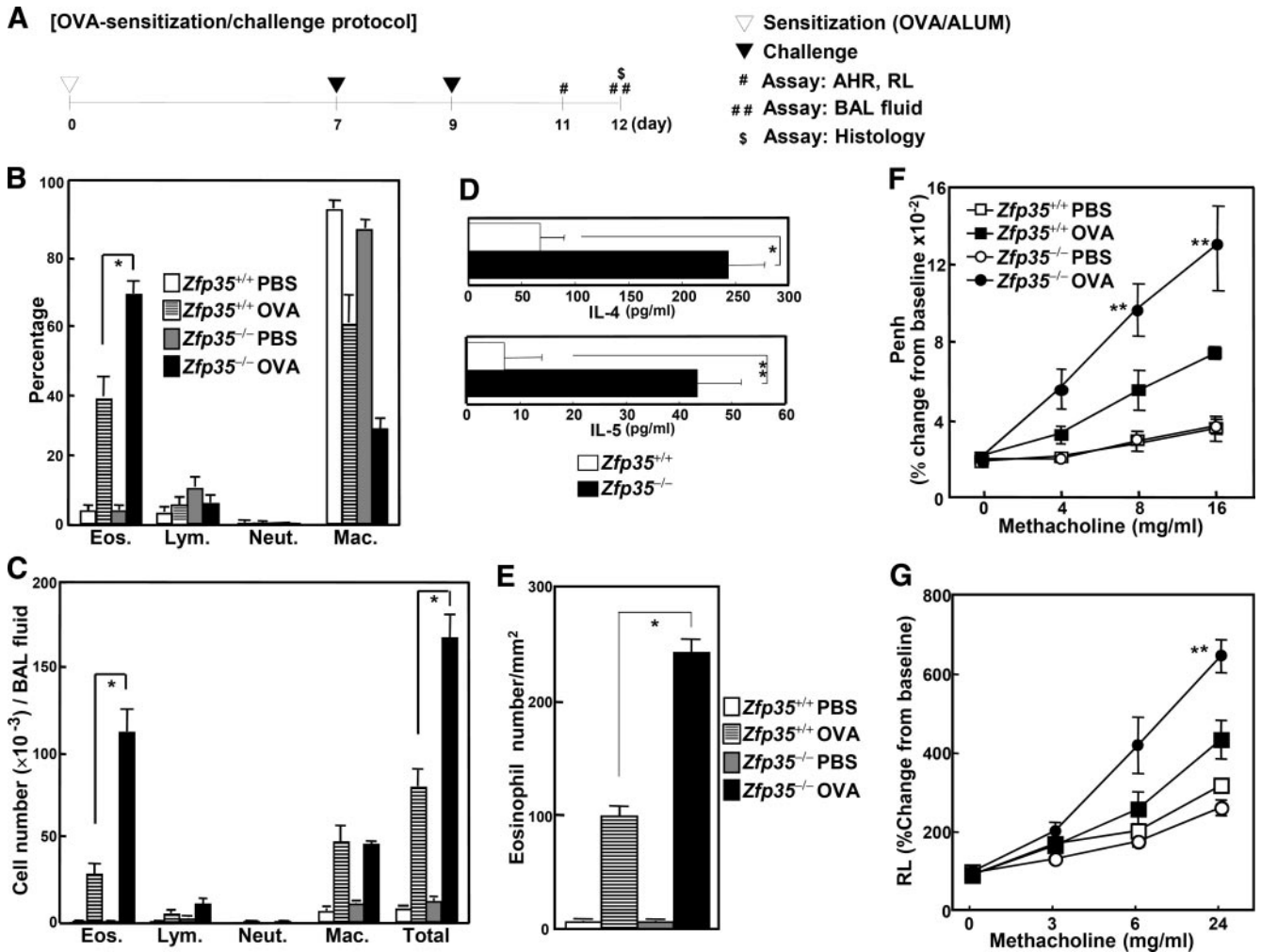


FIGURE 3. Increased OVA-induced airway inflammation and AHR in *Zfp35*^{-/-} mice. **A**, BALB/c mice were injected i.p. with 250 μ g of OVA in 4 mg of aluminum hydroxide gel on day 0. Mice were then treated intranasally with 100 μ g of OVA in 30 μ l of saline under anesthesia as described (challenge). On day 11, AHR and RL were assessed. On day 12, BAL fluid cells were prepared. Samples for histological analysis were prepared on day 12. The percentage (**B**) and absolute cell number (**C**) of eosinophils (Eos.), lymphocytes (Lym.), neutrophils (Neut.), and macrophages (Mac.) in BAL fluid are shown with SE. Four mice per group were used. *, $p < 0.01$. **D**, Six hours after the second intranasal challenge, cytokine concentrations in the BAL fluid were measured by ELISA. The values with SDs are shown. Three mice per group were used. *, $p < 0.01$ and **, $p < 0.05$. **E**, The lungs were fixed and stained with Luna. The eosinophil numbers in the peribronchiolar region (mean cell number per mm² with SDs) are also shown. Three mice per group were used. *, $p < 0.01$. AHR in response to increasing doses of methacholine was assessed by measuring enhanced pause (Penh; **F**) or airway resistance (RL; **G**). Mean values of the percent above baseline are shown with SD. A total of three independent experiments was performed with similar results. *, $p < 0.01$ and **, $p < 0.05$.

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⁺ CD44^{low}) 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-TCR β 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

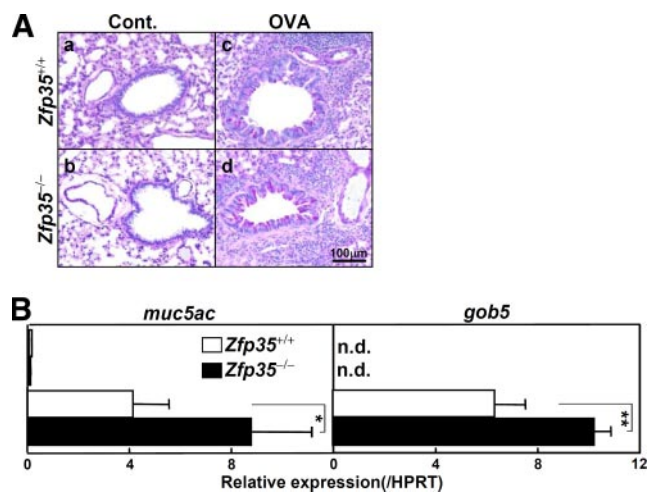


FIGURE 4. Hypersecretion of mucus in *Zfp35*^{-/-} mice. **A**, Ag-induced goblet cell hyperplasia was evaluated by periodic acid-Schiff staining. Representative photographic views of wild-type and *Zfp35*^{-/-} mice (BALB/c background) are shown (*a–d*). **B**, Total RNA was prepared from the lung of 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 **, $p < 0.05$). n.d., Not detected; Cont., control.

mouse increased significantly in *Zfp35*^{-/-} mice (Fig. 3, *B* and *C*). The cytokines (IL-4, IL-5, IL-13, and IFN- γ) in the BAL fluid were also measured by ELISA. Under our conditions, IL-4 and IL-5 were detectable and were significantly increased in the *Zfp35*^{-/-} mice (Fig. 3*D*). IL-13 and IFN- γ were undetectable in the BAL fluid. We evaluated the numbers of infiltrated eosinophils by Luna staining (Fig. 3*E*). The eosinophilic infiltration was observed to be enhanced in *Zfp35*^{-/-} mice and the infiltration extended to the surrounding regions in *Zfp35*^{-/-} mice (data not shown).

To evaluate the levels of AHR, OVA-immunized wild-type and *Zfp35*^{-/-} mice were exposed to intranasal-treated OVA. Then AHR was assessed by measuring the methacholine-induced airway obstruction in a whole-body plethysmograph and the respiratory system resistance using a computer-controlled small animal ventilator. As shown in Fig. 3, *F* and *G*, the augmented hyperresponsiveness to methacholine was detected in *Zfp35*^{-/-} mice compared with *Zfp35*^{+/+} control mice.

Specific staining of mucus-containing epithelial cells was not detected in wild-type or *Zfp35*^{-/-} mice when OVA was not challenged (Fig. 4*A*, 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. 4*A*, *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. 4*B*). 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 CD44^{high} or CD44^{low} splenic CD4 T cells, and no difference was detected between wild-type and *Zfp35*^{-/-} mice (Fig. 5*A*), 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. 5*B*). The number of CD25⁺ and CD69⁺ CD4 T cells in the lung slightly increased in the

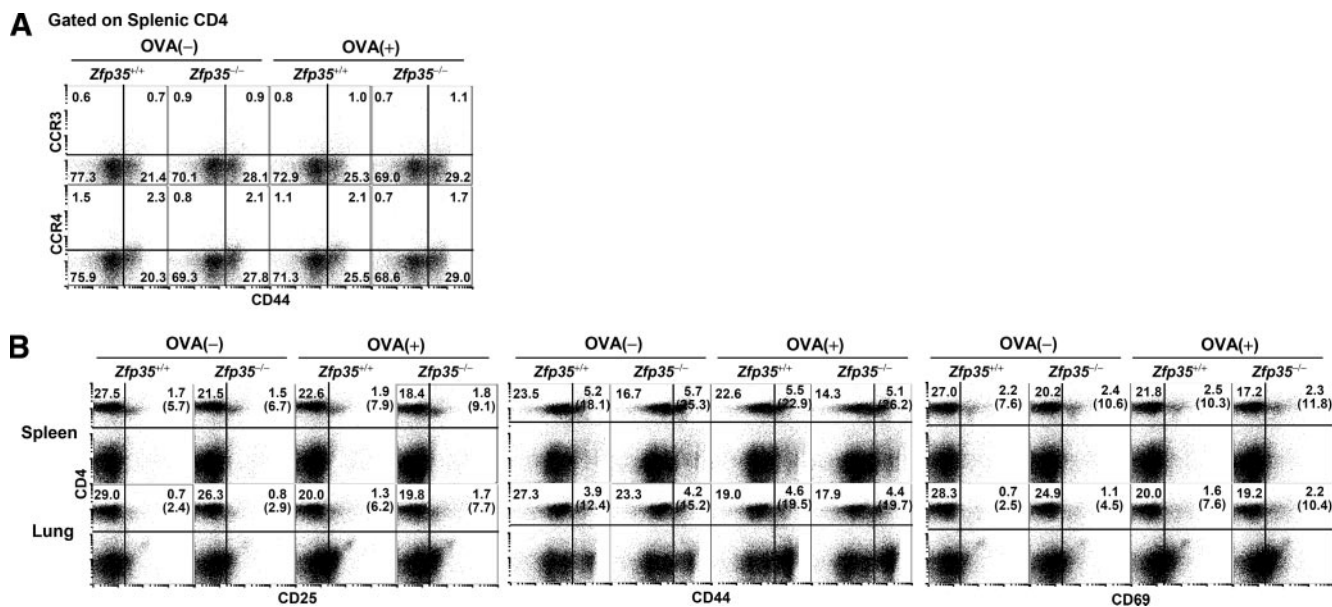


FIGURE 5. The expression of surface markers on CD4 T cells of OVA-immunized and -challenged *Zfp35*^{-/-} mice. The mice were injected i.p. with OVA/alum on day 0. On days 7 and 9, the mice were treated intranasally with OVA (challenge). On day 12, lung mononuclear cells and splenocytes were prepared. **A**, Splenocytes were stained with anti-CD4, anti-CD44, and anti-CCR3 mAb or anti-CCR4 mAb. **B**, Splenocytes and lung mononuclear cells were 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 CD4 T cells are given in parentheses. Three independent experiments were done with similar results.

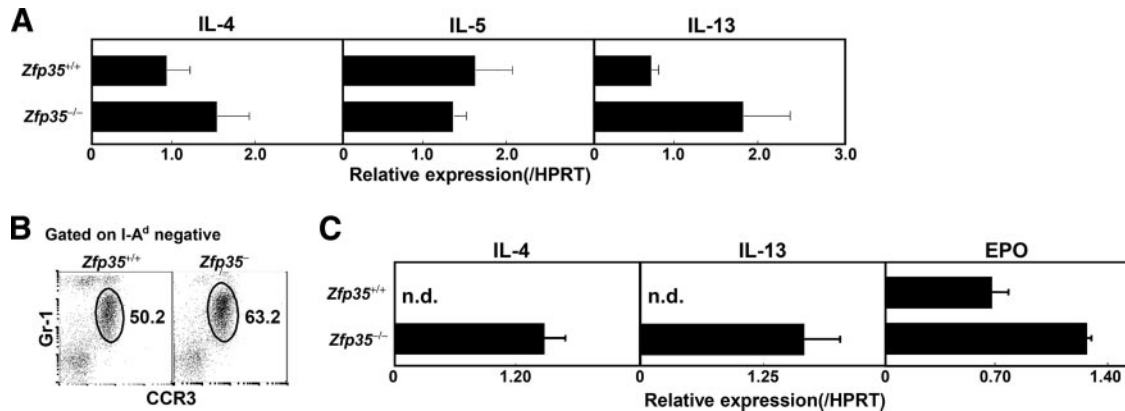


FIGURE 6. Increased Th2 cytokine production of CD4 T cells and eosinophils in the BAL fluid of OVA-immunized and -challenged *Zfp35*^{-/-} mice. The mice were immunized and challenged with OVA as described in Fig. 5. On day 12, BAL fluid cells were prepared and pooled from 10 mice per group. CD4 T cells and I-A^d-negative, Gr-1, and CCR3-positive eosinophils were isolated by a cell sorter. **A**, The mRNA expression of IL-4, IL-5, and IL-13 in the BAL fluid CD4 T cells was assessed by quantitative RT-PCR. **B**, Eosinophils in the BAL fluid were stained with anti-I-A^d, anti-Gr-1, and anti-CCR3 mAbs. Representative staining profiles of CCR3/Gr-1 on I-A^d-negative cells are shown. Percentages of CCR3⁺Gr-1⁺ cells in the BAL fluid are also indicated. **C**, The mRNA expression levels of IL-4, IL-13, and EPO in the eosinophils are shown. n.d., Not detected. Three independent experiments were done with similar results.

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 cell 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 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 Fig. 3, 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

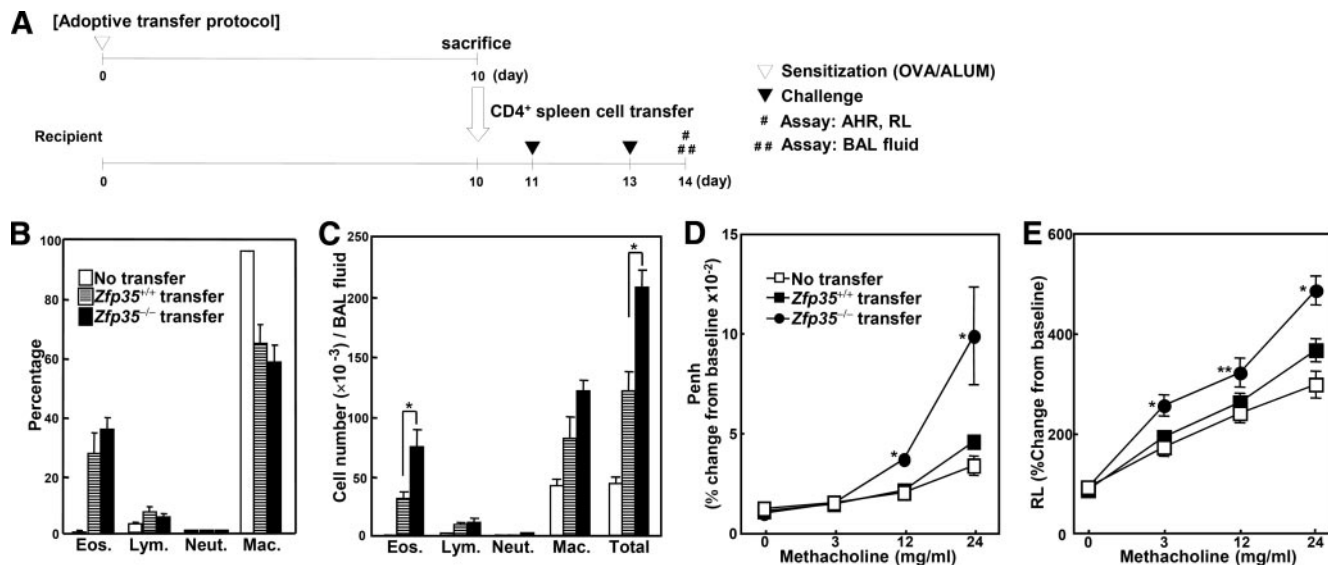


FIGURE 7. Increased airway inflammation and AHR induced by *Zfp35*^{-/-} CD4 T cell transfer. A, Donor mice (*Zfp35*^{+/+} or *Zfp35*^{-/-} mice, BALB/c background) and recipient mice (BALB/c) were sensitized i.p. with 250 μ g of OVA with alum on day 0. On day 10, splenic CD4 T cells were prepared from donor mice and transferred into recipient mice. The recipient mice were treated intranasally with 100 μ g of OVA in 30 μ l of saline under anesthesia on days 11 and 13. On day 14, AHR and RL were assessed. BAL fluid was collected on day 15. The percentage (B) and absolute cell number (C) of eosinophils (Eos.), lymphocytes (Lym.), neutrophils (Neut.) and macrophages (Mac.) in BAL fluid are shown. Four mice per group were used. AHR in response to increasing doses of methacholine was assessed by measuring enhanced pause (Penh; D) or airway resistance (RL; E). Mean values of the percentage above baseline are shown with SDs. A total of three independent experiments were performed with similar results. *, $p < 0.01$ and **, $p < 0.05$.

activator (63). 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 *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 *Zfp35*-deficient mice are not the result of an altered expression of the neighboring genes *znf191* and *znf397* in the same cluster.

The evolutionary relationship among amino acid sequences of different Zfps with phylogenetic tree analysis was reported (65). ZNF322 and ZNF328 are closely related to *Zfp35* in the phylogenetic tree (65, 66). ZNF322 contains nine zinc finger domains and regulates in a positive manner in MAPK signaling pathways. ZNF328 contains 15 zinc finger domains and 1 KRAB domain and regulates negatively the MAPK signaling pathways. Although no apparent effect of *Zfp35* on the activation of the ERK MAPK cascades was detected in Th2 cells (M. Kitajima and T. Nakayama, unpublished observation), *Zfp35* may also have a regulatory function on the MAPK signaling cascade in other cells.

ZNF^{EB} maps to human chromosome 18q12(62). The 18q12 region is known to be a hot spot for structural cytogenetic changes in numerous different malignancies (37). The mRNA expression of ZNF^{EB}, a human homolog of *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 ZNF^{EB} is a candidate key molecule involved in the pathogenesis of hematopoietic malignancies and immune disorders. Thus, *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 *Zfp35* containing 18 continuous zinc (72) finger motifs partly maintain intracellular zinc homeostasis, thereby controlling allergic asthma.

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Disclosures

The authors have no financial conflict of interest.

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