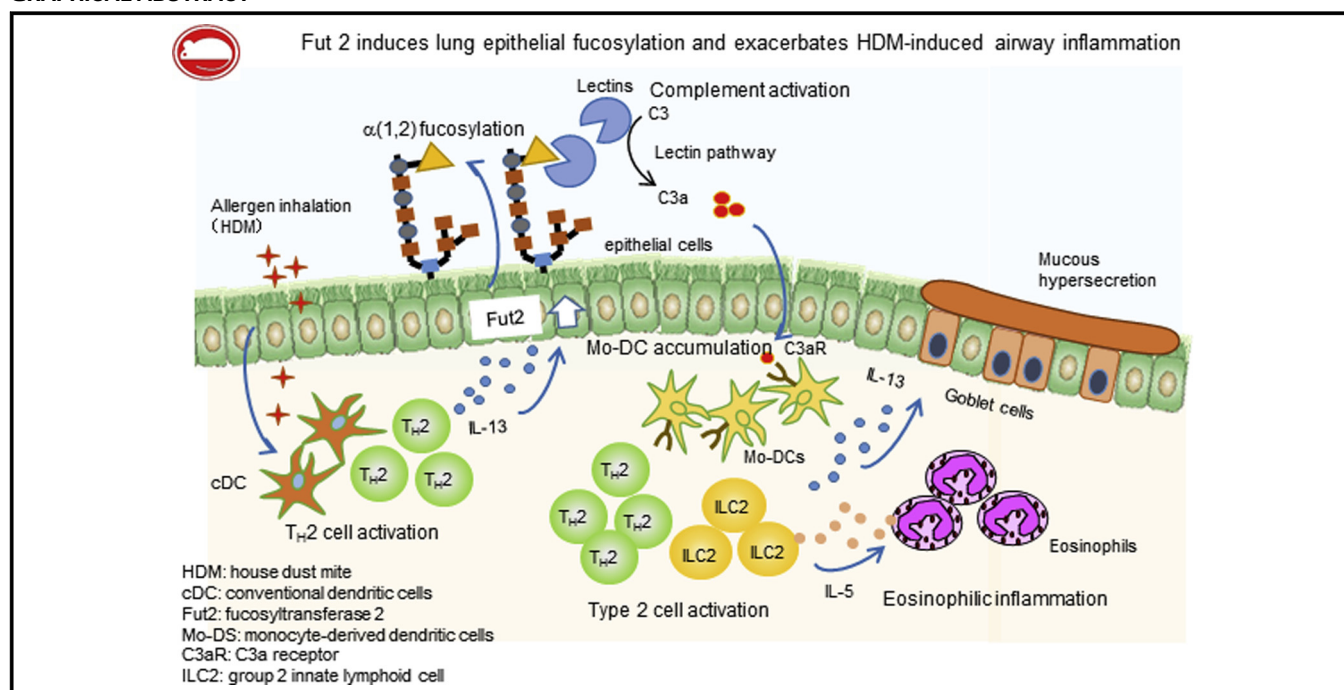


Fucosyltransferase 2 induces lung epithelial fucosylation and exacerbates house dust mite-induced airway inflammation



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GRAPHICAL ABSTRACT



Background: One of the pathognomonic features of asthma is epithelial hyperproduction of mucus, which is composed of a series of glycoproteins; however, it remains unclear how glycosylation is induced in lung epithelial cells from asthmatic patients and how glycan residues play a role in the pathogenesis of asthma. **Objective:** The objective of this study was to explore comprehensive epithelial glycosylation status induced by

allergic inflammation and reveal its possible role in the pathogenesis of asthma.

Methods: We evaluated the glycosylation status of lung epithelium using a lectin microarray. We next searched for molecular mechanisms underlying epithelial glycosylation. We also examined whether epithelial glycosylation is involved in induction of allergic inflammation.

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Results: On allergen inhalation, lung epithelial cells were heavily $\alpha(1,2)$ fucosylated by fucosyltransferase 2 (Fut2), which was induced by the IL-13-signal transducer and activator of transcription 6 pathway. Importantly, Fut2-deficient (*Fut2*^{-/-}) mice, which lacked lung epithelial fucosylation, showed significantly attenuated eosinophilic inflammation and airway hyperresponsiveness in house dust mite (HDM)-induced asthma models. Proteome analyses and immunostaining of the HDM-challenged lung identified that complement C3 was accumulated in fucosylated areas. Indeed, *Fut2*^{-/-} mice showed significantly reduced levels of C3a and impaired accumulation of C3a receptor-expressing monocyte-derived dendritic cells in the lung on HDM challenge.

Conclusion: Fut2 induces epithelial fucosylation and exacerbates airway inflammation in asthmatic patients in part through C3a production and monocyte-derived dendritic cell accumulation in the lung. (J Allergy Clin Immunol 2019;144:698-709.)

Key words: Asthma, epithelial fucosylation, fucosyltransferase 2, C3a, monocyte-derived dendritic cells

Asthma is the most prevalent chronic respiratory disease and is associated with eosinophilic inflammation, airway hyperresponsiveness, and mucus hypersecretion.^{1,2} Mucus, a multicomponent secretion that covers the surface of the airway epithelium, constitutes part of the immunoregulatory function of the airway epithelium. Although classical roles of respiratory mucus are to maintain the hydration of the airway and trap and expel particulate matter, such as inhaled allergens or pathogens,³ the function of respiratory mucus is now considered to include regulation of immune responses by retaining inhibitory molecules to pathogens/allergens and modulating cell differentiation and proliferation.⁴

Importantly, the mucus layer contains a series of glycoproteins, some of which have been shown to play a key role in the development of allergic airway inflammation.⁵ In addition, Muc5B and Muc5AC, major constituents of pulmonary mucins, have been shown to contribute to maintenance of immune homeostasis and development of airway hyperresponsiveness, respectively.^{6,7} Interestingly, a recent clinical study has demonstrated a difference in MUC5B glycosylation between patients with stable and those with unstable asthma,⁸ suggesting that not only glycoproteins in mucus but also glycans carried by glycoproteins are involved in the pathogenesis of asthma.

Involvement of epithelial glycosylation in immune regulation is further underscored by the fact that deficiency of O-glycans or fucosylation in the intestinal epithelium in mice results in the development of colitis, which is similar to human inflammatory bowel diseases.⁹⁻¹¹ Because mucus hyperproduction is one of the characteristics of asthmatic airways, glycosylation in the airway epithelium can also play a role in regulation of allergic airway inflammation in asthmatic patients. However, it remains largely unknown how glycosylation is induced in the airway epithelium and how each glycan residue is involved in the development of allergic inflammation.

Here we have sought to clarify glycan residues induced in the lung epithelium during allergic inflammation and their involvement in the pathogenesis of asthma. Our data demonstrate that epithelial fucosylation is a crucial regulator of allergic airway inflammation and mediates the communication among epithelial

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
C3aR:	C3a receptor
cDC:	Conventional dendritic cell
DC:	Dendritic cell
EpCAM:	Epithelial cell adhesion molecule
FACS:	Fluorescence-activated cell sorting
Fut2:	Fucosyltransferase 2
HDM:	House dust mite
ILC2:	Group 2 innate lymphoid cell
Mo-DC:	Monocyte-derived dendritic cell
OVA:	Ovalbumin
Rag2:	Recombination-activating gene 2
STAT6:	Signal transducer and activator of transcription 6
UEA-I:	<i>Ulex europaeus</i> agglutinin 1
WT:	Wild-type

cells, complement, and monocyte-derived dendritic cells (Mo-DCs).

METHODS

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Kanagawa, Japan). Fucosyltransferase 2 (*Fut2*)^{-/-} mice,¹² recombination-activating gene 2 (*Rag2*)^{-/-} mice, *T-bet*^{-/-} mice,¹³ signal transducer and activator of transcription 6 (*Stat6*)^{-/-} mice,¹⁴ and OT-II mice were housed in microisolator cages under specific pathogen-free conditions. The Chiba University Animal Care and Use Committee approved the animal procedures used in this study.

Murine models of airway inflammation

Mice were anesthetized with intraperitoneal injection of midazolam, butorphanol, and medetomidine, as described previously,¹⁵ and then sensitized intratracheally with 50 mg of house dust mite (HDM; Greer Laboratories, Lenoir, NC) in 25 mL of PBS. Seven days after sensitization, mice were challenged with 5 mg of HDM 4 times a week for 2 weeks (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Forty-eight hours after the last challenge, the glycosylation status of lung epithelial cells was evaluated by using a lectin array, lectin staining, and fluorescence-activated cell sorting (FACS). Sensitized mice were challenged for 4 consecutive days to evaluate allergic inflammation (see Fig E1, B). Numbers of inflammatory cells in bronchoalveolar lavage fluid (BALF), cytokine production from inflammatory cells, histologic analysis, airway hyperresponsiveness, and levels of antibodies were evaluated, as described previously.^{16,17} Other protocols are available in the Methods section in this article's Online Repository at www.jacionline.org.

FACS analysis

Single-cell suspensions of the lung were stained with *Ulex europaeus* agglutinin 1 (UEA-I), anti-CD45, anti-CD31, anti-epithelial cell adhesion molecule (EpCAM), and 4'-6-diamidino-2-phenylindole dihydrochloride. CD45⁺CD31⁻EpCAM⁺ panepithelial cells and each subpopulation of lung epithelial cells were isolated, as described previously.^{18,19} In some experiments cells were analyzed for expression of C3a receptor (C3aR), as described previously.²⁰ For intracellular staining of CD4⁺ T cells, BALF cells were stimulated and stained with indicated antibodies against surface molecules, followed by intracellular cytokine staining, as described previously.^{17,21} Data were collected with a FACSCanto II (BD Biosciences, San Jose, Calif) and analyzed by using FlowJo software (TreeStar, Ashland, Ore).

ELISA

Amounts of IL-5, IL-13, IL-17A, IFN- γ , thymic stromal lymphopoietin, IL-25, and IL-33 were determined by using ELISA kits from R&D Systems (Minneapolis, Minn). Levels of C3a and albumin were determined by using ELISA kits from LifeSpan BioSciences (Seattle, Wash) and Bethyl Laboratories (Montgomery, Tex), respectively. Levels of total IgE were evaluated by using an ELISA kit from BD Biosciences.

Lectin microarray analysis

Lung epithelial areas were microdissected and analyzed by using a lectin microarray, as described previously.²² The net intensity value for each spot was calculated by subtracting the background value from the signal intensity values of 3 spots and then normalized by the signal intensity of wheat germ agglutinin.

Detection of fucosylation and immunostaining of the lung

Lungs were fixed and embedded in OCT compound, as described previously.²³ Lung sections (5 mm) were stained with UEA-I rhodamine (Vector Laboratories, Burlingame, Calif) or wheat germ agglutinin–fluorescein isothiocyanate (Vector Laboratories). Where indicated, sections were stained together with a polyclonal rabbit anti-human C3d antibody (Dako, Santa Clara, Calif), which cross-reacts with mouse C3d; a polyclonal rabbit anti-murine CCSP antibody (Merck, Kenilworth, NJ); an anti-murine Muc5AC antibody (clone A85-1; Invitrogen, Carlsbad, Calif); or an anti-murine Ki67 antibody (clone SolA15; eBioscience, San Diego, Calif), as described previously.^{24,25} All immunofluorescent histologic images were acquired by using a confocal laser microscope (LSM710; Carl Zeiss, Oberkochen, Germany) and analyzed with Zen software (Carl Zeiss).

Quantitative RT-PCR analysis

Total RNA was prepared by using an RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) from lung epithelial cells, as described previously.¹⁸ Quantitative RT-PCR was performed with a standard protocol on an ABI PRISM 7300 instrument (Applied Biosystems, Foster City, Calif) with SYBR Green reagent (Power SYBR Green PCR Master Mix; Applied Biosystems). Levels of target genes were normalized to levels of β -actin. Sequences of PCR primers are available in the Methods section in this article's Online Repository.

Proteome analysis of murine lung epithelial cells

UEA-I⁺ epithelial areas were microdissected from serial sections of frozen lung tissue from HDM-challenged mice and analyzed by using the liquid chromatography–mass spectrometry system, as described previously.^{26–28} In brief, one of the sections was subjected to UEA-I staining to determine the fucosylated epithelial areas, and corresponding areas of sequential sections were isolated by using the Leica LMD 6500 (Leica, Wetzlar, Germany) for proteome analysis. Among the proteins identified, putative glycoproteins were selected according to prediction of a signal peptide or transmembrane segments.²⁷

Data analysis

Data are summarized as means $\pm$ SEMs, unless otherwise indicated. Statistical analysis of the results was performed by using the unpaired *t* test, the Mann-Whitney *U* test, or ANOVA with GraphPad software (GraphPad Software, La Jolla, Calif). *P* values of less than .05 were considered significant.

RESULTS

Allergic airway inflammation induces epithelial fucosylation

To gain insight into the roles of epithelial glycosylation in the pathogenesis of allergic airway inflammation, we first evaluated

the glycosylation status of the lung epithelial area on allergen exposure using a lectin microarray, a method that enables us to identify the comprehensive profiling of glycosylation status.^{29,30} Consistent with mucus hypersecretion in asthmatic patients, airways isolated from HDM-challenged mice exhibited greater signals for the most lectins than those from PBS-challenged control mice (Fig 1, A). Importantly, HDM challenge significantly increased signals for fucose-binding lectins, such as UEA-I, *Trichosanthes japonica* agglutinin II, and *Aspergillus oryzae* L-fucose-specific lectin, with the signal for UEA-I, a specific lectin for α (1,2)fucose, being most strongly induced (Fig 1, A and B).

The findings of the lectin microarray were confirmed by lectin staining of the lung sections. Lung of HDM-challenged mice but not control mice contained UEA-I⁺ epithelial cells (Fig 1, C), especially in Muc5AC⁺ or CCSP⁺ secretory epithelium of the conducting airways (Fig 1, D). Consistently, FACS analysis revealed that CD45⁺EpCAM⁺ lung epithelial cells of HDM-challenged mice showed significantly increased levels of UEA-I⁺ cells than those of control mice, whereas no UEA-I⁺ cells were found in CD45⁺EpCAM⁺ hematopoietic cells, even after HDM challenge (Fig 1, E). Although a few CD31⁺ endothelial cells were positive for UEA-I staining (see Fig E2, A, in this article's Online Repository at www.jacionline.org), which is in agreement with a previous report,³¹ the frequency of UEA-I⁺ cells in lung endothelial cells was significantly lower than that in lung epithelial cells (see Fig E2, A). Furthermore, albumin levels in BALF were comparable between wild-type (WT) and *Fut2*^{−/−} mice (see Fig E2, B), suggesting that *Fut2* is irrelevant for vascular permeability.

Kinetic analyses demonstrated that the frequency of UEA-I⁺ cells among lung epithelial cells was increased during HDM challenge and then decreased to basal levels 2 weeks after challenge cessation (Fig 1, F). On the other hand, only a few UEA-I⁺ epithelial cells in HDM-challenged mice were positive for Ki-67, a marker for proliferating cells, suggesting that these cells were largely in a quiescent state (see Fig E3 in this article's Online Repository at www.jacionline.org). Similar to HDM-induced allergic inflammation, an ovalbumin (OVA)-induced asthma model (see Fig E1, C) also showed lung epithelial fucosylation (Fig 1, G), suggesting that lung epithelial α (1,2)fucosylation is a general phenomenon observed in the setting of allergic inflammation.

Fut2 is responsible for induction of epithelial α (1,2)fucosylation

Fucosylated glycans are synthesized by fucosyltransferases. It is well known that among the 13 fucosyltransferases identified in the murine genome, *Fut1*, *Fut2*, and *Sec1* mediate α (1,2)fucosylation.³² As shown in Fig 2, A, quantitative RT-PCR analysis revealed that CD45⁺EpCAM⁺ lung epithelial cells expressed mRNA for *Fut2* but not for *Fut1* or *Sec1*. mRNA expression for *Fut2* was significantly increased by HDM challenge, whereas dendritic cells (DCs), CD4⁺ T cells, CD8⁺ T cells, or B cells did not express *Fut2* mRNA (Fig 2, A and B). Among the epithelial subpopulations fractionated by expression levels of EpCAM and MHC class II, ¹⁹ EpCAM^{hi} MHC class II[−] population, which mainly consists of *FoxJ1*⁺ ciliated cells and *Sgblal*⁺ Clara cells, expressed the highest levels of *Fut2* mRNA (Fig 2, C), which is in agreement with immunostaining data (Fig 1, D). Consistent with

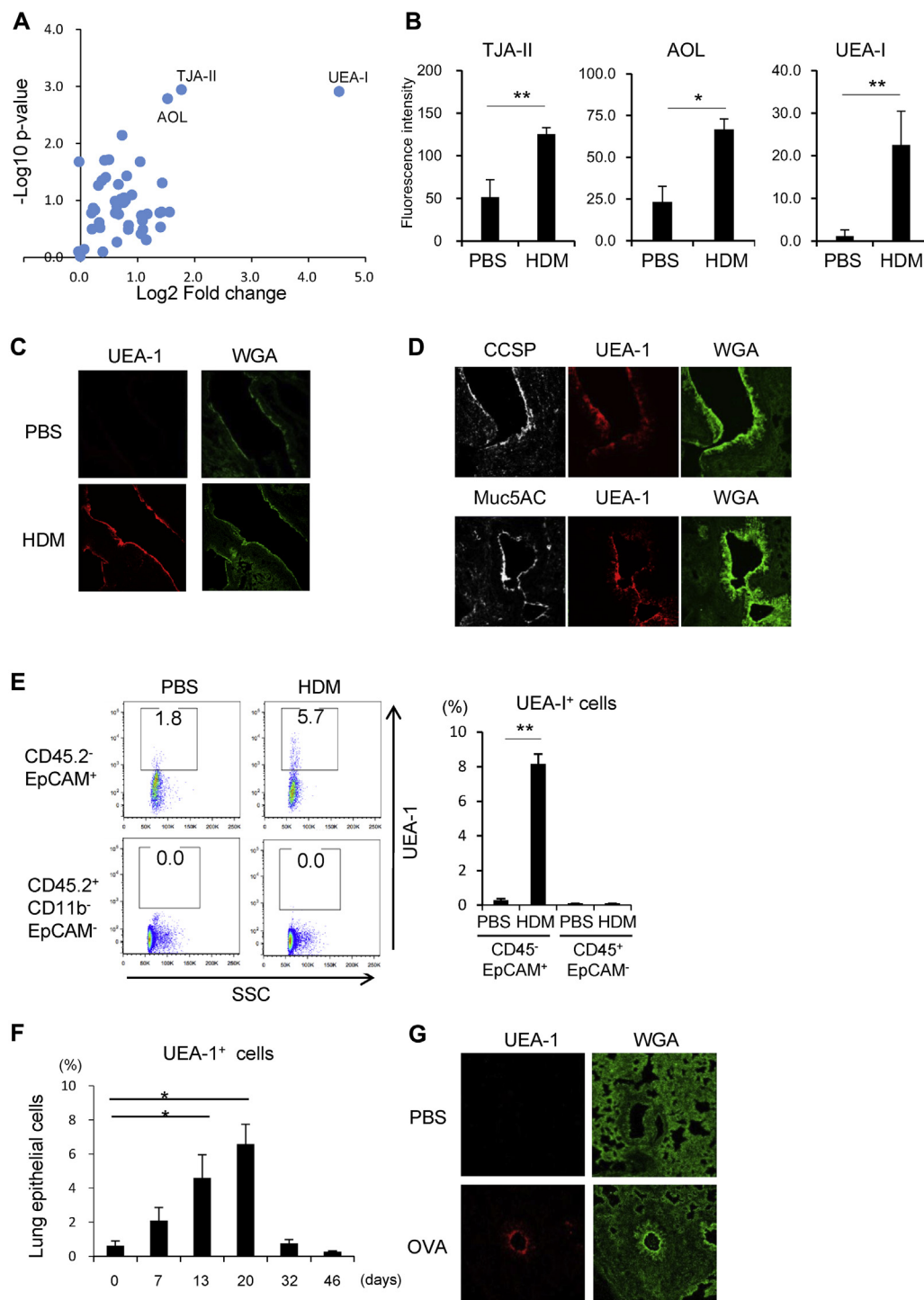


FIG 1. Allergic airway inflammation induces epithelial fucosylation. **A-E**, WT mice were sensitized and challenged with HDM or PBS (as a control; see Fig E1, A). Fig 1, A and B, Glycosylation status of lung epithelial area isolated by means of laser microdissection was analyzed with a lectin microarray. Fig 1, A, Dot plot of lectin profile based on $\log_2$ fold change (x -axis) and $-\log_{10}$ P value (y -axis) derived from 3 independent experiments. Fig 1, B, Fluorescence intensities of *Trichosanthes japonica* agglutinin II (TJA-II), *Aspergillus oryzae* L-fucose-specific lectin (AOL), and UEA-I. Data are means $\pm$ SEMs for 3 independent experiments. **C-G**, Lung tissue was analyzed by means of lectin staining and FACS. Fig 1, C and D, Representative microphotographs of lectin staining and immunostaining of CCSP and Muc5AC of lung sections. Fig 1, E, FACS plots with percentages of UEA-I⁺ cells in CD45⁻ EpCAM⁺ lung epithelial cells and CD45⁺ EpCAM⁻ hematopoietic cells. Data are means $\pm$ SEMs for 9 mice from 3 independent experiments. Fig 1, F, Shown are kinetics of the frequency of UEA-I⁺ cells in CD45⁻ EpCAM⁺ lung epithelial cells. Data are means $\pm$ SEMs for 3 mice. Fig 1, G, BALB/c mice were sensitized and challenged with OVA or PBS (see Fig E1, C). Lung tissue was analyzed by means of lectin staining. Shown are representative microphotographs of lung sections. WGA, Wheat germ agglutinin. * $P < .05$ and ** $P < .01$.

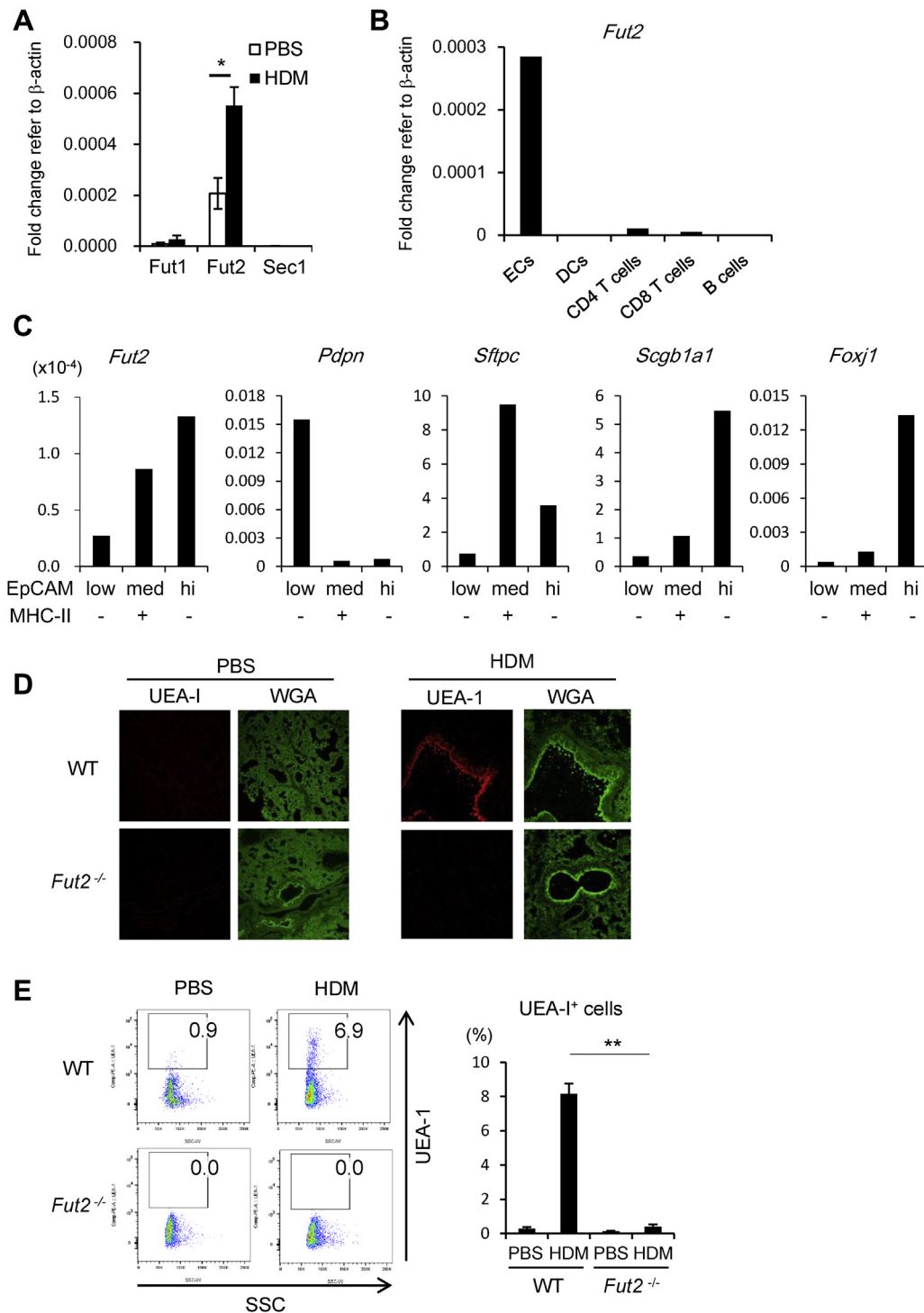


FIG 2. *Fut2* is responsible for induction of epithelial $\alpha(1,2)$ fucosylation. **A–C**, WT mice were sensitized and challenged with HDM or PBS (see Fig E1, A). Expression levels of *Fut1*, *Fut2*, and *Sec1* mRNA in CD45⁺EpCAM⁺ lung epithelial cells were evaluated by means of quantitative RT-PCR. Shown are means $\pm$ SEMs for 4 mice from 3 independent experiments. Fig 2, B, Expression levels of *Fut2* mRNA in CD45⁺EpCAM⁺ lung epithelial cells (ECs), DCs, CD4⁺ T cells, CD8⁺ T cells, and B cells in the lung were evaluated by means of quantitative RT-PCR. Shown are representative data from 2 independent experiments. Fig 2, C, Expression levels of mRNA for *Fut2*, *Pdpn* (a marker for type 1 alveolar epithelial cells), *Sftpc* (type 2 alveolar epithelial cells), *Scgb1a1* (Clara cells), and *Foxj1* (ciliated cells) in each subpopulation of lung epithelial cells. Shown are representative results from 2 independent experiments. **D** and **E**, WT mice and *Fut2*^{-/-} mice were sensitized and challenged with HDM or PBS (see Fig E1, A). Lungs were analyzed by means of lectin staining or FACS. Shown are representative microphotographs of lectin staining of lung sections (Fig 2, D) and representative FACS profiles of lung cells with percentages of UEA-I⁺ cells in CD45⁺EpCAM⁺ lung epithelial cells (Fig 2, E). SSC, Side scatter. Data are means $\pm$ SEMs for WT mice (n = 9) and *Fut2*^{-/-} mice (n = 3). WGA, Wheat germ agglutinin. **P* < .05 and ***P* < .01.

the finding that Fut2, but not Fut1 or Sec1, is expressed in lung epithelial cells (Fig 2, A), lectin staining and FACS analysis of lung epithelial cells confirmed the lack of UEA-I⁺ cells in *Fut2*^{-/-} mice (Fig 2, D and E). These results suggest that Fut2 is responsible for induction of epithelial $\alpha(1,2)$ fucosylation in the setting of allergic airway inflammation.

The IL-13–STAT6 pathway is required for epithelial $\alpha(1,2)$ fucosylation

We next explored the molecular mechanisms underlying epithelial $\alpha(1,2)$ fucosylation. We first examined the requirement of acquired immunity for $\alpha(1,2)$ fucosylation by using *Rag2*^{-/-} mice. Lectin staining and FACS analyses revealed that *Rag2*^{-/-} mice showed significantly lower levels of UEA-I⁺ cells in the epithelium than WT mice on HDM challenge (Fig 3, A–C). Consistently, antibody-mediated depletion of CD4⁺ T cells significantly attenuated lung epithelial $\alpha(1,2)$ fucosylation (data not shown). These results suggest that acquired immune responses are crucial.

Because IL-22 induces epithelial $\alpha(1,2)$ fucosylation in the intestine^{24,33,34} and CD4⁺ T cells are the major cellular sources of IL-22 in allergic airway inflammation,¹⁷ we next assessed whether IL-22 is involved in HDM-induced lung epithelial fucosylation. Unexpectedly, T-bet-deficient (*T-bet*^{-/-}) mice, which have decreased numbers of IL-22-producing cells,^{35,36} as well as T_H1 cells,¹³ showed comparable levels of UEA-I⁺ cells to WT mice (Fig 3, A–C). Furthermore, mice treated with anti-IL-22 antibody exhibited similar levels of UEA-I⁺ cells to control antibody-treated mice on HDM challenge (data not shown). These results indicate that IL-22 is dispensable for the development of lung epithelial $\alpha(1,2)$ fucosylation in allergic airway inflammation.

In contrast, we found that *Stat6*-deficient (*Stat6*^{-/-}) mice lacked the ability to induce lung epithelial $\alpha(1,2)$ fucosylation on HDM challenge (Fig 3, A–C). We also found that intratracheal administration of IL-13 (see Fig E1, D) induced epithelial $\alpha(1,2)$ fucosylation, even in *Rag2*^{-/-} mice (Fig 3, D and E), and induced mRNA expression of Fut2 in lung epithelial cells (Fig 3, F). Consistent with the fact that STAT6 is the main signal transducer of IL-13,³⁷ *Stat6*^{-/-} mice did not have epithelial $\alpha(1,2)$ fucosylation on IL-13 administration (Fig 3, G and H). Collectively, these results suggest that STAT6 in lung epithelial cells is crucial for induction of $\alpha(1,2)$ fucosylation.

Fut2 exacerbates HDM-induced eosinophilic airway inflammation

We next examined whether Fut2 is involved in the development of HDM-induced allergic airway inflammation. Because epithelial fucosylation starts to develop at the first week of HDM challenge (Fig 1, F), we evaluated the role of Fut2 in induction of allergic inflammation at 48 hours after the first week of HDM challenge (see Fig E1, B). As shown in Fig 4, A, *Fut2*^{-/-} mice exhibited significantly attenuated infiltration of eosinophils, neutrophils, and CD4⁺ T cells in BALF compared with WT mice. *Fut2*^{-/-} mice also showed less inflammatory, eosinophil-rich lesions around bronchi and blood vessels (Fig 4, B) and fewer periodic acid–Schiff–positive goblet cells (Fig 4, C) than WT mice. Moreover, *Fut2*^{-/-} mice exhibited significantly attenuated airway hyperresponsiveness to methacholine and low levels of

HDM-specific IgG₁ compared with WT mice (Fig 4, D and E). Total IgE levels also tended to be lower in *Fut2*^{-/-} mice (Fig 4, F). Taken together, these results suggest that Fut2-mediated lung epithelial $\alpha(1,2)$ fucosylation is associated with exacerbation of HDM-induced allergic inflammation.

Fut2 enhances HDM-induced T_H2 and T_H17 responses in the lung

We next evaluated immune responses against HDM in *Fut2*^{-/-} mice. Consistent with the reduced eosinophilic and neutrophilic inflammation in *Fut2*^{-/-} mice, levels of IL-13, IL-5, and IL-17A were significantly lower in *Fut2*^{-/-} mice than in WT mice, whereas IFN- γ levels were comparable (Fig 5, A). Intracellular cytokine staining revealed that *Fut2*^{-/-} mice had significantly fewer IL-5-, IL-13-, or IL-17A-producing CD4⁺ T cells in BALF than WT mice, whereas numbers of IFN- γ - or IL-22-producing cells were comparable between *Fut2*^{-/-} and WT mice (Fig 5, B and C). Consistently, *Fut2*^{-/-} mice contained lower numbers of GATA-3⁺CD4⁺ T cells and retinoic acid-related orphan receptor γ ⁺CD4⁺ T cells in BALF than WT mice (Fig 5, D). In contrast, numbers of T-bet⁺CD4⁺ T cells and FoxP3⁺CD4⁺ T cells were comparable between these mice (Fig 5, D). Although fucosylated glycans are reported to be involved in inflammatory cell recruitment,³² OVA-specific T_H2 cell–transferred *Fut2*^{-/-} mice and WT mice had similar levels of eosinophil and CD4⁺ T-cell infiltration into BALF on OVA inhalation (see Fig E4 in this article's Online Repository at www.jacionline.org). Taken together, these results suggest that Fut2-mediated lung epithelial $\alpha(1,2)$ fucosylation is involved in induction of HDM-induced T_H2 and T_H17 responses in the lung but not in activation and recruitment of pre-existing T_H2 cells.

We also examined innate immune responses against HDM in *Fut2*^{-/-} mice. As shown in Fig 5, E and F, levels of IL-33 and numbers of group 2 innate lymphoid cells (ILC2s) in the lung were reduced in *Fut2*^{-/-} mice compared with those in WT mice. In contrast, accumulation of ILC2s and eosinophils into the lung by means of single intranasal administration of IL-33 was similarly induced in *Fut2*^{-/-} and WT mice (see Fig E5 in this article's Online Repository at www.jacionline.org). These results suggest that Fut2 is involved in HDM-induced IL-33 production but not in initial responses of ILC2s to IL-33.

Fut2 is involved in local activation of complement and accumulation of Mo-DCs in the lung

Lung tissues rich in UEA-I⁺ epithelium were microdissected from HDM-challenged WT mice and subjected to proteomic analysis to further clarify the underlying mechanisms of the attenuated allergic airway inflammation in *Fut2*^{-/-} mice. Among 650 proteins identified in UEA-I⁺ lung tissues, we searched for candidate proteins as secretory proteins or membrane-associated proteins because fucosylated proteins are likely to be secreted or membrane bound. In agreement with immunostaining data (Fig 1, D), a representative goblet cell–related protein, Clca1, and the Clara cell–specific protein Scgblal were identified as relatively abundant proteins (peptide fragment > 3) in UEA-I⁺ lung epithelium (see Table E1 in this article's Online Repository at www.jacionline.org). Among the relatively abundant glycoproteins (see Table E1), we focused on complement component C3 because it has been shown that activation of C3 is involved in

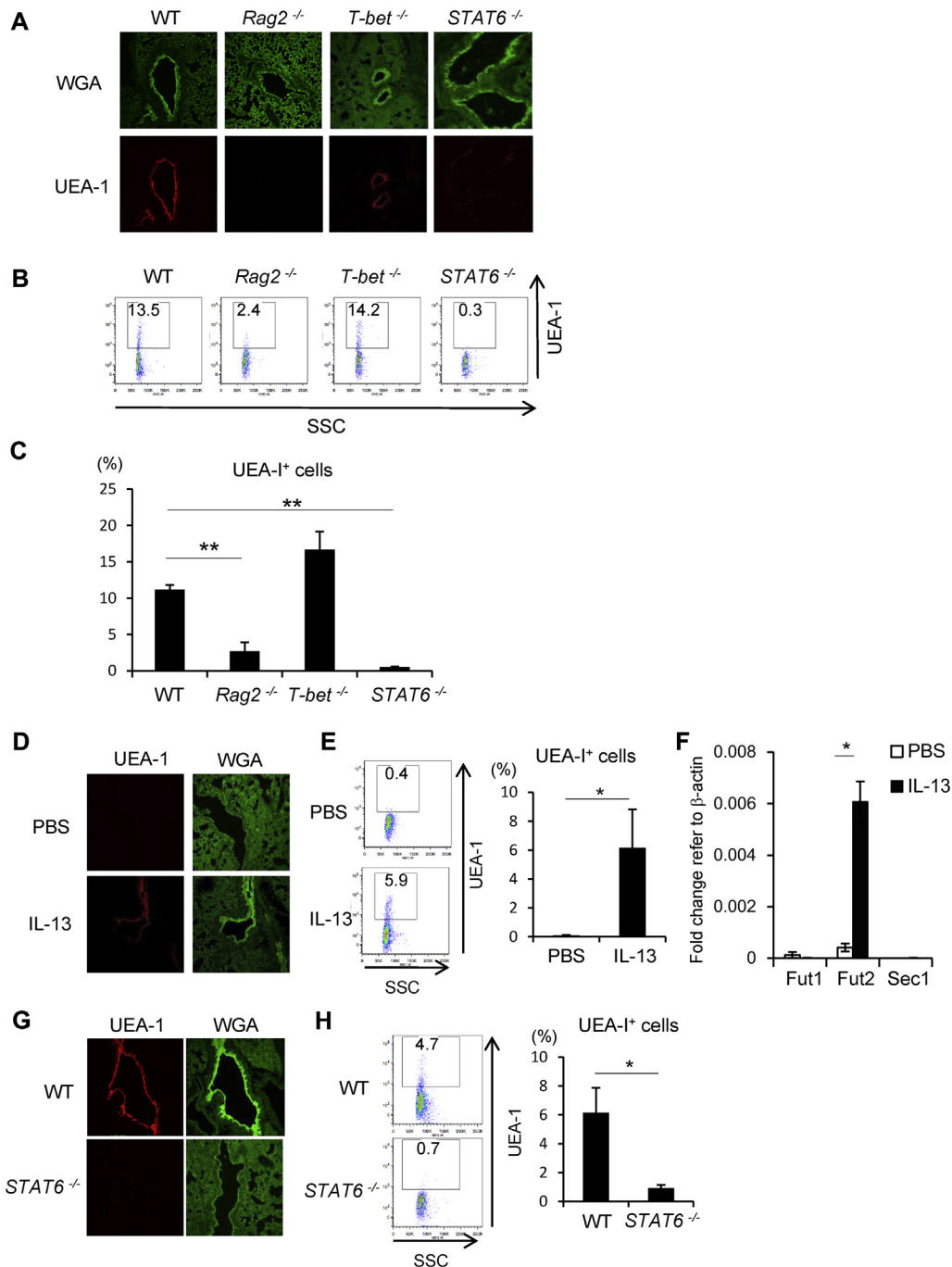


FIG 3. The IL-13-STAT6 pathway is required for epithelial $\alpha(1,2)$ fucosylation. **A-C**, WT mice, *Rag2*^{-/-}, *T-bet*^{-/-}, and *Stat6*^{-/-} mice were sensitized and challenged with HDM (see Fig E1, A). Representative microphotographs (Fig 3, A) and FACS profiles (Fig 3, B) with percentages of UEA-1⁺ cells in CD45⁺ EpCAM⁺ cells (Fig 3, C) are shown. Data are means $\pm$ SEMs for 3 mice. **D-F**, Recombinant IL-13 or PBS was intratracheally administered to *Rag2*^{-/-} mice (see Fig E1, D). Shown are representative microphotographs (Fig 3, D), FACS plots with percentages of UEA-1⁺ cells in CD45⁺ EpCAM⁺ cells (Fig 3, E) and mRNA levels of fucosyltransferases in CD45⁺ EpCAM⁺ lung epithelial cells (Fig 3, F). Data are means $\pm$ SEMs for 3 mice. **G** and **H**, Recombinant IL-13 was intratracheally administered to WT mice and *Stat6*^{-/-} mice (see Fig E1, D). Shown are representative microphotographs (Fig 3, G) and FACS plots with percentages of UEA-1⁺ cells in CD45⁺ EpCAM⁺ cells (Fig 3, H). Data are means $\pm$ SEMs for 3 mice. SSC, Side scatter; WGA, wheat germ agglutinin. **P* < .05 and ***P* < .01.

the pathogenesis of asthma³⁸ and that epithelial fucosylation or secretor status is involved in lectin-mediated activation of the complement pathway.^{25,39-41} In accordance with this notion,

immunostaining for C3d, a covalently bound fragment after C3 activation, showed that C3d was localized to the fucosylated epithelium in WT mice and that C3d levels were markedly

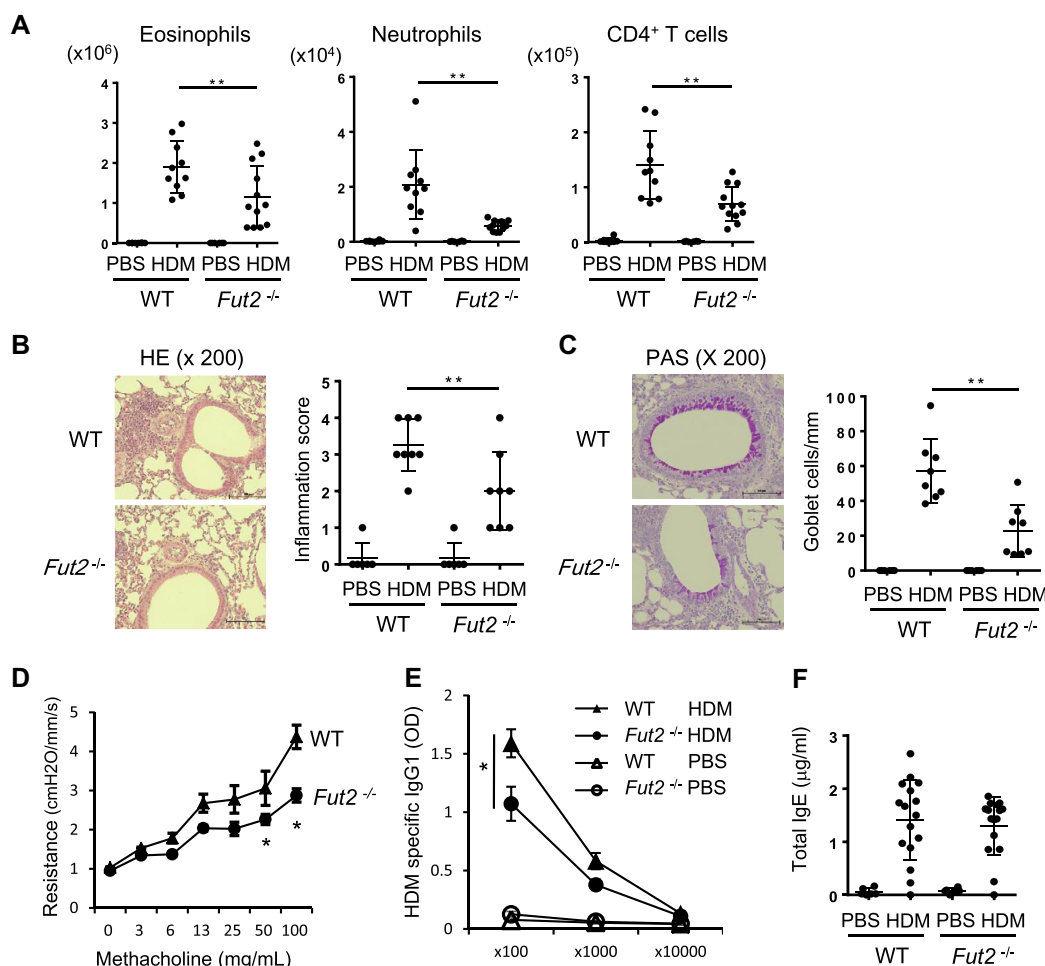


FIG 4. *Fut2* exacerbates HDM-induced eosinophilic airway inflammation. WT and *Fut2*^{-/-} mice were sensitized and challenged with HDM or PBS (see Fig E1, B). **A**, Absolute numbers of eosinophils, neutrophils, and CD4⁺ T cells in BALF were evaluated at 48 hours after the last challenge. Data are means $\pm$ SEMs for 12 mice in each group from 3 independent experiments. **B** and **C**, Representative photomicrographs of hematoxylin and eosin (HE)-stained lung sections with histologic inflammation scores (Fig 4, B) and those with periodic acid-Schiff (PAS) staining and numbers of goblet cells (Fig 4, C). Data are means $\pm$ SEMs for 4 to 7 mice in each group from 2 independent experiments. Bar = 50 μ m. **D**, Airway resistance to methacholine was measured at 48 hours after the last HDM challenge. Data are means $\pm$ SEMs for 7 mice in each group. **E** and **F**, Serum levels of HDM-specific IgG₁ (Fig 4, E) and total IgE (Fig 4, F). Data are means $\pm$ SEMs for 18 mice in each group from 3 independent experiments. * $P < .05$ and ** $P < .01$.

reduced in *Fut2*^{-/-} mice (Fig 6, A). Consistently, lung lysates of *Fut2*^{-/-} mice contained significantly lower levels of C3a than those of WT mice (Fig 6, B). In contrast, expression levels of mRNA for C3 in lung epithelial cells were comparable between WT and *Fut2*^{-/-} mice (Fig 6, C), suggesting that local activation rather than local production of C3 is affected by *Fut2*.

Given that C3a is likely to be involved in allergic airway inflammation by recruiting and/or activating inflammatory cells that express C3aR, we next searched for C3aR-expressing cells in the lung. FACS analysis of single-cell suspensions of the lung revealed that Mo-DCs expressed C3aR, especially after HDM challenge, whereas CD11b⁺ conventional dendritic cells (CD11b⁺ cDCs), CD103⁺ DCs, or CD4⁺ T cells did not express C3aR, even after HDM challenge (Fig 6, D). Consistently, lungs of HDM-challenged *Fut2*^{-/-} mice contained significantly lower numbers of Mo-DCs than those of HDM-challenged WT mice (Fig 6, E), whereas numbers of CD11b⁺ cDCs and CD103⁺

DCs were comparable between these mice (Fig 6, E). On the other hand, expression of MHC class II and CD86 on Mo-DCs was similar between these mice (see Fig E6 in this article's Online Repository at www.jacionline.org). mRNA levels of GM-CSF, a representative cytokine that stimulates Mo-DCs, in lung epithelial cells were also similar between these mice (see Fig E7 in this article's Online Repository at www.jacionline.org). Because Mo-DCs have been shown to contribute to the development of allergic airway inflammation,⁴² these results suggest that *Fut2* induces lung epithelial fucosylation and exacerbates allergic airway inflammation, possibly through production of C3a and subsequent accumulation of Mo-DCs in the lung.

DISCUSSION

In this study we show that airway epithelium is heavily $\alpha(1,2)$ fucosylated on induction of allergic inflammation. Airway

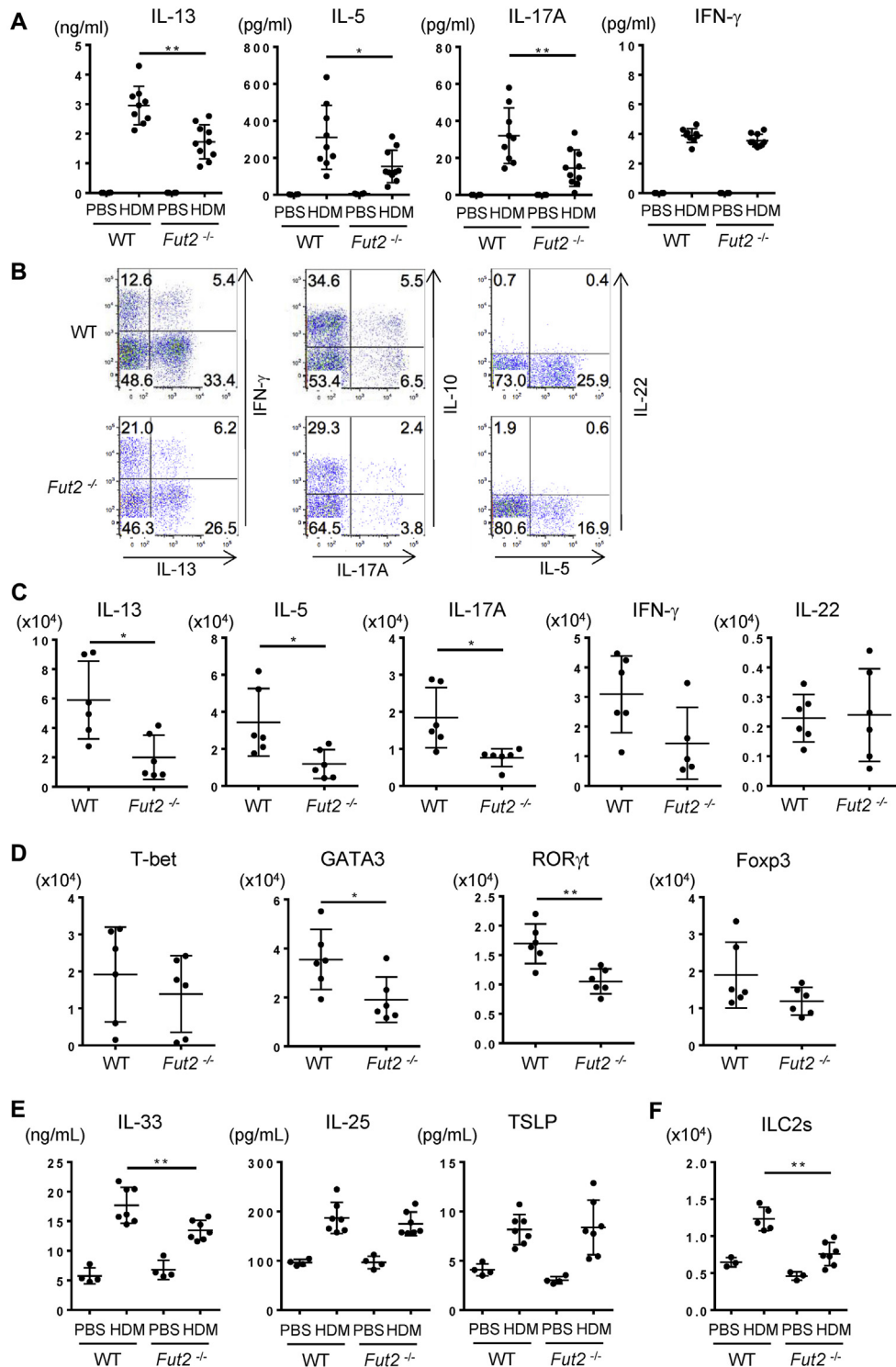


FIG 5. *Fut2* enhances HDM-induced TH2 and TH17 responses in the lung. WT and *Fut2*^{-/-} mice were sensitized and challenged with HDM (see Fig E1, B). **A**, Cytokine production in supernatants of single-cell suspensions of the lung restimulated with HDM for 5 days. Data are means $\pm$ SEMs for 10 mice in each group from 3 independent experiments. **B** and **C**, Shown are representative cytokine profiles of CD4⁺CD3e⁺ cells (Fig 5, B) and numbers of IL-13-, IL-5-, IL-17A-, IFN- γ -, or IL-22-producing CD4⁺CD3e⁺ cells in BALF (Fig 5, C). Data are means $\pm$ SEMs for 5 to 6 mice in each group from 2 independent experiments. **D**, Numbers of CD4⁺CD3e⁺ cells expressing T-bet, GATA3, retinoic acid-related orphan receptor γ t (ROR γ t), or Foxp3 in BALF are shown. Data are means $\pm$ SEMs for 6 mice in each group from 2 independent experiments. **E**, Levels of IL-33, thymic stromal lymphopoietin, and IL-25 in lung homogenates were analyzed by means of ELISA. Data are means $\pm$ SEMs for 7 mice in each group. **F**, Numbers of ILC2s (Lin⁻Thy1.2⁺ST2⁺ cells) in the lung were shown. Data are means $\pm$ SEMs for 6 mice in each group from 2 independent experiments. **P* < .05 and ***P* < .01.

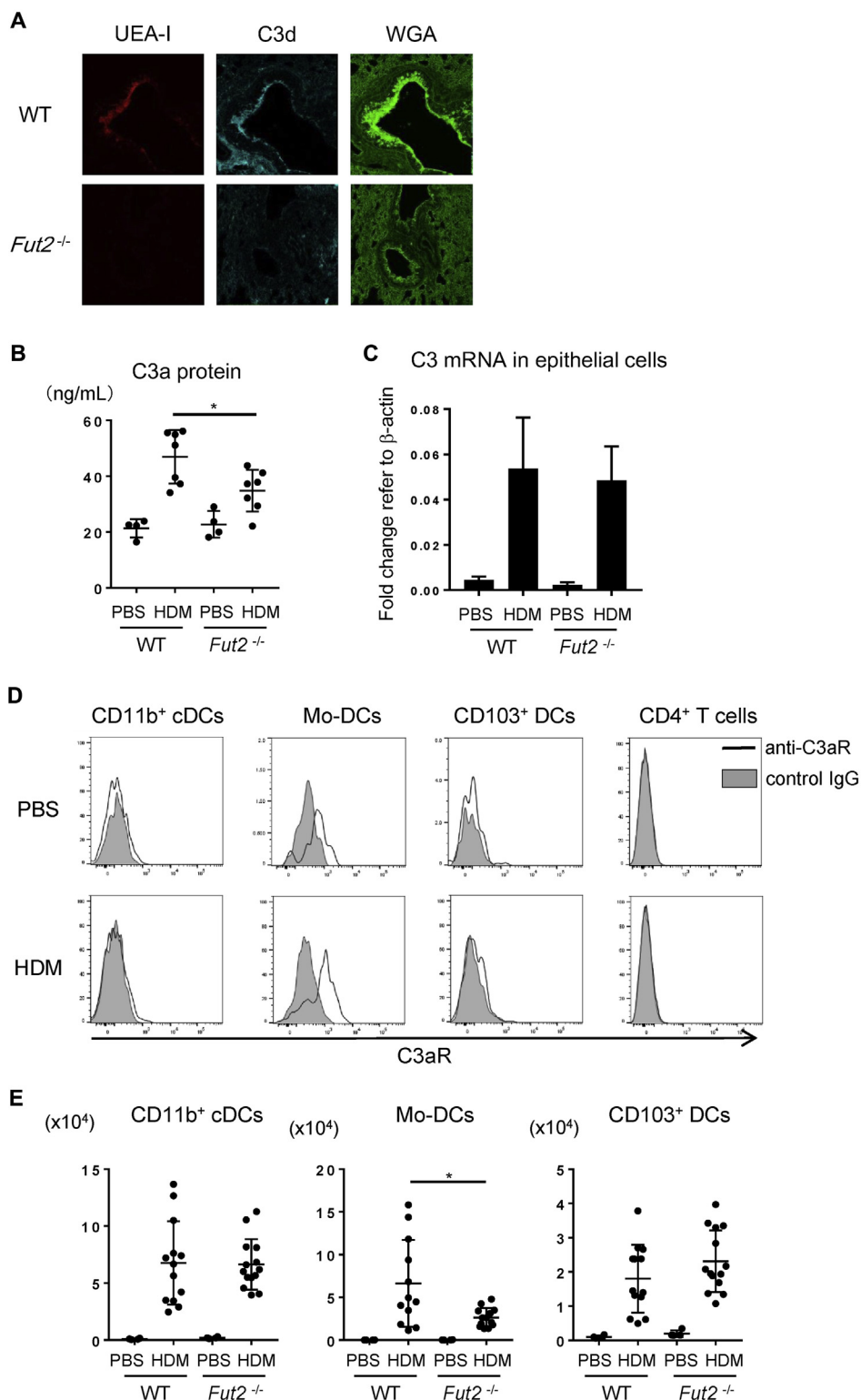


FIG 6. *Fut2* is involved in local activation of complement and accumulation of Mo-DCs in the lung. WT mice and *Fut2*^{-/-} mice were sensitized and challenged with HDM or PBS (see Fig E1, B). **A**, Lung sections were subjected to immunostaining for C3d and lectin staining. Shown are representative immunostaining for C3d and lectin staining. **B**, Levels of C3a in lung homogenates were analyzed by means of ELISA. Data are means $\pm$ SEMs for 7 mice in each group. **C**, mRNA levels of C3 in CD45⁺EpCAM⁺ lung epithelial cells were evaluated by using quantitative RT-PCR. **D**, C3aR expression in each DC subset and CD4⁺ T cells in WT mice was evaluated by means of FACS. **E**, Numbers of CD103⁺ DCs, Mo-DCs, and CD11b⁺ cDCs in the lung were evaluated by using flow cytometry. Data are means $\pm$ SEMs for 13 mice in each group from 4 independent experiments. WGA, Wheat germ agglutinin. * $P < .05$.

epithelium is covered with mucus, a dense carbohydrate-rich matrix that contains a series of glycoproteins, and mucus synthesis and secretion are increased in the airways of asthmatic patients.⁴³ However, the function of each carbohydrate residue in the development of allergic airway inflammation remains unknown. By using a lectin microarray, we found that among carbohydrate modifications, $\alpha(1,2)$ fucosylation is the most prominent epithelial glycosylation of the lung with allergic inflammation (Fig 1). It has been reported that terminal fucosylation is increased in lung epithelial cells in patients with cystic fibrosis, a genetic disease that is often associated with respiratory tract infection.^{44,45} Furthermore, several case-control studies have revealed that genetic polymorphisms in the *FUT2* gene are associated not only with susceptibility to the onset of asthma in children but also with the frequency of exacerbation in adult asthmatic patients.^{46,47} These findings suggest that epithelial fucosylation could be involved in the pathogenesis of respiratory diseases in human subjects, possibly by modulating the balance between immune homeostasis and inflammation in the lung.

Regarding the mechanisms underlying lung epithelial $\alpha(1,2)$ fucosylation, we show that HDM-induced epithelial $\alpha(1,2)$ fucosylation is lacking in *Rag2*^{-/-} and *Stat6*^{-/-} mice (Fig 3, A-C). We also show that administration of IL-13 induces epithelial $\alpha(1,2)$ fucosylation (Fig 3, D and E) and *Fut2* mRNA expression in epithelial cells, even in *Rag2*^{-/-} mice (Fig 3, F), suggesting that the IL-13-STAT6 pathway in epithelial cells induces $\alpha(1,2)$ fucosylation through *Fut2* induction. Given that Muc5AC⁺ goblet cells are one of the major fucosylated subpopulations in lung epithelial cells (Fig 1, D), various factors, such as Notch and inducers of epidermal growth factor signaling, are also likely to be involved in epithelial fucosylation by directly inducing goblet cell differentiation and/or by promoting T_H2 cell differentiation and subsequent IL-13 production.⁴⁸⁻⁵⁰

Intriguingly, kinetics analysis revealed that epithelial fucosylation disappeared within 2 weeks after HDM challenge cessation (Fig 1, F). In contrast, only a few fucosylated lung epithelial cells expressed Ki67 (see Fig E3), a marker for actively proliferating cells, which is in agreement with a recent study that elucidates slow turnover of lung epithelial cells in steady-state conditions.⁵¹ Although a precise turnover rate of lung epithelial cells under allergic inflammation still needs to be clarified, these results suggest that loss of epithelial fucosylation does not result from cell renewal but from other mechanisms, including the short half-life of fucosylated proteins.

Our findings suggest that epithelial fucosylation enhances the magnitude of immune responses to inhaled allergens. Mechanistically, our analysis revealed that epithelial fucosylation is associated with local activation of C3 (Fig 6). These results are consistent with recent findings that stress-induced L-fucose triggers complement activation^{25,41} and that salivary agglutinin modulates complement activation in a secretor status-dependent manner.^{39,40} Because C3a has been shown to be involved in the pathogenesis of asthma,²⁸ it is possible that epithelial fucosylation exacerbates allergic airway inflammation partly through local production of C3a.

Furthermore, consistent with the finding that Mo-DCs in the lung express C3aR (Fig 6, D), accumulation of Mo-DCs was significantly attenuated by the absence of *Fut2* (Fig 6, E). On the other hand, expression of GM-CSF in lung epithelial cells, as well as expression of MHC class II and CD86 on Mo-DCs, was not affected by the absence of *Fut2* (see Fig E6). It has

been shown that Mo-DCs possess the capacity to present allergens to promote T_H2 cell differentiation.¹⁶ In addition, Mo-DCs have been shown to play a proinflammatory role in allergic inflammation through production of various chemokines, including CCL2 and CCL24,¹⁶ which are known to attract monocytes and eosinophils, respectively.⁵² Moreover, Mo-DCs have been shown to express OX40 ligand,¹⁶ which promotes T_H2 cell-mediated immunity in the lung.⁵³ Taken together with previous findings, our findings support the notion that *Fut2*-mediated epithelial fucosylation could exacerbate allergic inflammation by enhancing accumulation of Mo-DCs in the lung through C3a production.

Although our data suggest a causative role of lung epithelial fucosylation in allergic airway inflammation, it is still possible that other mechanisms are involved in *Fut2*-mediated enhancement of allergic inflammation. One possibility is that the lack of epithelial fucosylation alters the microbiota and that the alteration of microbiota influences allergic airway inflammation because intestinal fucose has been shown to be involved in host-microbial symbiosis.²⁴ In this regard we explored the possibility that free fucose liberated from lung epithelium modulates allergic inflammation. However, in preliminary experiments intratracheal administration of L-fucose to *Fut2*^{-/-} mice had a negligible effect on HDM-induced eosinophilic airway inflammation (data not shown). These results make involvement of the lung microbiota less likely; however, we cannot exclude the possibility that other microbiota, such as those in the intestines, are involved in these mechanisms. Further studies using mice lacking *Fut2* specifically in lung epithelial cells are needed to exclude this possibility. Although there are several technical difficulties, further studies to identify an epithelial protein that is specifically fucosylated by *Fut2* in the lung are also needed to enhance our understanding of the mechanisms by which *Fut2* enhances allergic airway inflammation.

In conclusion, we show that lung epithelium is fucosylated by *Fut2* on allergen exposure, possibly through the IL-13-STAT6 pathway, and that *Fut2* enhances T_H2 cell-mediated allergic airway inflammation. Although further studies are needed, our data suggest that *Fut2* plays a key role in immune responses in the lung and could be a novel therapeutic target of asthma.

We thank Drs K. Suzuki and K. Ikeda for valuable discussion, Ms K. Nemoto for technical support, and Ms J. Iwata for animal care.

Key messages

- Allergic airway inflammation is associated with epithelial fucosylation, which is dependent on *Fut2*.
- *Fut2*^{-/-} mice, which lack the capacity to induce epithelial fucosylation, exhibit attenuated allergic airway inflammation.
- Epithelial fucosylation is associated with C3 activation and Mo-DC accumulation in the lung.

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METHODS

Murine models of airway inflammation

OVA-induced asthma model. BALB/c mice were immunized and challenged with OVA, as described previously (Fig E1, C).^{E1}

IL-13-induced airway inflammation. C57BL/6, *Rag2*^{-/-}, or *Stat6*^{-/-} mice were intratracheally administered murine IL-13 (5 µg; Pepro-Tech), as described elsewhere (Fig E1, D).^{E2}

T_H2 transfer model. OVA-specific T_H2 cells were generated from splenocytes of OT-II mice, as described previously,^{E3} and intravenously transferred into WT or *Fut2*^{-/-} mice (2 × 10⁶ cells per mouse). Twenty-four hours after the transfer, mice were challenged with OVA inhalation 3 times at 24-hour intervals.^{E4} Forty-eight hours after the last inhalation, eosinophils and CD4⁺ T cells in BALF were evaluated (see Fig E1, E).

IL-33 administration. WT and *Fut2*^{-/-} mice were intranasally administered once with murine IL-33 (500 ng; PeproTech; Fig E1, F).

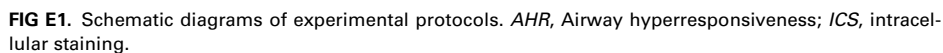
RT-PCR primers

β-Actin: forward gctctggctcctagcaccat, reverse gccaccgatccacacagagt; *Fut1*: forward tacctcatcattgcagacatct, reverse ctctgggggtgattgtccaag; *Fut2*:

forward tgtgacttcaccatcatcc, reverse tctgacagggtttggagctt; *Sec1*: forward gacgacgacgtgaaacct, reverse cttctctggctacacacctgag; *C3*: forward aagcatcaacacaccaaca, reverse cttgagctccattcgtgaca; *Pdpn*: forward accgtgccagtgtgttctg, reverse agcacctgtggtgttatttctg; *Sfip*: forward atggacatgagtagcaaagaggt, reverse cagcatgagaaggcgtttgag; *Scgb1a1*: forward atgaagatgccatcacaatcac, reverse ggatgccacataaccagactct; *Foxj1*: forward ccctgacgacgtggactatG, reverse gccgacagagtgtcttggt; and GM-CSF: forward ggccttggaagcatgtagagg, reverse ggagaactcgttagagacgactt.

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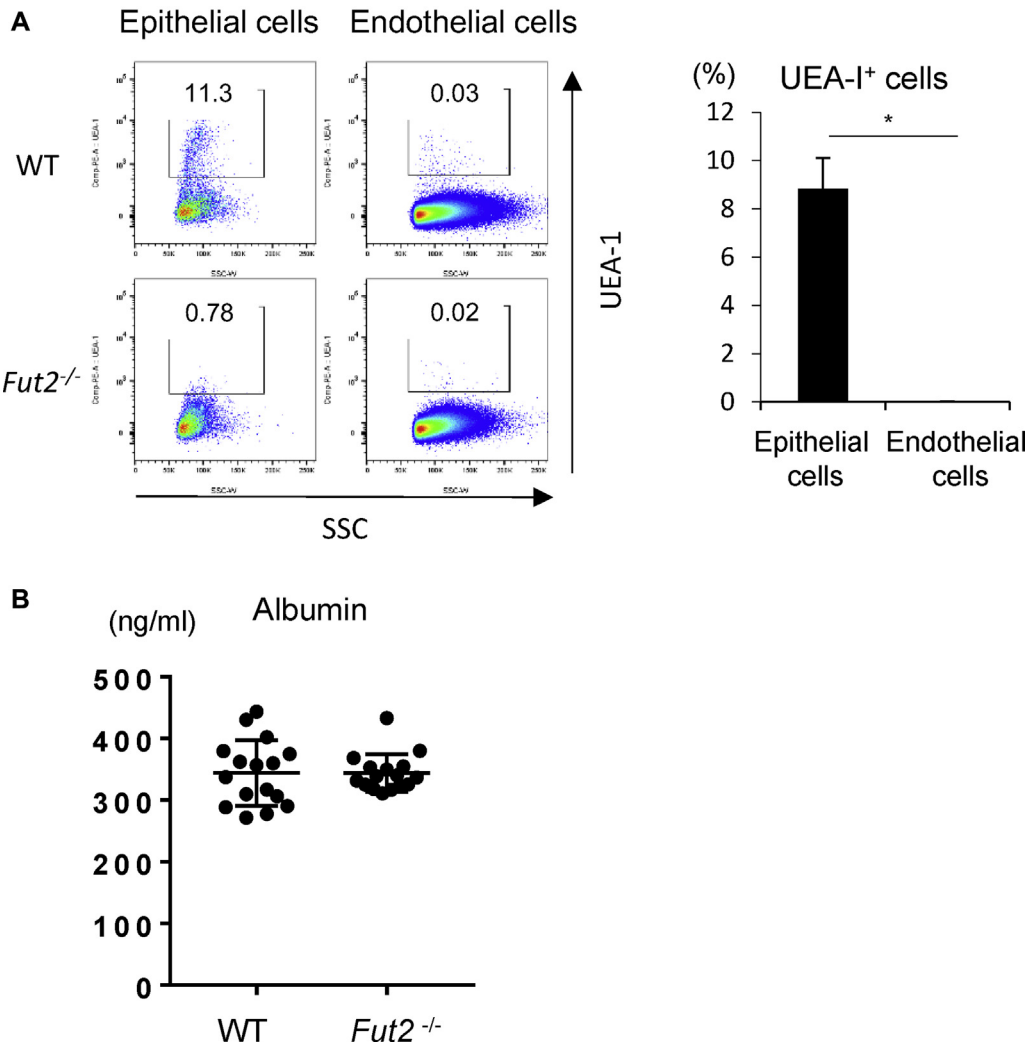


FIG E2. $\alpha(1,2)$ Fucosylation of lung endothelial cells. WT and *Fut2*^{-/-} mice were challenged with HDM or PBS (Fig E1, A). **A**, Shown are representative FACS plots with percentages of UEA-I⁺ cells in CD45⁺EpCAM⁺CD31⁻ lung epithelial cells and CD45⁺EpCAM⁻CD31⁺ endothelial cells. SSC, Side scatter. **B**, Albumin levels in BALF. Data are means $\pm$ SEMs for 16 mice from 4 independent experiments. **P* < .05.

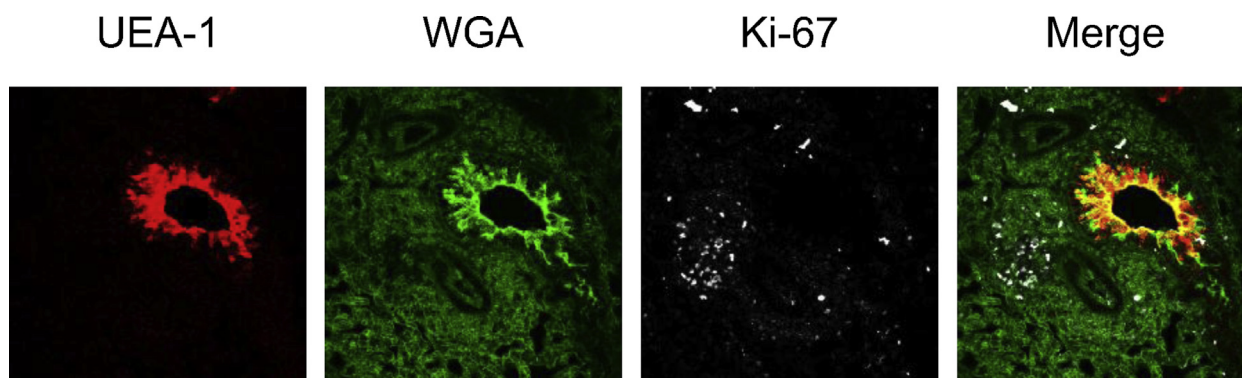


FIG E3. Ki67 expression in fucosylated lung epithelial cells. WT mice were sensitized and challenged with HDM (Fig E1, A). Forty-eight hours after the last HDM challenge, lung tissue was subjected to immunostaining for Ki67 and lectin staining. Shown are representative microphotographs of lungs stained with UEA-I (red) for fucosylation, WGB (green) as a marker for epithelial cells, and Ki67 (white) for actively proliferating cells. WGA, Wheat germ agglutinin.

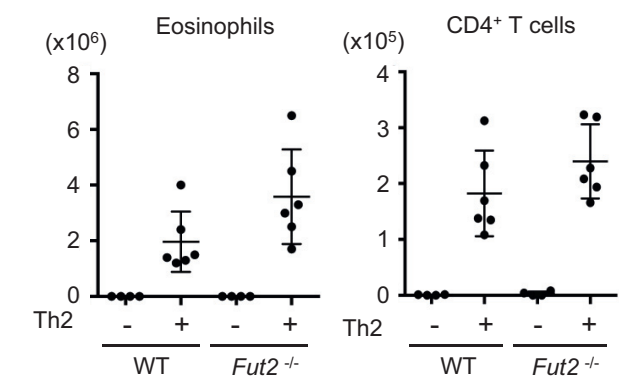


FIG E4. Responses of adaptively transferred antigen-specific T_H2 cells in *Fut2*^{-/-} mice. WT and *Fut2*^{-/-} mice were injected with OVA-specific T_H2 cells or PBS and challenged with OVA inhalation (Fig E1, E). Shown are numbers of eosinophils and CD4⁺ cells in BALF. Data are means $\pm$ SEMs for 4 to 6 mice from 2 independent experiments.

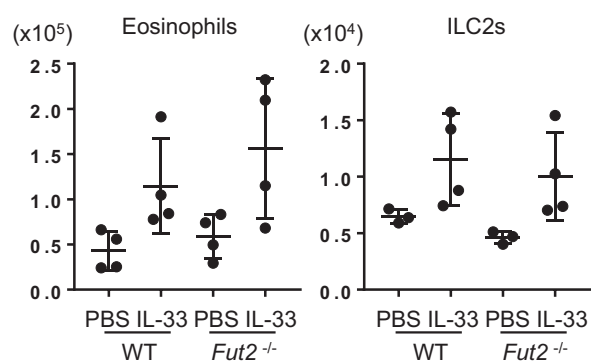


FIG E5. Initial responses on IL-33 administration. WT and *Fut2*^{-/-} mice were intranasally administered once with IL-33 (Fig E1, F). Twenty-four hours after administration, numbers of eosinophils and ILC2s in the lung were evaluated. Data are means ± SEMs for 4 mice.

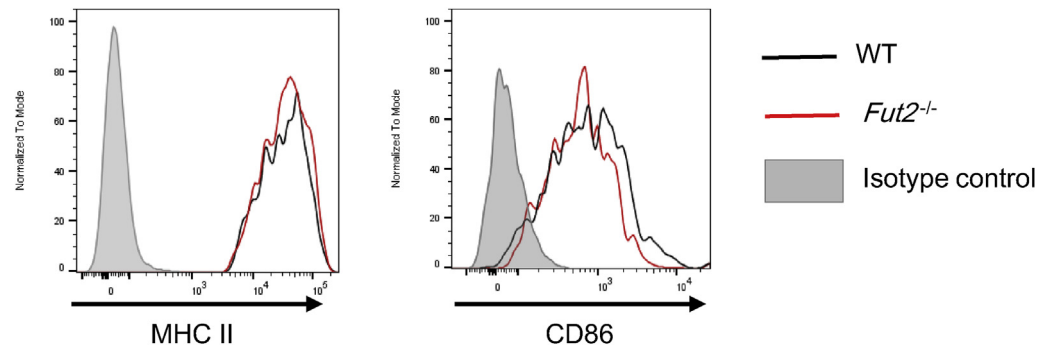


FIG E6. Expression levels of CD80 and MHC class II on Mo-DCs. WT and *Fut2*^{-/-} mice were sensitized and challenged with HDM or PBS (Fig E1, B). Forty-eight hours after the last HDM challenge, expression of CD86 and MHC class II on Mo-DCs was examined by using flow cytometry. Shown are representative histograms for CD86 and MHC class II expression on Mo-DCs.

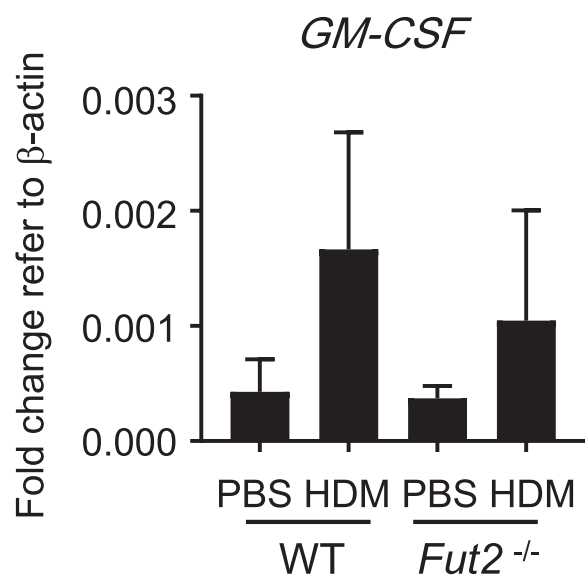


FIG E7. Expression levels of GM-CSF mRNA in lung epithelial cells. WT and *Fut2*^{-/-} mice were sensitized and challenged with HDM or PBS (Fig E1, A). Expression levels of mRNA for GM-CSF in lung epithelial cells were evaluated. Data are means $\pm$ SEMs for 4 mice from 2 independent experiments.

TABLE E1. Possible glycoproteins identified in lung tissues rich in UEA-I⁺ epithelium

Abundance	Protein accession no.	Gene name	Protein description
57	Q9D7Z6	<i>Clca1</i>	Calcium-activated chloride channel regulator 1
36	Q91Z98	<i>Chil4</i>	Chitinase-like protein 4
30	P20029	<i>Hspa5</i>	Endoplasmic reticulum chaperone BiP
27	Q61114	<i>Bpifb1</i>	BPI fold-containing family B member 1
23	P09103	<i>P4hb</i>	Protein disulfide-isomerase
24	P07724	<i>Alb</i>	Serum albumin
21	Q8VCT4	<i>Ces1d</i>	Carboxylesterase 1D
21	P27773	<i>Pdia3</i>	Protein disulfide-isomerase A3
13	P08113	<i>Hsp90b1</i>	Endoplasmic
14	P37040	<i>Por</i>	NADPH-cytochrome P450 reductase
10	P33267	<i>Cyp2f2</i>	Cytochrome P450 2F2
12	O35744	<i>Chil3</i>	Chitinase-like protein 3
10	Q06318	<i>Scgb1a1</i>	Uteroglobin
8	P01027	<i>C3</i>	Complement C3
7	Q9Z331	<i>Krt6b</i>	Keratin, type II cytoskeletal 6B
8	P14211	<i>Calr</i>	Calreticulin
7	O09049	<i>Reg3g</i>	Regenerating islet-derived protein 3-gamma
7	Q61362	<i>Chi3l1</i>	Chitinase-3-like protein 1
6	O88312	<i>Agr2</i>	Anterior gradient protein 2 homolog
6	Q92111	<i>Tf</i>	Serotransferrin
6	Q922R8	<i>Pdia6</i>	Protein disulfide-isomerase A6
5	P13020	<i>Gsn</i>	Gelsolin
5	Q8VDN2	<i>Atp1a1</i>	Sodium/potassium-transporting ATPase subunit alpha-1
4	Q99PL5	<i>Rrbp1</i>	Ribosome-binding protein 1
4	P08003	<i>Pdia4</i>	Protein disulfide-isomerase A4
4	P35242	<i>Sfipa1</i>	Pulmonary surfactant-associated protein A
4	P56395	<i>Cyb5a</i>	Cytochrome b5

Abundance is the number of peptide fragments attributed to each gene.