

# Exposure to nonmicrobial N-glycolylneuraminic acid protects farmers' children against airway inflammation and colitis



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**Background:** Childhood exposure to a farm environment has been shown to protect against the development of inflammatory diseases, such as allergy, asthma, and inflammatory bowel disease.

**Objective:** We sought to investigate whether both exposure to microbes and exposure to structures of nonmicrobial origin, such as the sialic acid N-glycolylneuraminic acid (Neu5Gc), might play a significant role.

**Methods:** Exposure to Neu5Gc was evaluated by quantifying anti-Neu5Gc antibody levels in sera of children enrolled in 2 farm studies: the Prevention of Allergy Risk factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study (n = 299) and the Protection Against Allergy Study in Rural Environments (PASTURE) birth cohort (cord blood [n = 836], 1 year [n = 734], 4.5 years [n = 700], and 6 years [n = 728]), and we associated them with asthma and wheeze. The effect of Neu5Gc was examined in murine airway inflammation and colitis models, and the role of Neu5Gc in regulating immune activation was assessed based on helper T-cell and regulatory T-cell activation in mice.

**Results:** In children anti-Neu5Gc IgG levels correlated positively with living on a farm and increased peripheral blood forkhead box protein 3 expression and correlated inversely with wheezing and asthma in nonatopic subjects. Exposure to Neu5Gc in mice resulted in reduced airway hyperresponsiveness and inflammatory cell recruitment to the lung. Furthermore, Neu5Gc administration to mice reduced the severity of a colitis model. Mechanistically, we found that Neu5Gc exposure reduced IL-17<sup>+</sup> T-cell numbers and supported differentiation of regulatory T cells.

**Conclusions:** In addition to microbial exposure, increased exposure to non-microbial-derived Neu5Gc might contribute to the protective effects associated with the farm environment. (*J Allergy Clin Immunol* 2018;141:382-90.)

**Key words:** *Farmers' children, nonmicrobial, N-glycolylneuraminic acid, airway inflammation, colitis, anti-inflammatory*

The hygiene hypothesis suggests that children growing up in an environment rich in microbes or microbial components have less allergy, autoimmune disease, and colitis.<sup>1-4</sup> Asthma, hay fever, and colitis are less prevalent in farmers' children.<sup>5-7</sup> The microbial load in the child's environment has been proposed to be the critical factor influencing the child's developing immune system and to confer protection against atopic diseases.<sup>8-11</sup> However, a farming lifestyle not only implicates increased exposure to microbes but also to nonmicrobial molecules potentially influencing the developing immune system.<sup>12,13</sup>

N-glycolylneuraminic acid (Neu5Gc) is a sialic acid specifically expressed on nonhuman mammalian cells and glycoproteins and not present in bacteria.<sup>14</sup> In contrast to many other mammals, including primates, human subjects lack the enzyme CMP-Neu5Ac hydroxylase (CMAH) because of a genetic mutation and are therefore not able to synthesize Neu5Gc from the precursor N-acetylneuraminic acid (Neu5Ac).<sup>15,16</sup> As a consequence, human subjects mount a humoral immune response by producing anti-Neu5Gc antibodies, which can be used as a surrogate marker for exposure to Neu5Gc.<sup>14,17,18</sup>

In the present study we investigated the role of exposure to Neu5Gc in the development of airway inflammation and colitis.

#### Abbreviations used

BAL:	Bronchoalveolar lavage
CMAH:	CMP-Neu5Ac hydroxylase
Foxp3:	Forkhead box protein 3
Neu5Ac:	N-acetylneuraminic acid
Neu5Gc:	N-glycolylneuraminic acid
PARSIFAL:	Prevention of Allergy Risk factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle
PASTURE:	Protection Against Allergy Study in Rural Environments
SCID:	Severe combined immunodeficiency
Treg:	Regulatory T

We determined the levels of anti-Neu5Gc antibodies in children's serum samples and correlated them with farm exposure. Moreover, we associated anti-Neu5Gc levels with the incidence of asthma and wheezing. Furthermore, we investigated the effect of Neu5Gc on the development of airway inflammation in a CMAH (Neu5Gc)-deficient murine model mimicking the human situation and examined the immune mechanisms in primary dendritic cells and T helper cells.

## METHODS

### Study design and population

The European cross-sectional Prevention of Allergy Risk factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study aimed to study the determinants of childhood asthma and allergies in farming and anthroposophic populations in 5 European countries, as described previously.<sup>19</sup> Parents of participating children were invited to fill out a questionnaire about farming lifestyle, farm exposures, child's diet, and allergic diseases.<sup>20-22</sup> Written informed consent was obtained from the children's parents for questionnaires, blood sampling, and environmental exposure assessment, and the research ethics committee of canton Basel approved the study. In the present study data of a sample of Swiss children (5-14 years old) with available blood samples were used (n = 299). Questions on health outcomes and farm exposure were derived from the internationally validated International Study of Asthma and Allergies in Childhood II<sup>23</sup> questionnaire and the Allergy and Endotoxin Study (ALEX).<sup>7</sup> Children with reported doctor-diagnosed asthma once or obstructive bronchitis more than once in their lifetime were regarded as having asthma. Obstructive bronchitis is commonly used to define the first occurrence of asthmatic symptoms. Reported wheezing during the past 12 months was considered wheeze. A child who lived on a farm and whose family ran the farm was coded as being a farmer's child.<sup>24</sup>

The Protection Against Allergy Study in Rural Environments (PASTURE) birth cohort is a prospective birth cohort involving children from rural areas in 5 European countries (Austria, Finland, France, Germany, and Switzerland) designed to evaluate risk factors and preventive factors for asthma and atopic diseases.<sup>25</sup> Pregnant women were recruited during the third trimester of pregnancy between August 2002 and March 2005 and divided into 2 groups. Women who lived on family-run farms where any kind of livestock was kept were assigned to the farm group. Women from the same rural areas but not living on a farm were in the reference group. In total, 1133 children were included in this birth cohort. All available serum samples of cord blood (n = 836) and samples at 1 year (n = 734), 4.5 years (n = 700), and 6 years (n = 728) were included in the study.

The research ethics committee of canton St Gallen approved the study, and written informed consent was obtained from all parents. Questionnaires were administered in interviews or self-administered to the mothers within the third trimester of pregnancy and when the children were 2, 12, 18, and 24 months of age and then yearly up to age 6 years. Information on parental atopic status, sex, and the duration of breast-feeding was recorded in questionnaires during

pregnancy, 2 months after birth, and at 1 year of age. Positive parental history of allergies was defined as ever having asthma, allergic rhinitis, or atopic dermatitis by one of the parents. Children were defined as having asthma when the parents reported in the 6-year questionnaire that the child had either a doctor-diagnosed asthma or at least 2 doctor-diagnosed episodes of obstructive bronchitis. In this birth cohort 5 phenotypes of wheeze could have been determined by using latent class analysis, similar to previous epidemiologic studies.<sup>26</sup> We used the phenotypes “intermediate,” “late-onset,” or “persistent” to define wheeze, and the phenotypes “no/infrequent” or “transient” wheeze were used as the reference group. Gene expression of forkhead box protein 3 (*Foxp3*) and *IL10* were measured with quantitative PCR in peripheral blood leukocytes.

### Anti-Neu5Gc antibody quantification

Serum levels of anti-Neu5Gc antibodies were determined by means of ELISA, as previously described.<sup>27</sup> Five hundred nanograms per well of Neu5Ac-polyacrylamide or Neu5Gc-polyacrylamide (GlycoTech, Gaithersburg, Md) were coated on a 96-well microtiter plate. After washing and blocking of the plate, 100  $\mu$ L of a 1:10 dilution of sera was incubated in duplicates on the plate. Bound antibodies were detected by using a horseradish peroxidase-conjugated mouse anti-human IgG (Sigma-Aldrich, Buchs, Switzerland). Measured values were normalized to a standard curve of normal human serum (Sigma-Aldrich) measured on the same plate. For background correction of anti-Neu5Gc IgG levels, anti-Neu5Ac IgG levels were subtracted.<sup>27</sup>

### Animals

CMAH knockout mice<sup>28</sup> were a kind gift from Ajit Varki (University of California, San Diego, Calif) and were bred at AO Research Institute in Davos, Switzerland. Female C57BL/6 mice, C.B17 severe combined immunodeficiency (SCID) mice, and BALB/c mice aged 6 to 8 weeks were obtained from Charles River (Sulzfeld, Germany) and housed at the AO Research Institute Davos. Mice were housed at 4 to 6 animals per cage in individually ventilated cages in a 12-hour/12-hour light/dark cycle, with vegetarian food and water available *ad libitum*. Mice of different genotypes were cohoused for at least 2 weeks before the start of the experiments. All experimental procedures were carried out in accordance with Swiss law and approved by the animal experiment commission of the canton Grisons, Switzerland.

### Allergic airway inflammation mouse model

**Ovalbumin model.** Mice were sensitized by means of intraperitoneal injection of 20  $\mu$ g of grade VI ovalbumin (Sigma-Aldrich) emulsified in 500  $\mu$ g of Alum (Pierce, Rockford, Ill) in 200  $\mu$ L of sterile 0.9% isotonic sodium chloride (NaCl) on days 0, 7, and 21, followed by 20 minutes of 1% grade V ovalbumin (Sigma-Aldrich) aerosol treatments on days 26, 27, and 28. In addition, mice received 50 mg/kg/d LPS-free Neu5Gc (Inalco Pharmaceuticals, San Luis Obispo, Calif) by means of gavage starting 5 days before the first ovalbumin injection. Analysis of mice occurred 24 hours after the last aerosol challenge.

**House dust mite model.** Mice were treated at day 0 with 1  $\mu$ g and on days 7 to 11 with 10  $\mu$ g of house dust mite extract (GREER Laboratories, Lenoir, NC) administered intranasally. Mice were analyzed on day 12. The mice received 50 mg/kg/d Neu5Gc by means of gavage starting 3 or 14 days before the first house dust mite application. Bronchoalveolar lavage (BAL) was performed with 1 mL of PBS containing 1 $\times$  protease inhibitor cocktail (Roche, Mannheim, Germany). The total number of leukocytes was counted with a Neubauer counting chamber. For differential cell counts, cytospin preparations were fixed and stained with Diff-Quick (Merz & Dade AG, Duding, Switzerland). Macrophages, lymphocytes, eosinophils, and neutrophils were identified by using standard morphologic criteria, and 100 to 200 cells were counted per cytospin preparation.

### SCID colitis

More information on SCID colitis can be found in Kjellef et al.<sup>29</sup> To induce colitis, CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>+</sup> cells were applied to SCID mice. These cells

were isolated from total spleen cells of BALB/c mice by using 2 rounds of AutoMACS separation with reagents from Miltenyi Biotec (Bergisch Gladbach, Germany). Three hundred eighty thousand cells were injected intraperitoneally into C.B17 SCID mice. Control animals were injected with sodium chloride. The severity of the disease was assessed by loss of body weight and a symptom score comprising the injection site, breathing, activity, fur condition, movement, body weight, and condition of the feces.

### Lung tissue and lymph node cell isolation and flow cytometric analysis

Dissociation kits for mice and gentleMACS (Miltenyi Biotec) were used according to the manufacturer's protocol to prepare single-cell suspensions from lung tissue or lymph nodes. All flow cytometric analyses were performed on the Gallios Flow Cytometer (Beckman Coulter, Brea, Calif). Anti-CD3 (145-2C11), anti-Helios (22F6), anti-IL-5 (TRFK5), and anti-CD4 (RM4-5) antibodies were obtained from BioLegend (San Diego, Calif). Anti-CD25 (PC61.5), anti-Foxp3 (FJK-16s), anti-IL-10 (JES5-16E3), anti-IL-17A (eBio17B7), anti-IFN- $\gamma$  (XMG1.2), anti-IL-13 (eBio13A), anti-CD127 (A7R34), and anti-IL-4 (11B11) antibodies were obtained from eBioscience (Vienna, Austria). Cells were stained with the fixable viability dye eFlour780 (eBioscience). For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin (50 and 500 ng/mL) for 4 hours at 37°C in a 5% CO<sub>2</sub> atmosphere in the presence of Brefeldin solution (eBioscience) to inhibit cytokine secretion. Cells were permeabilized with reagents from eBioscience.

### Immunoglobulins

Total IgE levels were assessed with a Milliplex kit (Merck Millipore, Billerica, Mass). Ovalbumin/house dust mite-specific IgE/IgG<sub>1</sub> levels in sera were measured by means of ELISA coated with ovalbumin/house dust mite and detected with anti-IgE/IgG<sub>1</sub> (BD Biosciences, Franklin Lakes, NJ).

### Lung function measurements

Mice were intubated after achievement of anesthesia, and airway resistance was assessed with the flexiVent system (SCIREQ, Montreal, Quebec, Canada). Airway resistance was measured in response to increasing concentrations of methacholine (Sigma-Aldrich).

### Primary cell isolation

CD14<sup>+</sup> monocytes were isolated from healthy human PBMCs by using AutoMACS and reagents from Miltenyi Biotec. CD14<sup>+</sup> monocytes were differentiated into monocyte-derived dendritic cells *in vitro* with IL-4 and GM-CSF for 5 days. Cells were stimulated with 3 mmol/L Neu5Gc (Inalco Pharmaceuticals). Murine dendritic cells and T helper cells were isolated from spleens of nontreated mice by using AutoMACS and reagents from Miltenyi Biotec. Cells were cocultured for 5 days in a 1:30 ratio. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin (50 and 500 ng/mL) for 24 hours at 37°C in a 5% CO<sub>2</sub> atmosphere in the presence of Brefeldin solution (eBioscience). T helper cell differentiation was assessed by means of flow cytometry.

### Statistical analysis

Anti-Neu5Gc IgG levels showed a skewed distribution and were therefore log-transformed, resulting in an approximately normal distribution. The proportions of nondetectable values in the PARSIFAL study were 14.7% less than the lower detection limit and 7.5% greater than the upper detection limit. In the PASTURE birth cohort sera were frequently less than the lower detection limit (cord blood, 45.5%; 1 year, 63.8%; 4.5 years, 45.4%; and 6 years, 42.0%; corresponding to the lowest tertile in the analyses) and less frequent above the upper detection limit (cord blood, 1.4%; 1 year, 0.8%; 4.5 years, 2.1%; and 6 years, 2.8%). To take these relevant proportions of

nondetectable values into account, Tobit regression was used with anti-Neu5Gc IgG levels as the dependent variable.<sup>30</sup> Geometric mean ratios along with 95% CIs were computed to describe the association between anti-Neu5Gc IgG levels and exposures. Logistic regression was used to explore the association between binomial health outcomes and anti-Neu5Gc IgG levels, and effects were expressed as odds ratios along with 95% CIs. In these models anti-Neu5Gc IgG levels were introduced as a categorical variable (tertiles) to avoid data loss because of nondetection.

To assess potential confounding for the association between anti-Neu5Gc IgG levels and asthma predefined variables (being a farm child, sex, age, body mass index, number of older siblings, parental history of allergies, parental education, and maternal smoking during pregnancy) were introduced into the regression models one by one. Variables that changed the odds ratios for anti-Neu5Gc IgG levels on asthma by more than 10% were used in the final models.

Two-sided *P* values of less than .05 were considered significant. In the PASTURE birth cohort all analyses were adjusted for farming status and center. Statistical analysis was performed with Stata/SE 10.1 software (StataCorp, College Station, Tex) and SAS 9.2 software (SAS Institute, Cary, NC).

Mouse and *in vitro* experiments were graphed and analyzed statistically with Prism 5 software (GraphPad Software, San Diego, Calif). Data were expressed as means ± SEMs and analyzed for significance by using the Mann-Whitney test. Samples or animals were only excluded because of technical problems.

## RESULTS

### Anti-Neu5Gc IgG levels are increased in farmers' children and inversely associated with nonatopic wheeze and asthma

To assess whether farmers' children are exposed to Neu5Gc, we measured anti-Neu5Gc antibody levels in the context of the PARSIFAL study and the PASTURE birth cohort (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). At all time points, farmers' children had higher levels of anti-Neu5Gc IgG compared with nonfarmers' children (Table 1). To assess whether exposure to Neu5Gc might affect the children's development of asthma and wheezing, we associated anti-Neu5Gc antibody levels with asthma and wheeze. Higher anti-Neu5Gc IgG levels were inversely associated with asthma and wheezing in a dose-dependent manner in the PARSIFAL study population (Fig 1, A).

In the longitudinal PASTURE birth cohort the sample size allowed us to divide the children into those with and without atopy (defined as allergic sensitization to any allergens at 6 years of age). We found a significant inverse association between anti-Neu5Gc IgG levels and wheezing among nonatopic children and a trend for an inverse association between anti-Neu5Gc IgG levels and asthma (Fig 1, B).

Next, we investigated potential associations between anti-Neu5Gc IgG levels and gene expression of regulatory T (Treg) cell marker in children's peripheral blood leukocytes. Increasing anti-Neu5Gc IgG levels were associated with increased gene expression of *Foxp3*, and a positive nonstatistically significant trend was shown with *IL10* gene expression in 6-year-old children of the longitudinal study (Table II). No other significant associations were found between anti-Neu5Gc IgG and gene expression of immunologic markers (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

In summary, farmers' children had increased anti-Neu5Gc IgG levels, which correlated with less wheezing and asthma in nonatopic children and increased expression of Treg cell markers.

**TABLE 1.** Anti-Neu5Gc IgG levels in farmers' children related to nonfarmers' children

	GMR (95% CI)
PASTURE birth cohort*	
Cord blood	<b>3.98 (1.82-8.72)</b> §
1 y	2.00 (0.62-6.49)
4.5 y	<b>6.73 (2.68-16.92)</b> §
6 y	<b>19.45 (8.16-46.4)</b> §
PARSIFAL study†	
School age	<b>3.36 (2.23-5.05)</b> ‡

GMR, Geometric mean ratio. Values in boldface are statistically significant.

\*GMR adjusted for center, parental atopy, sex, and duration of breast-feeding.

†Unadjusted GMR.

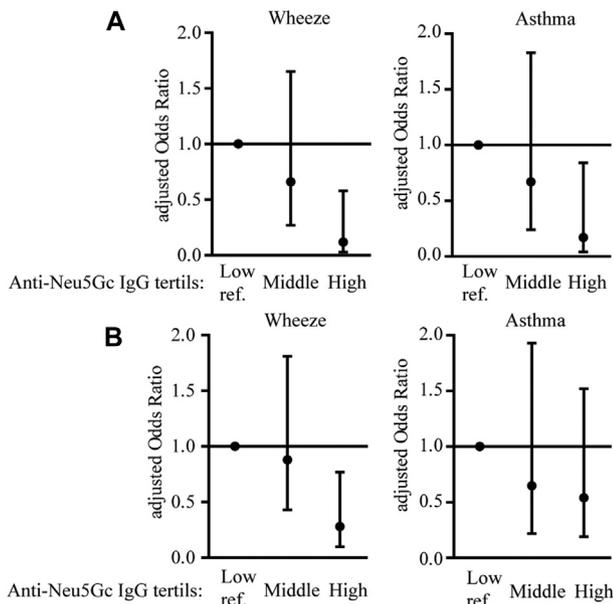
‡*P* < .01.

§*P* < .001.

### Airway inflammation severity in CMAH-deficient and wild-type mice is reduced by oral administration of Neu5Gc

To further investigate the functional role of Neu5Gc in the development of inflammatory airway diseases, we used a murine model with Neu5Gc deficiency.<sup>28</sup> CMAH-deficient mice lack Neu5Gc because of a mutation in the synthesis pathway resembling the human situation. Because murine inflammatory airway disease models do not cover all immunologic aspects compared with human disease, we applied 2 different models with CMAH-deficient mice, one with ovalbumin as the allergen and aluminum hydroxide as an adjuvant and one with house dust mite extract as the allergen. We assessed whether the severity of airway inflammation in CMAH-deficient mice could be reduced by long-term exposure (beginning 14 days before sensitization) to a dose of Neu5Gc that was comparable with the calculated exposure of a child living on a farm.<sup>31</sup> We found that airway resistance in response to methacholine and total cell, eosinophil, and neutrophil numbers in BAL fluid were reduced by Neu5Gc administration in both models (Fig 2, A and B). Furthermore, inflammatory cell infiltration and mucus production in tissue sections from the lungs of mice administered Neu5Gc were reduced (Fig 2, C and D). Shorter-term exposure (beginning 3 days before sensitization) to Neu5Gc did not significantly reduce total cell, eosinophil, and neutrophil numbers in BAL fluid, suggesting that a longer exposure time is required to achieve protection (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

To investigate further the underpinning mechanisms induced by Neu5Gc administration, we analyzed levels of cytokine production by lung T helper and lung Treg cells and immunoglobulin levels in sera. Flow cytometric analyses of lung CD3<sup>+</sup>CD4<sup>+</sup> cells revealed that Neu5Gc application reduced the percentage of IL-17–producing T helper cells in both murine models (Fig 2, E). The percentage of IL-4–, IFN-γ–, IL-10–, IL-13–, and IL-5–producing T helper cells was not altered (see Figs E2, A, and E3, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Although the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells was not significantly increased in lung tissues through exposure to Neu5Gc in the ovalbumin model, these cells produced more IL-10 (Fig 2, F). In the house dust mite model we observed more CD25<sup>+</sup>Foxp3<sup>+</sup> and CD25<sup>+</sup>Foxp3<sup>+</sup>CD127<sup>–</sup> Treg cells after Neu5Gc application (Fig 2, F, and see Fig E3, B). Neu5Gc administration did not have an effect on immunoglobulin levels in sera (see Figs E2, B, and E3, C).



**FIG 1.** Association between anti-Neu5Gc IgG levels in tertiles and wheeze or asthma. **A**, Cross-sectional PARSIFAL study (school-aged children): adjusted odds ratio for the incidence of asthma or wheezing of tertiles of anti-Neu5Gc IgG levels related to the lowest tertile as reference. **B**, Longitudinal PASTURE birth cohort (at age of 6 years): adjusted odds ratio for the incidence of nonatopic asthma or wheezing of tertiles of anti-Neu5Gc IgG levels related to the lowest tertile as reference. Odds ratios are adjusted for farming status, center, atopic parents, and sex.

**TABLE II.** Association of anti-Neu5Gc IgG levels with expression of *Foxp3* and *IL10* at 6 years of age

	GMR (95% CI)
PASTURE birth cohort	
<i>Foxp3</i>	<b>1.24 (1.05-1.47)*</b>
<i>IL10</i>	1.16 (0.99-1.32)

GMR, Geometric mean ratio. Values in boldface are statistically significant.

\* $P < .05$ .

To investigate whether exogenously added Neu5Gc had an effect only in the absence of endogenously produced Neu5Gc, we applied Neu5Gc to wild-type mice and investigated whether it was able to reduce the severity of house dust mite extract–induced airway inflammation. Similar to the findings of CMAH-deficient mice, we found in wild-type mice reduced airway resistance in response to methacholine; reduced total cell, eosinophil, and neutrophil numbers in BAL fluid; less IL-17, IL-4, and IL-13 production by lung T helper cells; and more lung CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells producing IL-10 (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

In summary, administration of Neu5Gc to both CMAH-deficient or wild-type mice ameliorated the symptoms of airway inflammation, enhanced Treg cells, and reduced IL-17 production by T helper cells in lungs.

### Colitis severity is reduced by oral administration of Neu5Gc

Next, we assessed whether the severity of another inflammatory disease was ameliorated by exposure to Neu5Gc. Therefore

we applied daily Neu5Gc orally to SCID mice that received CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>+</sup> cells to induce colitis and assessed the severity of the disease by scoring symptoms, assessing body weight, and measuring the ratio between weight and length of the colon. We found that an increase in symptom score and body weight loss was prevented by Neu5Gc administration compared with that seen in nontreated mice (Fig 3, A and B). Furthermore, the weight/length ratio of the colon was significantly improved (Fig 3, C). Additionally, we found that mesenteric lymph node T helper cells produced less IL-17 and that the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells was enhanced in response to Neu5Gc administration (Fig 3, D and E). However, the percentage of IL-4<sup>-</sup>, IFN- $\gamma$ <sup>-</sup>, IL-10<sup>-</sup>, IL-13<sup>-</sup>, and IL-5–producing T helper cells was not influenced by Neu5Gc administration, whereas assessment of total inflammatory cell infiltration in the gut by means of hematoxylin and eosin staining was also not significantly changed by Neu5Gc (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

In summary, administration of Neu5Gc during induction of colitis in mice ameliorated symptoms, enhanced Treg cells, and reduced IL-17 production by T helper cells in mesenteric lymph nodes.

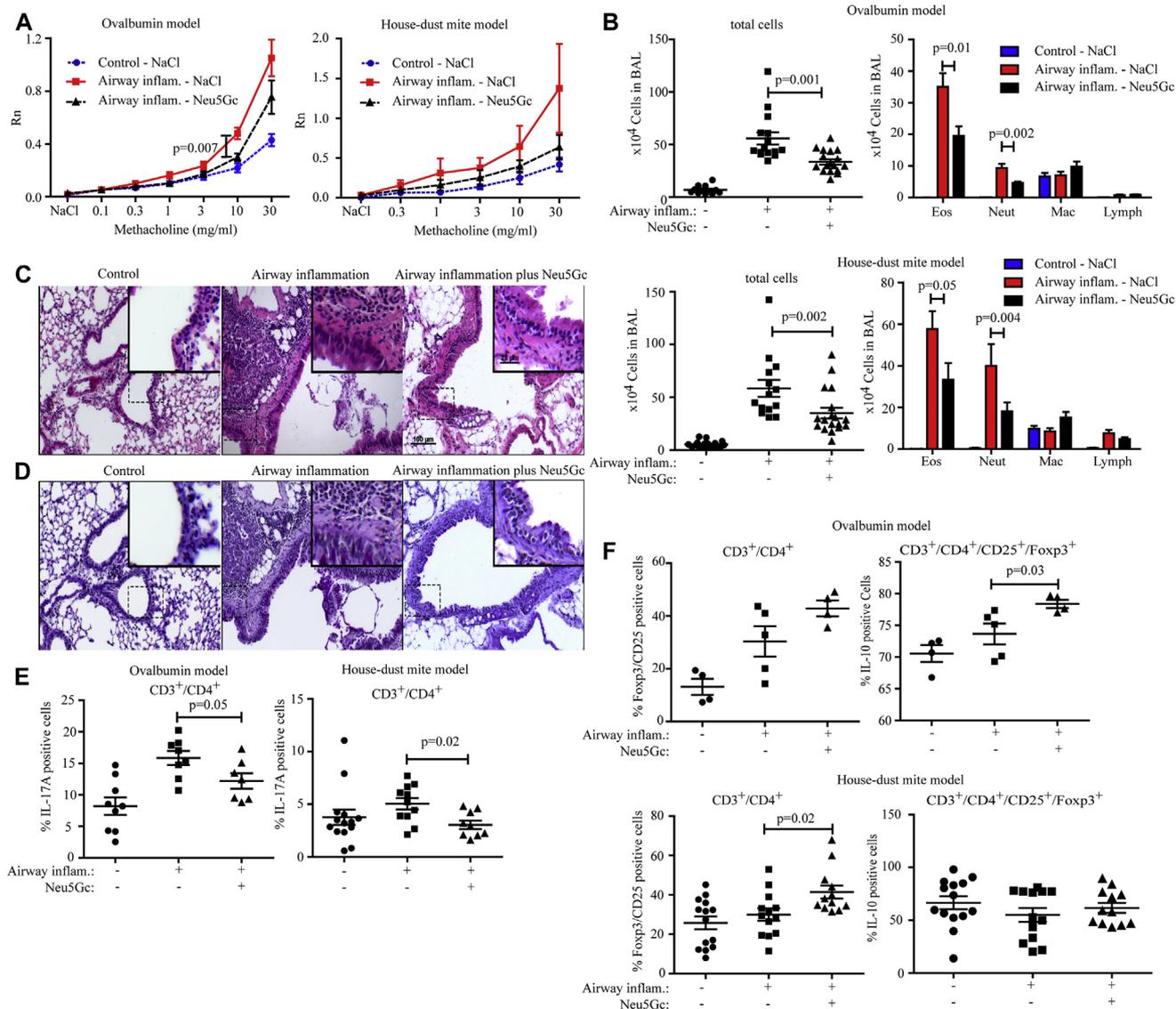
### Neu5Gc induces a regulatory phenotype in dendritic cells, and coculture of Neu5Gc-expressing T helper cells with dendritic cells leads to less IL-17–producing T helper cells and induction of Treg cells

Because the murine studies suggested a direct effect of Neu5Gc on immune cells, leading to less IL-17 production of T helper cells and enhanced Treg cells, we examined the effects of Neu5Gc on primary human and murine immune cells. We measured expression of regulatory molecules in human monocyte-derived dendritic cells that were stimulated with Neu5Gc. Indoleamine 2,3-dioxygenase and retinaldehyde dehydrogenase 2 gene expression, as well as IL-10 secretion, were increased. All of these molecules are known to be involved in Treg cell differentiation (Fig 4, A).<sup>32</sup>

Next, murine dendritic cells and naive T helper cells were isolated from either wild-type or CMAH-deficient spleens and cocultured. Cocultures of wild-type cells led to lower levels of IL-17–producing T helper cells and higher levels of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells compared with cultures of cells isolated from CMAH-deficient mice (Fig 4, B and C). Cocultures of wild-type T helper cells or dendritic cells with CMAH-deficient dendritic cells or T helper cells showed intermediate IL-17 production by T helper cells and medium-level CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell polarization (Fig 4, B and C).

### DISCUSSION

Our data show that environmental exposure to Neu5Gc is associated with less nonatopic asthma and wheezing in children and has anti-inflammatory effects in murine models of airway and gut inflammation, regardless of whether Neu5Gc is endogenously present. Human subjects are able to take up Neu5Gc through fluid pinocytosis and specific lysosomal transporters and incorporate it in newly synthesized glycoproteins.<sup>27,28,33</sup> Therefore Neu5Gc administration in human subjects might be protective, independent of whether diet-derived Neu5Gc is already present on cells. This suggests that not only microbial but also nonmicrobial



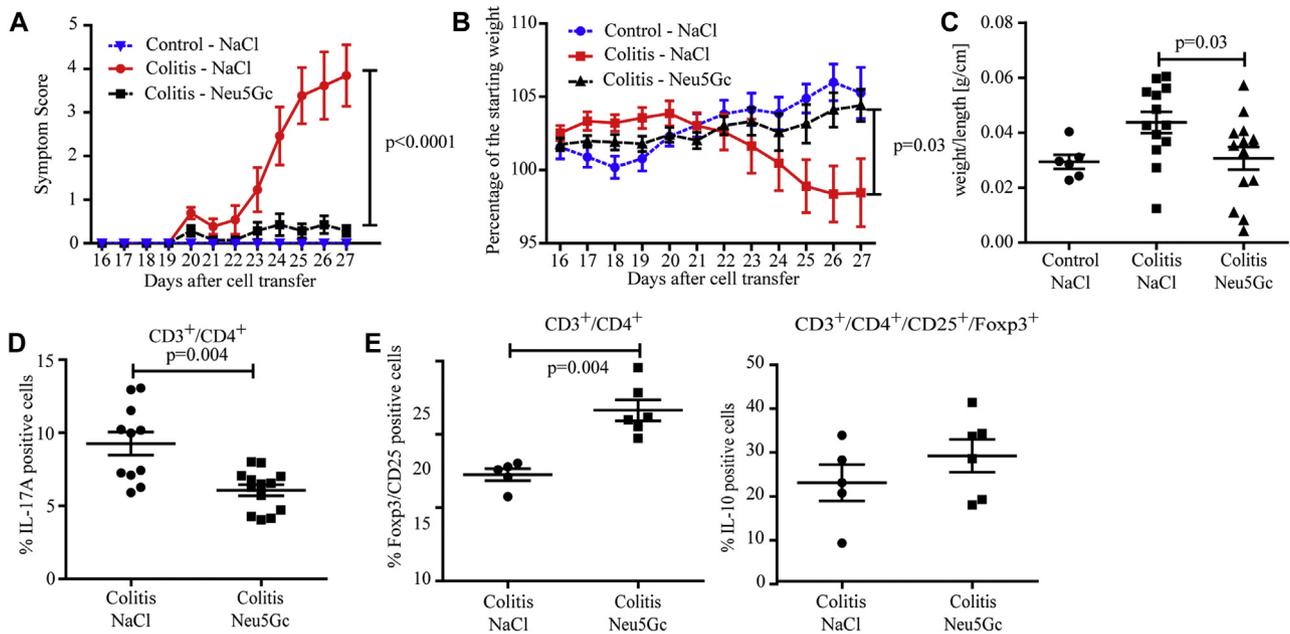
**FIG 2.** Oral application of Neu5Gc to CMAH-deficient mice reduced the severity of airway inflammation. **A**, Airway resistance in response to increasing doses of methacholine. **B**, Total and differential cell counts in BAL fluid. **C**, Representative hematoxylin and eosin (H&E)-stained lung tissue. **D**, Representative periodic acid-Schiff (PAS)-stained lung tissue. **E**, Quantification of IL-17 secretion by lung CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells. **F**, Quantification of lung CD3<sup>+</sup>/CD4<sup>+</sup>/CD25<sup>+</sup>/Foxp3<sup>+</sup> Treg cells and their IL-10 secretion. Each dot represents an individual animal. Data were assessed in 4 (ovalbumin model) and 4 (house dust mite model) independent experiments (means and SEMs). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neut*, neutrophils.

components in the environment have anti-inflammatory effects, adding a new aspect to the hygiene hypothesis.

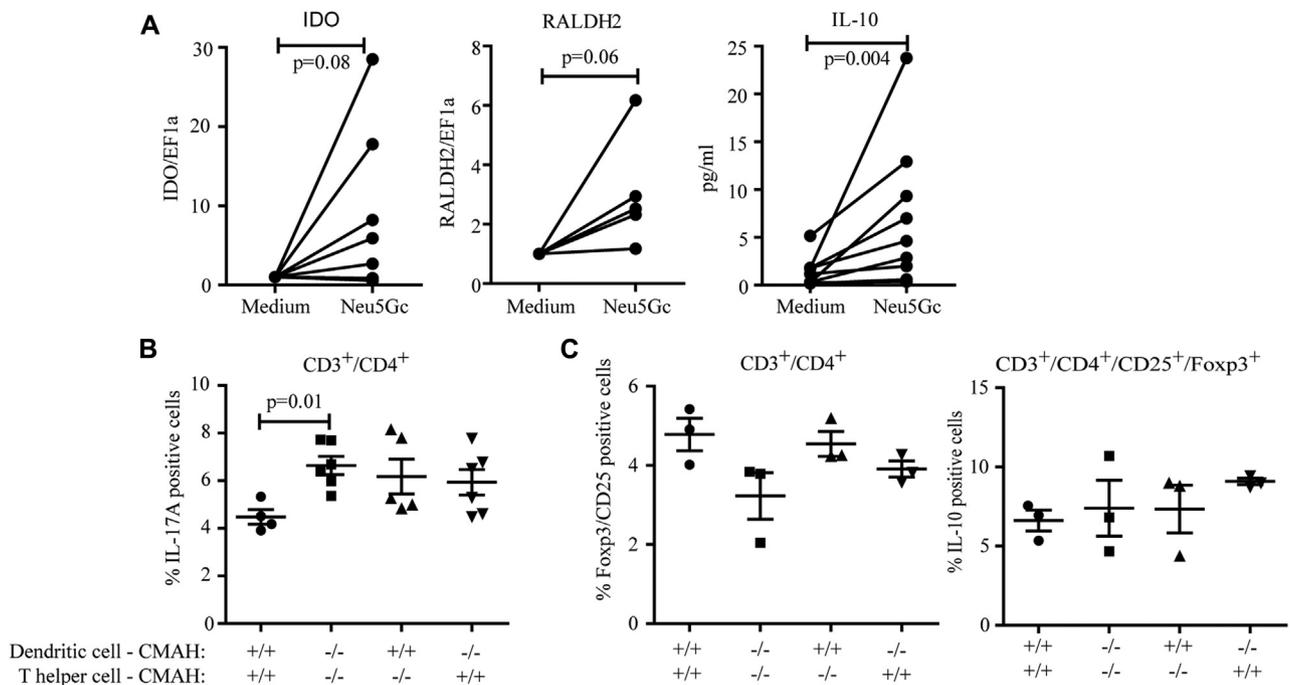
Various environmental exposures have been shown to reduce the child's risk of atopic disease and asthma.<sup>2</sup> The microbial diversity and load in a child's environment has been suggested to be the principal factor in driving maturation of the child's immune system toward a nonatopic phenotype.<sup>8-10</sup> The relevance of such exposures is supported by the observation that farmers' children express higher levels of CD14 and TLR (ie, innate immune receptors recognizing pathogen-associated molecular patterns signaling danger to the immune system).<sup>8,24,34,35</sup>

Neu5Gc can be regarded as an example of a non-microbial-associated molecular pattern. The beneficial effect of Neu5Gc seems to be anti-inflammatory and anti-T<sub>H</sub>17. Treg cell numbers

were increased and IL-17 secretion of T helper cells was decreased after Neu5Gc administration. Moreover, epidemiologic data showed positive associations between anti-Neu5Gc IgG levels and expression of the Treg cell markers Foxp3 or IL-10 in children's white blood cells. T<sub>H</sub>17 and Treg cell subsets have a dichotomous character influenced by several cytokines. T<sub>H</sub>17 cells are critical for the immune response against bacterial and fungal infections, and increased levels in peripheral blood and lesions are associated with pathology in patients with multiple sclerosis, rheumatoid arthritis, psoriasis, Crohn disease, and ulcerative colitis.<sup>36</sup> Treg cells are known to control several inflammatory diseases without influencing the immune response against pathogens.<sup>37,38</sup> Finally, Neu5Gc treatment of dendritic cells induced a regulatory phenotype. Regulatory dendritic cells have



**FIG 3.** Oral application of Neu5Gc reduced the severity of colitis in SCID mice. **A**, Symptom score. **B**, Body weight. **C**, Weight/length ratio of the colon. **D**, Quantification of IL-17 secretion by mesenteric lymph node CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells. **E**, Quantification of mesenteric lymph node CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and their IL-10 secretion. Each dot represents an individual animal. Data were assessed in 2 independent experiments (means and SEMs).



**FIG 4.** Neu5Gc induces a regulatory phenotype in dendritic cells and leads to less IL-17-producing T helper cells and induction of Treg cells in T helper cell-dendritic cell cocultures. **A**, Expression of indoleamine 2,3-dioxygenase (*IDO*) and retinaldehyde dehydrogenase 2 (*RALDH2*) genes and secretion of IL-10 by human monocyte-derived dendritic cells stimulated with Neu5Gc. **B**, IL-17 production of T helper cells. **C**, Quantification of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and their IL-10 secretion. Each dot represents an individual donor/animal. Data were assessed in 2 independent experiments (means and SEMs).

been described to play a role in resolution of chronic inflammation.<sup>32</sup> The anti-inflammatory effects of Neu5Gc resemble the effects previously described for short-chain fatty acids, anti-inflammatory polyunsaturated fatty acids, or certain biogenic amines.<sup>39-42</sup> Whether the effects of Neu5Gc are also mediated by G protein-coupled receptor signaling or epigenetic mechanisms needs to be investigated in future experiments.

Several studies have previously shown that dietary exposure to Neu5Gc in combination with anti-Neu5Gc antibodies was associated with inflammation and cancer.<sup>31,43-45</sup> In contrast, the results of our murine models show a significant protective effect on airway and gut inflammatory responses. The contrasting findings could be related to the fact that we applied Neu5Gc in a highly purified form, which did not induce an antibody response in CMAH-deficient mice, which was also previously shown by others.<sup>14</sup> Because of the lack of an antibody response in mice and the absence of antibodies in the *in vitro* dendritic cell and lymphocyte models, we propose that it is the Neu5Gc molecule itself and not the antibody response to the sialic acid that is anti-inflammatory. Indeed, the influence of anti-Neu5Gc antibodies on tumor progression is dose dependent, with low levels promoting and high levels inhibiting tumor growth.<sup>46</sup> To our knowledge, there is no study showing that farmers' children have an increased risk of cancer.<sup>47</sup>

Our data suggest that not only immunologic danger signals derived from microbes but also non-microbial-associated molecular patterns can be protective environmental exposures.

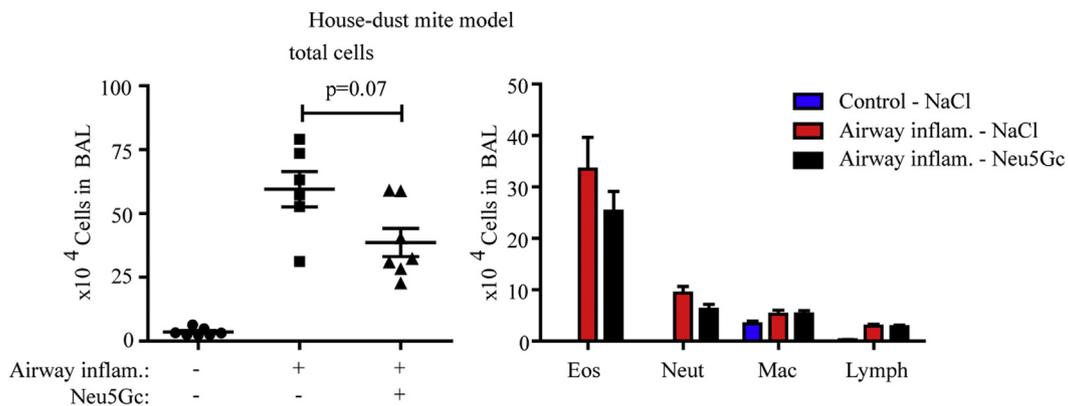
#### Key messages

- Anti-Neu5Gc antibody levels correlate with living on a farm and increased peripheral blood Foxp3 expression and are inversely associated with nonatopic asthma and wheezing in children.
- Exposure to Neu5Gc reduces the severity of airway and intestinal inflammation in mice.
- Exposure to Neu5Gc induced Treg cells and reduced IL-17<sup>+</sup> T helper cells in the lungs of mice.

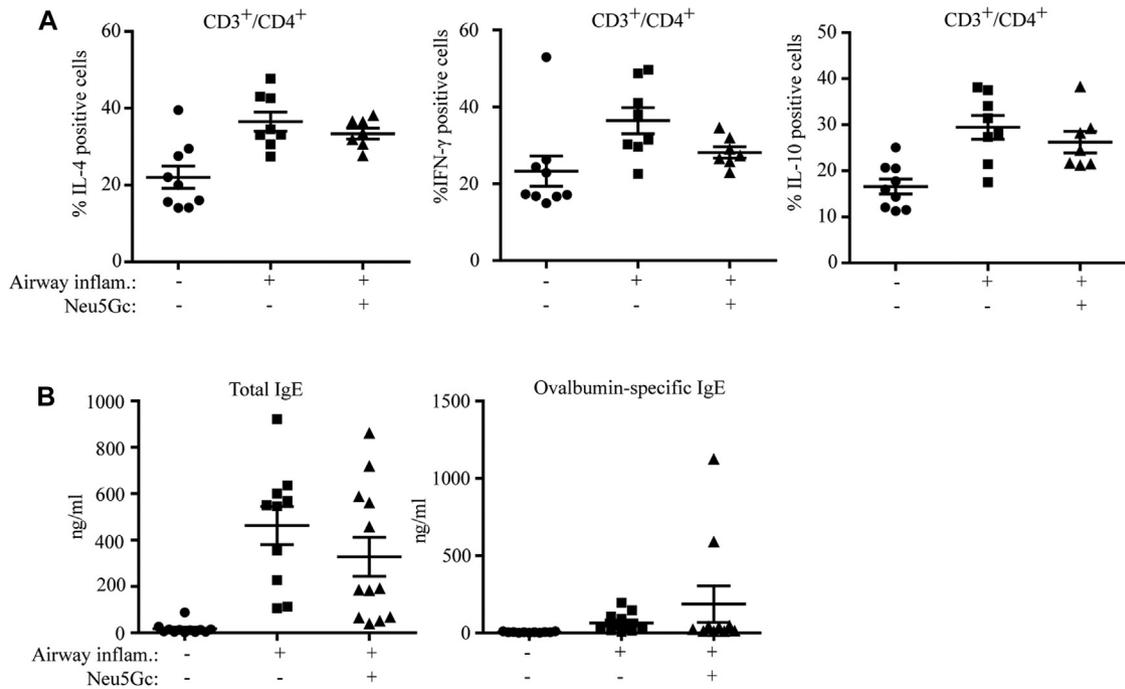
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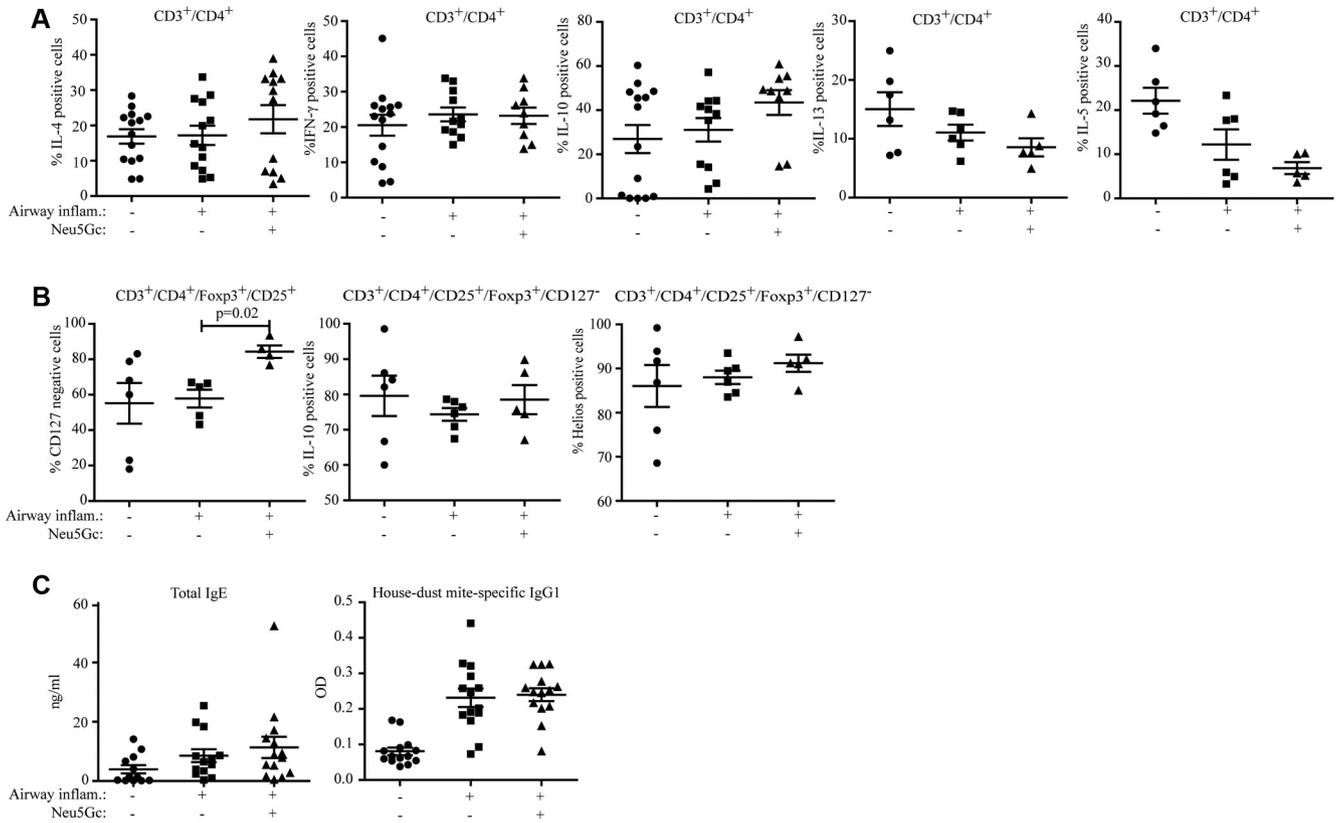
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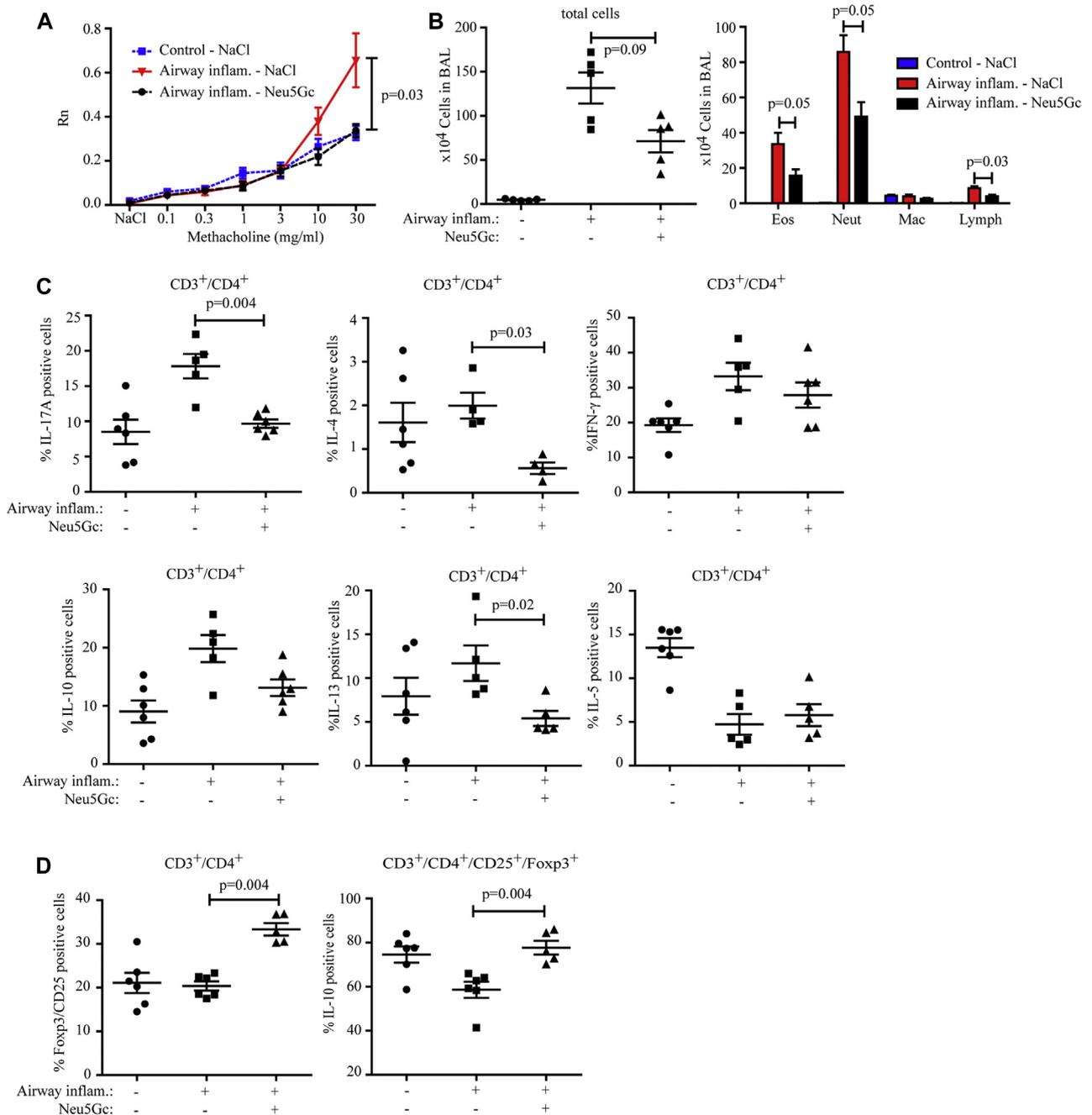
**FIG E1.** Short-term oral application of Neu5Gc to CMAH-deficient mice did not reduce the severity of airway inflammation. Total and differential cell counts in BAL fluid. Each *dot* represents an individual animal. Data were assessed in 1 experiment (mean and SEMs). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neut*, neutrophils.



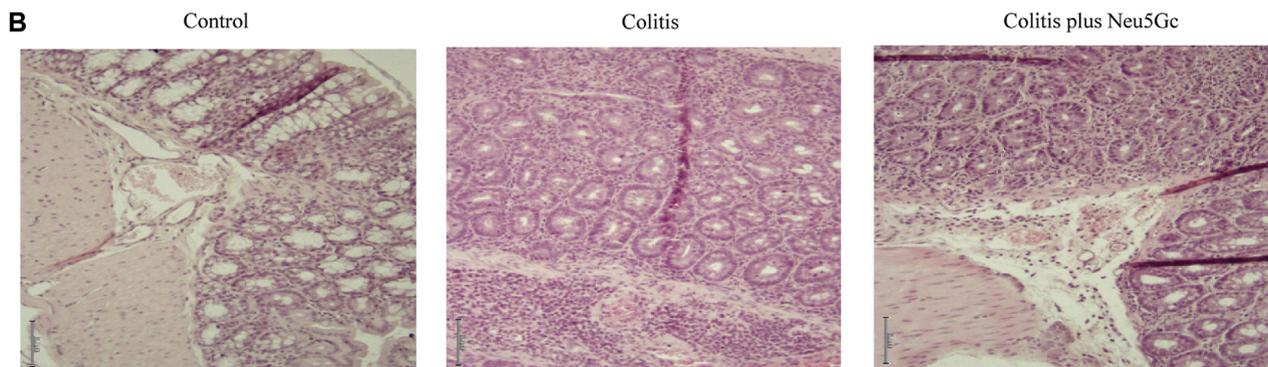
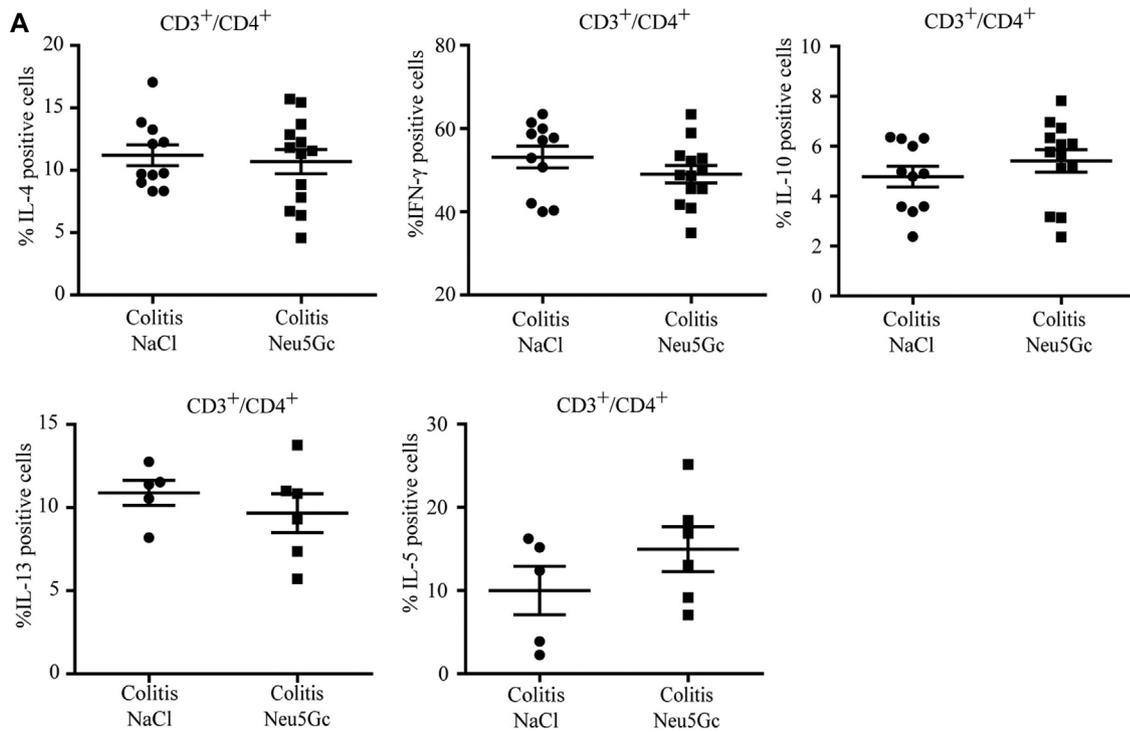
**FIG E2.** Oral application of Neu5Gc to CMAH-deficient mice reduced the severity of airway inflammation in a model using ovalbumin as an allergen. **A**, Quantification of cytokine secretion by lung CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells. **B**, Quantification of total and ovalbumin-specific IgE in sera. Each dot represents an individual animal. Data were assessed in 2 independent experiments (means and SEMs).



**FIG E3.** Oral application of Neu5Gc to CMAH-deficient mice reduced the severity of airway inflammation in a model using house dust mite as an allergen. **A**, Quantification of cytokine secretion by lung CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells. **B**, Quantification of lung CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell subsets and their IL-10 secretion. **C**, Quantification of total IgE and house dust mite-specific IgG<sub>1</sub> in sera. Each dot represents an individual animal. Data were assessed in 2 independent experiments (means and SEMs).



**FIG E4.** Oral application of Neu5Gc to wild-type mice reduced the severity of airway inflammation in a model using house dust mite as an allergen. **A**, Airway resistance in response to increasing doses of methacholine. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophages; *Neut*, neutrophils. **B**, Total and differential cell counts in BAL fluid. **C**, Quantification of cytokine secretion by lung CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells. **D**, Quantification of lung CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells and their IL-10 secretion. Each dot represents an individual animal. Data were assessed in 2 independent experiments (means and SEMs).



**FIG E5.** Oral application of Neu5Gc prevented the onset of colitis in SCID mice. **A**, Quantification of cytokine secretion by mesenteric lymph node CD3<sup>+</sup>CD4<sup>+</sup> T helper cells. **B**, Representative hematoxylin and eosin (H&E)-stained gut tissue. Each dot represents an individual animal. Data were assessed in 2 independent experiments (means and SEMs).

**TABLE E1.** Characteristics of the PASTURE birth cohort and the PARSIFAL study

	PASTURE birth cohort								PARSIFAL study	
	With blood samples at birth (n = 836)		With blood samples at 1 y (n = 734)		With blood samples at 4.5 y (n = 700)		With blood samples at 6 y (n = 728)		School age (n = 299)	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Center										
Austria	172	20.6	139	18.9	90	12.9	107	14.7		
Switzerland	177	21.2	140	19.1	145	20.7	167	22.9	299	100
France	140	16.8	122	16.6	151	21.6	153	21		
Germany	157	18.8	154	21	164	23.4	150	20.6		
Finland	190	22.7	179	24.4	150	21.4	151	20.7		
Farmer	382	45.7	367	50	346	49.4	350	48.1	170	56.9
Sex										
Female	412	49.8	361	49.2	331	47.4	348	47.9	149	49.8
Age (y)										
5-6									35	11.7
7-8									81	27.1
9-10									77	25.8
11-12									89	29.8
13-14									17	5.7
Atopic parents										
No	382	46.4	333	45.8	310	44.5	315	43.6	200	67.1
Yes	441	53.6	394	54.2	387	55.5	408	56.4	98	32.9
Atopic sensitization (specific IgE result $\geq 0.35$ kU/L against common inhalant and/or food allergens)										
Yes	92	11.2	211	28.8	399	57.1	389	53.5	81	27.2
No	731	88.8	522	71.2	300	42.9	338	46.5	217	72.8
Asthma										
Yes							86	13.1	21	7.1
No							568	86.9	276	92.9

**TABLE E2.** Association between gene expression of immunologic markers and anti-Neu5Gc IgG levels in the cross-sectional PARSIFAL study

Immunologic marker	aGMR/aOR* (95% CI)
PARSIFAL study	
IFN- $\gamma$	0.92 (0.41-2.07)
IL-13 <sup>†</sup>	0.75 (0.30-1.83)
IL-4 <sup>†</sup>	0.79 (0.47-1.35)
IL-10	1.47 (0.77-2.83)
TLR1	1.13 (0.90-1.43)
TLR2	0.92 (0.74-1.14)
TLR4_1	1.03 (0.82-1.28)
TLR4_2	1.07 (0.84-1.36)
TLR5	0.89 (0.67-1.20)
TLR6	1.01 (0.81-1.25)
TLR7	1.15 (0.91-1.47)
TLR8_1	1.12 (0.86-1.46)
TLR8_2	1.13 (0.86-1.50)
TLR9_1	1.04 (0.77-1.41)
TLR9_2	0.99 (0.76-1.29)
TLR10	0.80 (0.49-1.30)

Linear and logistic (\*) regression for the association between immunologic markers (target variable) and exposure variables is shown. Average odds ratios across tertiles of anti-Neu5Gc levels are shown.

\_1 and \_2, Isoforms of the respective gene; *aGMR*, adjusted geometric mean ratio; *aOR*, adjusted odds ratio.

\*Geometric means ratios/odds ratios adjusted for being a farmers' child, sex, and age. CIs adjusted for multiple testing by using the Bonferroni method.

<sup>†</sup>IL-13 and IL-4 were dichotomized because of the high proportion of nondetectable values.