

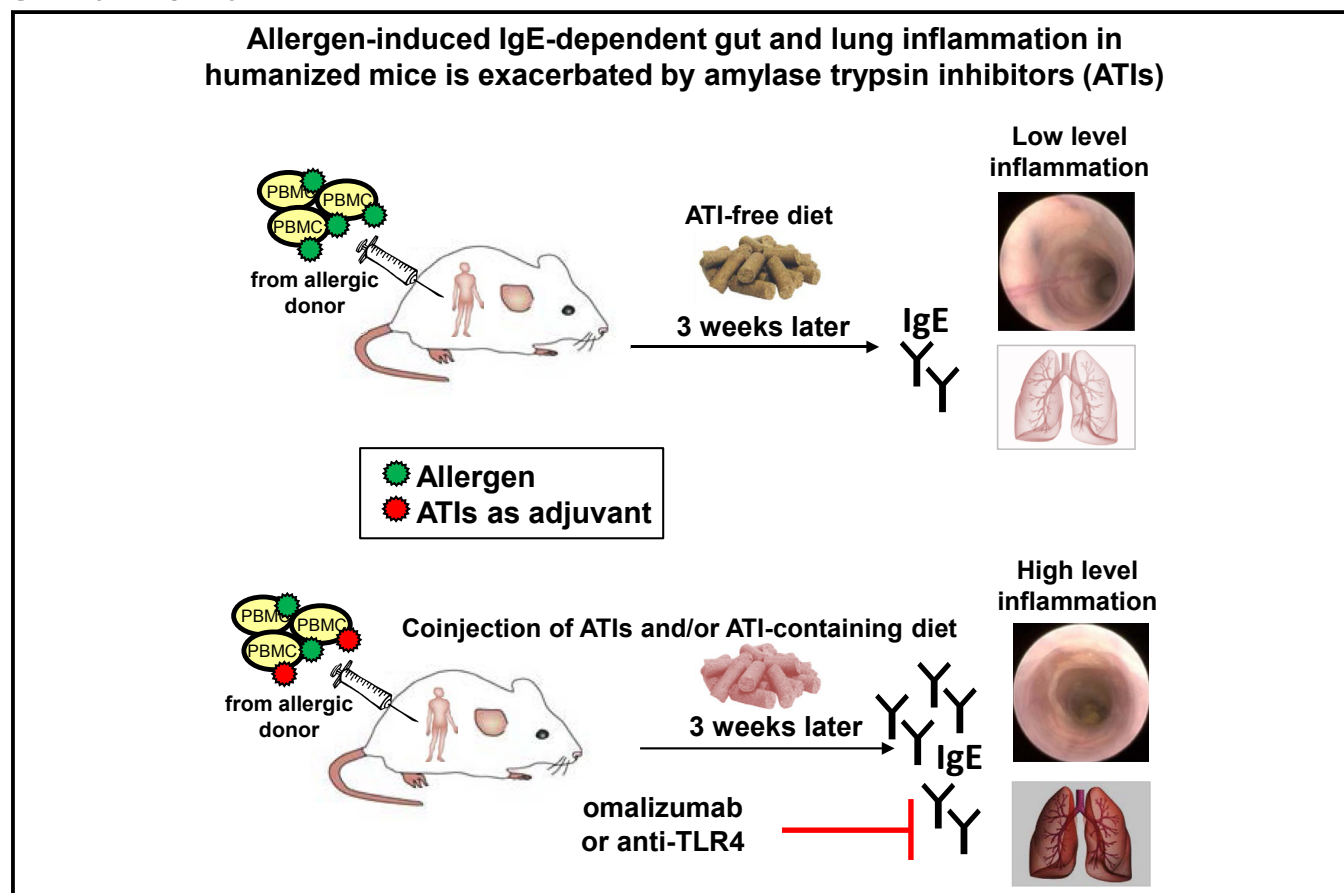
Wheat amylase-trypsin inhibitors exacerbate intestinal and airway allergic immune responses in humanized mice



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GRAPHICAL ABSTRACT



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Background: Amylase-trypsin inhibitors (ATIs) in wheat and related cereals are potent activators of myeloid innate immune cells via engagement of TLR4. Furthermore, ATIs have been shown to serve as adjuvants in experimental intestinal inflammatory diseases.

Objective: The aim of this study was to analyze whether ATIs are also modifiers of allergic inflammation.

Methods: Therefore, CD4⁺ T cells from donors sensitized to grass or birch pollen were stimulated with autologous allergen-pulsed dendritic cells in the presence or absence of ATIs or the control storage protein zein from corn. To analyze allergen-induced gut and lung inflammation, immunodeficient mice were engrafted with PBMCs from these allergic donors plus the respective allergen, and fed with selected diets. Three weeks later, inflammation was induced by rectal or intranasal allergen challenge and monitored by mini endoscopy or airway hyperreactivity, respectively.

Results: Allergen-specific T-cell proliferation and cytokine production was significantly exacerbated by ATIs and not by zein. *In vivo*, allergen-specific human IgE level was strongly elevated in sera of mice receiving an ATI-containing diet compared with mice that were fed gluten-free and thus ATI-free diet. Importantly, allergen-induced IgE-dependent colitis and airway hyperreactivity were also enhanced in ATI-fed mice. Gut inflammation was further increased in mice receiving an additional ATI injection and even detectable in the absence of the aeroallergen, whereas zein had no such effect. Injection of anti-human TLR4 mAbs or the anti-human IgE mAb omalizumab completely abolished ATI-induced allergic inflammation.

Conclusions: These results underline that wheat ATIs are important nutritional activators and adjuvants of allergy, which might be exploited for nutritional therapeutic strategies. (J Allergy Clin Immunol 2019;143:201-12.)

Key words: Humanized mice, amylase-trypsin inhibitors, allergen, IgE, colon, lung, celiac disease, gluten, wheat sensitivity

The prevalence of inhalative or food allergies and of most autoimmune diseases, including celiac disease, has greatly increased over the last few decades. While the immunological mechanisms of IgE-mediated (atopic) allergic diseases, that is, allergic rhinitis, asthma, atopic eczema, as well as intestinal inflammation during food allergy, are characterized by a decrease in allergen-specific regulatory T cells in favor of T_H2 cells,¹ celiac disease, another food sensitivity, is dominated by an HLA-DQ2– or HLA-DQ8–restricted intestinal T_H1 T-cell response toward ingested gluten peptides from wheat, barley, and rye and a resultant autoimmune response to the autoantigen tissue transglutaminase.²⁻⁴ Besides celiac disease and wheat allergy, a third form of gluten-related diseases exists, the nonceliac (nonallergy) wheat sensitivity (NCWS). Patients with NCWS have intestinal complaints such as diarrhea, obstruction, or pain, or extraintestinal symptoms such as headache, joint pain, fatigue, depression, or worsening of preexistent chronic diseases after eating gluten-containing foods, which improve on a gluten-free diet. NCWS can be diagnosed only when wheat allergy (wheat-specific IgE antibodies and skin reactions) and celiac disease (HLA-DQ2/DQ8, or intestinal inflammation, villous atrophy, crypt hyperplasia, and positive autoantibodies to tissue transglutaminase after long-term gluten consumption) have been ruled out.⁵⁻⁷ Our recent data

Abbreviations used

AHR: Airway hyperreactivity
APC: Antigen-presenting cell
ATI: Amylase-trypsin inhibitor
DC: Dendritic cell
IMDM: Iscove modified Dulbecco medium
MCP-1: Monocyte chemotactic protein-1
NCWS: Nonceliac (nonallergy) wheat sensitivity

indicate that NCWS is an innate immune reaction to wheat caused by the protein class of amylase-trypsin inhibitors (ATIs), whereas gluten proteins show no such activity.^{8,9} Wheat ATIs activate myeloid, antigen-presenting cells (APCs) such as monocytes, macrophages, and dendritic cells (DCs) of the intestinal mucosa to produce inflammatory cytokines and chemokines, for example, IL-8, TNF- α , or monocyte chemotactic protein-1 (MCP-1), via engagement of TLR4.⁸ Wheat ATIs are a family of up to 17 water-soluble globulins with similar secondary but variant primary structure that display molecular weights around 15 kD as monomers and represent 2% to 4% of the wheat protein (compared with 80%-90% for gluten). ATIs may play a major role in grain maturation, but can also inhibit parasite amylase and trypsin-like activities. While other plants express similar proteins, the ATIs of only gluten-containing cereals have relevant TLR4-stimulating activity. Notably, they resist intestinal proteolytic digestion, serving as orally active TLR4 activators that are ingested in a normal, wheat-based diet, which contains an estimated 0.7 to 1.5 g of ATIs per day.^{5,6} ATIs exist as monomers, as non-covalently-linked dimers (named 0.19 or 0.54), or as tetramers (named CM2 and CM3), and the main ATI species in wheat, 0.19 and CM3, activate TLR4 at comparable levels.^{6,8,10,11} Once activated by ATIs, APCs quickly migrate to peripheral lymph nodes where they promote ongoing (adaptive) immune activation in experimental intestinal diseases.⁹ The aim of the current study was to study whether nutritional ATIs can also modify human allergen-specific immune responses *in vitro* and allergen-induced gut and lung inflammation *in vivo* using a recently developed humanized mouse model of PBMC-engrafted immunodeficient mice.¹²⁻¹⁴

METHODS

Patients

Heparinized blood was obtained from 17 allergic donors suffering from allergic rhinoconjunctivitis and/or asthma to grass or birch pollen or nonallergic healthy controls. Specific sensitization was documented by positive skin prick test result and detection of allergen-specific IgE in the sera (t3 or Gx1 CAP class ≥ 4 measured with the ImmunoCAP specific IgE blood test; Thermo Fisher Scientific, Phadia GmbH, Freiburg, Germany). Ten donors were also weakly sensitized against wheat (f4, Phadia GmbH), among them 7 against ATIs as detected by streptavidin ImmunoCAP (o212, Phadia GmbH) coupled with 20 μ g biotinylated ATIs. Biotinylation of ATIs was performed with Biotin Labeling Kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Detailed patient characteristics are presented in Table E1 in this article's Online Repository at www.jacionline.org. This study was approved by the local ethics committee. Informed consent was obtained from all blood donors before the study.

Mice and defined diets

Four- to 8-week-old male or female nonobese diabetic CB17-Prkdc^{scid}/J γ c^{-/-} mice (Jackson Laboratory, Bar Harbour, Me) were kept under specific pathogen-

free conditions in the Translational Animal Research Center of the University Medical Center of the Johannes Gutenberg University in Mainz and fed with a normal protein-containing diet. Three weeks before starting the experiment, mice were fed with a carbohydrate, lipid, and trace element defined gluten-free diet based on 20.3% milk casein as protein source. In the ATI-challenge diet, 25% of casein was replaced by commercial gluten (Sigma-Aldrich, Taufkirchen, Germany), which contains approximately 3% (wt/wt) ATIs, as determined by bioactivity assay using THP-1 macrophages, mass spectrometry, and Western blotting.⁹ Commercial gluten was used as the easiest available, defined ATI-containing protein source. As shown by us before, gluten alone (de-enriched from ATI by further salt extractions) had no TLR4 and innate immunity-stimulating effect.^{8,9} Diets were prepared by Sniff (Soest, Germany). All experiments were performed in accordance with current federal, state, and institutional guidelines (authorization no. 23 177-07/G 13-1-011).

Isolation of ATIs from wheat

ATIs were isolated as described previously.⁹ Briefly, full-kernel flour was sieved (250 μ m) and defatted with methanol/diethylether, followed by centrifugation. The dried pellet was extracted with 50 mM ammonium bicarbonate, 0.3 mol NaCl, pH 7.8, at 4°C yielding approximately 80% total ATI protein in the supernatant, which was fractionated with ammonium sulfate. The protein fraction precipitating between 1.8 and 4 mol was redissolved and extensively dialyzed against 10 mM ammonium bicarbonate, cleared by centrifugation, filtered through a 0.22- μ m membrane, and freeze-dried, to yield an approximately 60% preparation with mainly isoforms CM3, 0.19, and 0.28, as determined by quantitative Western blotting using ATI isoform-specific antibodies and mass spectrometry. If needed, full purification was performed by HPLC as described previously.⁹ All ATI batches were tested for endotoxin content, which was substantially below the endotoxin-free limit of 0.1 ng/mg as measured by the LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Darmstadt, Germany).⁹

Generation of monocyte-derived allergen-pulsed DCs

PBMCs were isolated from heparinized blood by Ficoll-Paque 1.077 g/mL (Biochrom GmbH, Berlin, Germany) density centrifugation. To enrich CD14⁺ monocytes, 5×10^6 PBMCs per well were incubated for 45 minutes in a 12-well plate (Greiner, Frickenhausen, Germany) in Iscove modified Dulbecco medium (IMDM) containing L-glutamine and 25 mM HEPES (Lonza, Basel, Switzerland) supplemented with an antibiotic-antimycotic solution containing 100 μ g/mL streptomycin, 100 U/mL penicillin, and 250 ng/mL amphotericin B (Sigma-Aldrich) and 3% heat-inactivated autologous plasma at 37°C. After washing the nonadherent cells with prewarmed PBS, the remaining monocytes (purity, >90%) were incubated in IMDM (1.5 mL/well) supplemented with 1% heat-inactivated autologous plasma, IL-4 (1000 U/mL; Miltenyi Biotec, Bergisch Gladbach, Germany), and GM-CSF (200 U/mL; Leukine, Immunex Corp, Seattle, Wash). On day 6, the resulting immature DCs were pulsed with birch pollen or grass pollen allergen extract (10 μ g/mL; ALK-Abello, Hamburg, Germany), HPLC-purified ATIs (20 μ g/mL), or the alcohol-soluble storage protein zein from corn (20 μ g/mL; Sigma-Aldrich), and further stimulated with TNF- α (1000 U/mL; Miltenyi Biotec), IL-1 β (2000 U/mL; Miltenyi Biotec), and prostaglandin E₂ (1 μ g/mL; Cayman Chemical, Ann Arbor, Mich) to induce their full maturation. Mature DCs were harvested 48 hours after stimulation, washed twice, and used for T-cell stimulation assays. Mature DCs expressed more than 90% of CD80, CD83, CD86, and MHC-class II molecules as assessed by flow cytometry.

In some experiments, immature DCs were stimulated with 20 μ g/mL ATIs or zein 30 minutes after addition of 5 μ g/mL anti-human TLR4 mAb (HTA125) or the mouse IgG2a (MOPC-173) isotype control (BioLegend, San Diego, Calif) and supernatants were collected 48 hours later.

Purification of T cells

Autologous CD4⁺ T cells were obtained from human donor PBMCs or PBMC-engrafted murine spleens using antibody-coated paramagnetic MultiSort MicroBeads (MACS; Miltenyi Biotec) according to the manufacturer's protocol. Separation was confirmed by flow cytometry (purity, >98% CD4⁺ T cells).

Coculture of T cells and autologous allergen-pulsed DCs

For proliferation assays, 1×10^5 CD4⁺ T cells were cocultured in 96-well plates (Greiner) in triplicate with 1×10^4 autologous unpulsed or allergen-pulsed DCs in 200 μ L IMDM supplemented with 5% heat-inactivated autologous plasma. After 5 days, the cells were pulsed with 37 kBq/well of [³H]-thymidine (ICN, Irvine, Calif) for 6 hours, and [³H]-thymidine incorporation was evaluated in a beta counter (1205 Betaplate; LKB Wallac, Turku, Finland). For cytokine production assays, 5×10^5 CD4⁺ T cells were cocultured in 48-well plates in the presence of 5×10^4 autologous unpulsed or allergen-pulsed DCs in 1 mL IMDM supplemented with 5% heat-inactivated autologous plasma. On day 7, T cells were restimulated with 5×10^4 newly generated DCs, and supernatants were collected 24 hours later. In some experiments, ATIs or zein (50 μ g/mL) was added to the cocultures.

Engraftment of mice with PBMCs and measurement of gut inflammation after rectal allergen challenge

Unless otherwise indicated, mice were fed with gluten-free and thus ATI-free diet at least 3 weeks before starting the experiments. Then, mice were injected intraperitoneally with 1×10^7 PBMCs from donors allergic to grass or birch pollen (see Table E1) in the presence or absence of the respective allergen extract (20 μ g; ALK-Abello) and with or without the addition of ATIs or zein (100 μ g), LPS (10 ng; Sigma-Aldrich) in 500 μ L 0.9% NaCl, and, in some experiments, with or without the addition of 5 μ g anti-human TLR4 mAb (HTA125) or the mouse IgG2a (MOPC-173) isotype control (BioLegend). Mice either continued with the gluten-free diet or were fed the 25% gluten/ATI-containing diet. Eight days later, mice received an additional intraperitoneal boost of the same amount of allergen, ATI or zein, and mAbs or isotype controls in 200 μ L 0.9% NaCl. Control groups received 0.9% NaCl alone without PBMCs. On day 21, mice were anesthetized with ketamine/xylazine and examined with a high-resolution video endoscope ("Coloview system"; Karl Storz, Tuttlingen, Germany) before and 2 hours after challenge with 20 μ g allergen in 100 μ L 0.9% NaCl rectally as described earlier.¹² Briefly, a 1.9-mm outer diameter mini endoscope was introduced via the anus and the colon was carefully inflated with an air pump. Endoscopic pictures obtained are of high quality and allow the monitoring and grading of inflammation of the colon up to 4 cm (~30% of colon length). Thereafter, endoscopic scoring of 5 parameters (translucent structure, granularity, fibrin, vascularity, and stool consistency), called murine endoscopic index of colitis severity, from 0 to 3, resulting in an overall score ranging from 0 (no change) to 15 (severe colitis), was performed.¹⁵

In some experiments, mice were intraperitoneally treated with 250 μ g of the humanized anti-IgE mAb omalizumab (Xolair; Novartis, Hünigues, France) or human IgG control (Intratec; Biotest Pharma GmbH, Dreieich, Germany) in 200 μ L 0.9% NaCl 3 days and 1 hour before challenge.

Measurement of airway hyperreactivity by invasive body plethysmography

In some experiments, mice were further challenged intranasally with 20 μ L allergen in 20 μ L 0.9% NaCl on days 22 and 23. Airway hyperreactivity (AHR) was measured 24 hours later by administering increasing doses (3.125–50 mg/mL) of methacholine into pentobarbital anesthetized and intubated mice using an invasive whole-body plethysmograph (Buxco Electronics, Inc, Wilmington, NC). Data are reported as airway resistance above the baseline value taken before receiving methacholine. After measurement of AHR, lungs were lavaged via the tracheal tube with 1 mL PBS. The number of bronchoalveolar lavage cells was counted after trypan blue exclusion. Differential cell counts were made from cytocentrifuged preparations stained with DiffQuick (Dade Behring AG, Düringen, Switzerland).

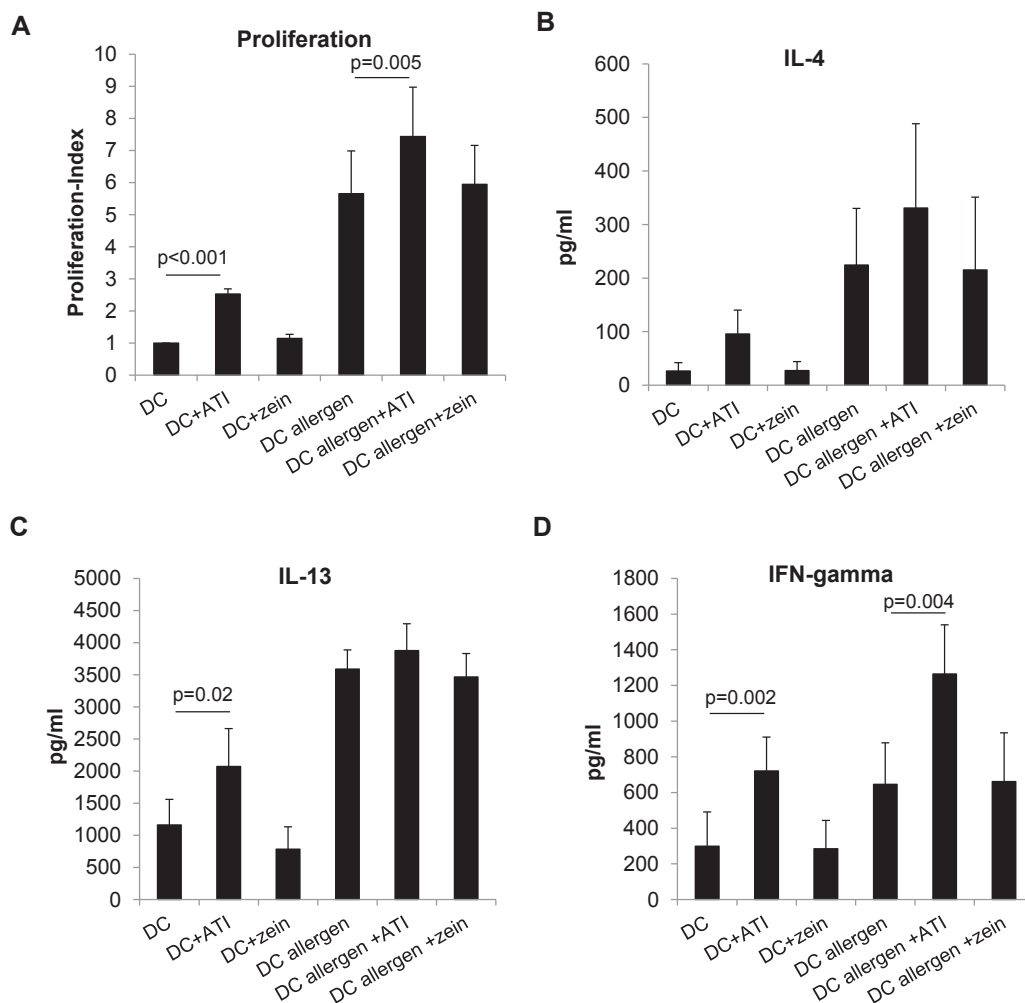


FIG 1. Enhanced proliferation and cytokine production of cocultures of CD4⁺ T cells and mature monocyte-derived DCs in the presence of ATIs but not zein. CD4⁺ T cells were cocultured with autologous mature allergen-pulsed or unpulsed DCs in the presence or absence of ATIs or zein (50 μ g/mL) for 5 days (proliferation measured by [³H]-thymidine incorporation, **A**) or for 1 week and restimulated with freshly generated DCs for 24 hours (IL-4, IL-13, and IFN- γ production measured in the supernatants by ELISA, **B-D**). Proliferation index was calculated in relation to the proliferation induced by unpulsed DCs. Shown are the means $\pm$ SEM from 12 independent experiments.

Sera collection, cell preparation, and histology

After endoscopy or measurement of AHR, sera were collected and stored at -20°C until determination of allergen-specific and total IgE level by ImmunoCAP specific/total IgE blood test (Phadia GmbH).

Then, spleens from each group were pooled and dispersed through a 70- μ m nylon cell strainer (BD Biosciences, Heidelberg, Germany). After lysis of erythrocytes, cells were washed twice with PBS and used for flow cytometric staining and isolation of human CD4⁺ T cells.

For histology, colon and lungs were removed and placed in formalin for paraffin sections. Staining was performed with hematoxylin and eosin (Sigma-Aldrich), periodic acid-Schiff (Merck, Darmstadt, Germany), or mouse anti-human CD45 (2B11 and PD7/26; DakoCytomation, Glostrup, Denmark) and visualized with the Dako REAL EnVision Detection System, Peroxidase/DAB+, Mouse/Rat (DakoCytomation) according to the manufacturer's instructions. Slides were examined with a microscope (BX40; Olympus, Hamburg, Germany) using the color view soft imaging system camera and software (Soft Imaging System GmbH, Münster, Germany). Quantification of mucus-containing cells per millimeter basement membrane was performed in the primary bronchus and

medium-sized bronchi using a microscope equipped with AxioCam ICc5 camera and ZEN 2012 (blue edition) software (Zeiss, Jena, Germany) in a blinded fashion.

Flow cytometric analysis

Surface phenotyping was performed by staining 5×10^5 CD4⁺ human T cells, 5×10^5 splenocytes, or 5×10^4 human DCs with specific mAbs for 20 minutes at 4°C . Cells were washed, and analyzed by BD Accuri C6 Flow Cytometer (BD Biosciences). The following mAbs were used: fluorescein isothiocyanate-conjugated anti-human HLA-DR (L243), and CD3 (UCHT1); phycoerythrin-conjugated anti-human CD80 (L307.4), CD83 (HB15e), CD86 (2331(FUN-1)), and CD45 (HI30), allophycocyanin-conjugated anti-human CD19 (HIB19), and mouse and rat IgG isotype controls (all BD Biosciences); Alexa Fluor 647-conjugated anti-human CD4 (MT310; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif); allophycocyanin-conjugated anti-human CD8 (MEM-31; EuroBioSciences, Friesoythe, Germany); and fluorescein isothiocyanate-conjugated anti-human FoxP3 (PCH101; eBioscience, San Diego, Calif).

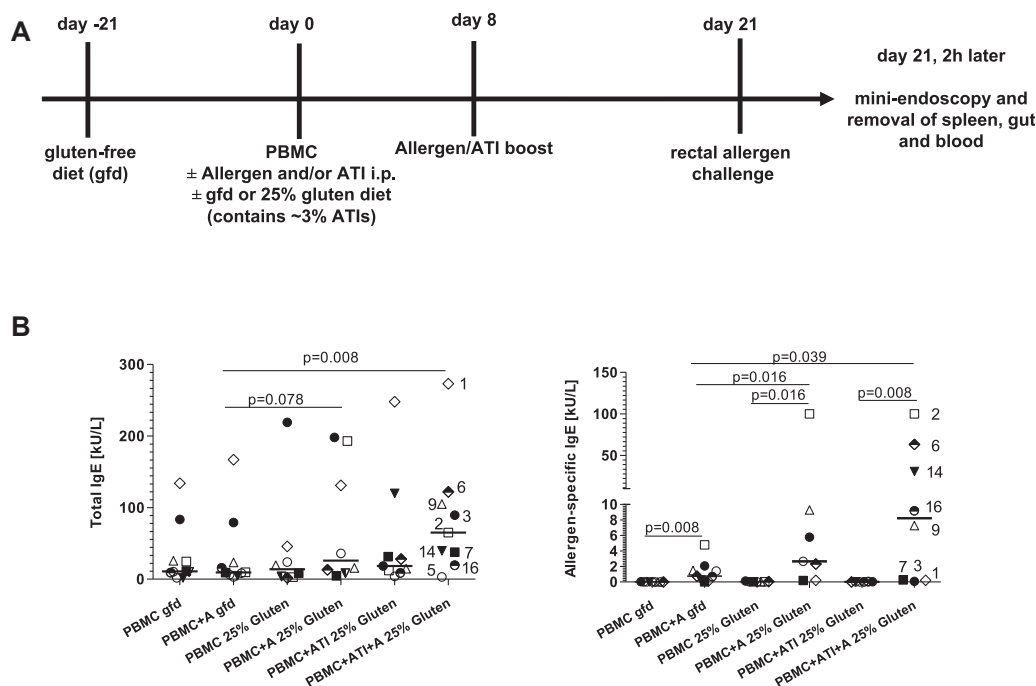


FIG 2. Enhanced total and allergen-specific human IgE production in humanized NSG mice that were fed the gluten/ATI-containing diet. **A**, Treatment scheme: Before starting the experiment, all mice received a gluten-free diet over 3 weeks. Then, 1×10^7 PBMCs from highly sensitized birch or grass pollen-allergic donors were injected i.p. with or without 20 μ g of the respective allergen $\pm$ 100 μ g ATI. Mice either continued with the gluten-free diet or received a diet in which 25% of the protein (casein) was replaced by gluten containing approximately 3% ATIs. On day 8, mice were boosted with the allergen and ATI. Two weeks later, mini endoscopy was performed 2 hours after rectal allergen challenge (20 μ g), and spleen, gut, and blood were collected. **B**, Total and allergen-specific human IgE level was measured in mouse sera by ImmunoCAP blood test. Shown are the single values + median of 9 (8 for allergen-specific IgE) independent experiments. Numbers represent the patient ID from Table E1. **A**, Allergen; **gfd**, gluten-free diet; **i.p.**, intraperitoneally; **NSG**, nonobese diabetic-severe combined immunodeficiency gamma chain knockout.

Quantification of cytokine production and ATI-specific IgE by ELISA

Human IL-1 β , IL-6, TNF- α (R&D Systems, Wiesbaden, Germany), IL-17 (BioLegend), and IL-4, IL-10, IL-12p40, IL-13, and IFN- γ (BD Biosciences) were measured by ELISA according to the manufacturer's instructions. ATI-specific IgE level was measured using 20 μ g/mL of ATIs for coating and biotinylated anti-human IgE for detection (G7-26, BD Biosciences).

Statistical analysis

ANOVA was used to determine the levels of difference between experimental groups. Student 2-tailed paired *t* test and nonparametric Wilcoxon matched-pairs signed rank test (for nonnormally distributed) were used to test the statistical significance of the results. A Tukey honest significant difference test for unequal samples was used in post hoc analysis. A *P* value below .05 was considered to indicate statistical significance. Statistical analysis was carried out in GraphPad Prism 5.0 software (GraphPad Software, La Jolla, Calif). The number of experiments is indicated at the end of each figure legend.

RESULTS

ATIs enhance immune responses in cocultures of human CD4⁺ T cells and autologous mature monocyte-derived DCs

To analyze whether ATIs can modify human allergen-specific immune responses *in vitro*, we first added either ATIs or the control storage protein zein from corn to

cocultures of CD4⁺ T cells from donors allergic to grass or birch pollen and autologous allergen-pulsed DCs or directly pulsed the DCs with ATIs or zein together with the respective allergen. In both cases, ATIs significantly enhanced allergen-specific T-cell proliferation as well as T_H1 and T_H2 cytokine production (production of IL-10 was only rarely detected), whereas zein even at higher concentrations had no effect (Fig 1, A-D, and data not shown). Interestingly, a similar ATI-related increase could also be observed when T cells were stimulated with ATI-pulsed DCs without grass or birch pollen allergen or after addition of ATIs to cocultures of CD4⁺ T cells and unpulsed DCs, illustrating the innate stimulatory capacity of ATIs *per se*. Again, this effect was not observed by the control protein zein (Fig 1, A-D).

In confirmation of our previous data in intestinal inflammation,⁸ addition of ATIs but not zein strongly activated immature DCs to produce proinflammatory cytokines in a TLR4-dependent manner (see Fig E1 in this article's Online Repository at www.jacionline.org).

Enhanced total and allergen-specific human IgE production in ATI-fed humanized mice

Next, we investigated whether ATIs also modulate allergic inflammation *in vivo* using a recently developed humanized mouse model of allergy. In this model, immunodeficient nonobese diabetic-severe combined immunodeficiency gamma chain

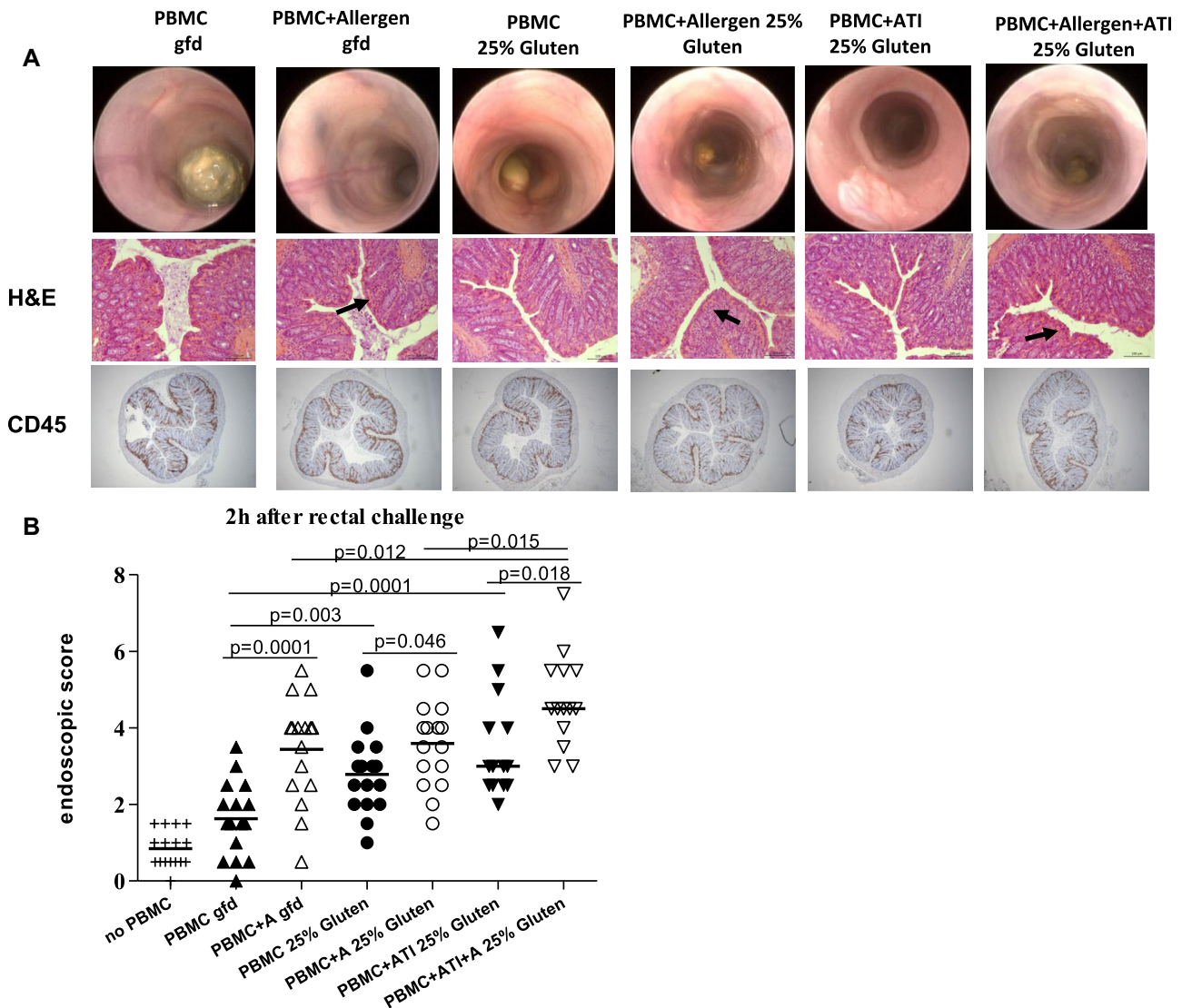


FIG 3. Enhanced allergen-induced gut inflammation in ATI-fed humanized NSG mice. Mice were treated as described in Fig 2, A. Endoscopic scoring (from 0 to 3) of 5 parameters (translucent structure, granularity, fibrin, vascularity, and stool) was performed, resulting in an overall score ranging from 0 (no change) to 15 (severe colitis). Shown are representative endoscopic pictures and the histology (hematoxylin and eosin [magnification $\times 200$] and anti-human CD45 staining [magnification $\times 40$]) (A) of colonic tissue and the overall endoscopic score (B) of 7 independent experiments with at least 2 mice per group, leading to 14 to 16 mice per data set. Black arrows show thickening of the mucosal wall. Values are expressed as single dots + mean. A, Allergen; gfd, gluten-free diet; H&E, hematoxylin and eosin; NSG, nonobese diabetic-severe combined immunodeficiency gamma chain knockout.

knockout mice are engrafted with human PBMCs from highly sensitized birch or grass pollen-allergic donors together with the respective allergen and IgE-dependent gut and lung inflammation is measured 3 weeks later after rectal or intranasal allergen challenge. Importantly, transfer of PBMCs from nonallergic or only weakly sensitized allergic donors does not lead to human allergen-specific IgE production and subsequent inflammatory responses of the intestine or the lung.^{12,13} In the present study, all mice received a lipid and carbohydrate defined, gluten-free (milk casein as protein source) and thus ATI-free diet at least 3 weeks before PBMC injection. Then, mice either continued with the gluten-free diet or were fed the same diet where 25% of the protein was replaced by commercial gluten (that contains ~3% ATIs). Some mice were additionally treated with ATIs by

intraperitoneal injection (Fig 2, A, and Fig 4, A). When analyzing total and grass or birch pollen-specific human IgE in mouse sera, we could demonstrate that both were significantly increased in mice receiving the ATI-containing diet in the presence or absence of additional ATI injection compared with mice that continued with the gluten-free diet. Of note, allergen-specific human IgE level was detectable only in PBMC plus allergen-treated mice (Fig 2, B).

Enhanced allergen-induced IgE-dependent gut inflammation in ATI-fed humanized mice

As previously published, allergen-induced IgE-dependent gut inflammation can be initiated by rectal allergen challenge 3 weeks after PBMC transfer from highly sensitized allergic donors and

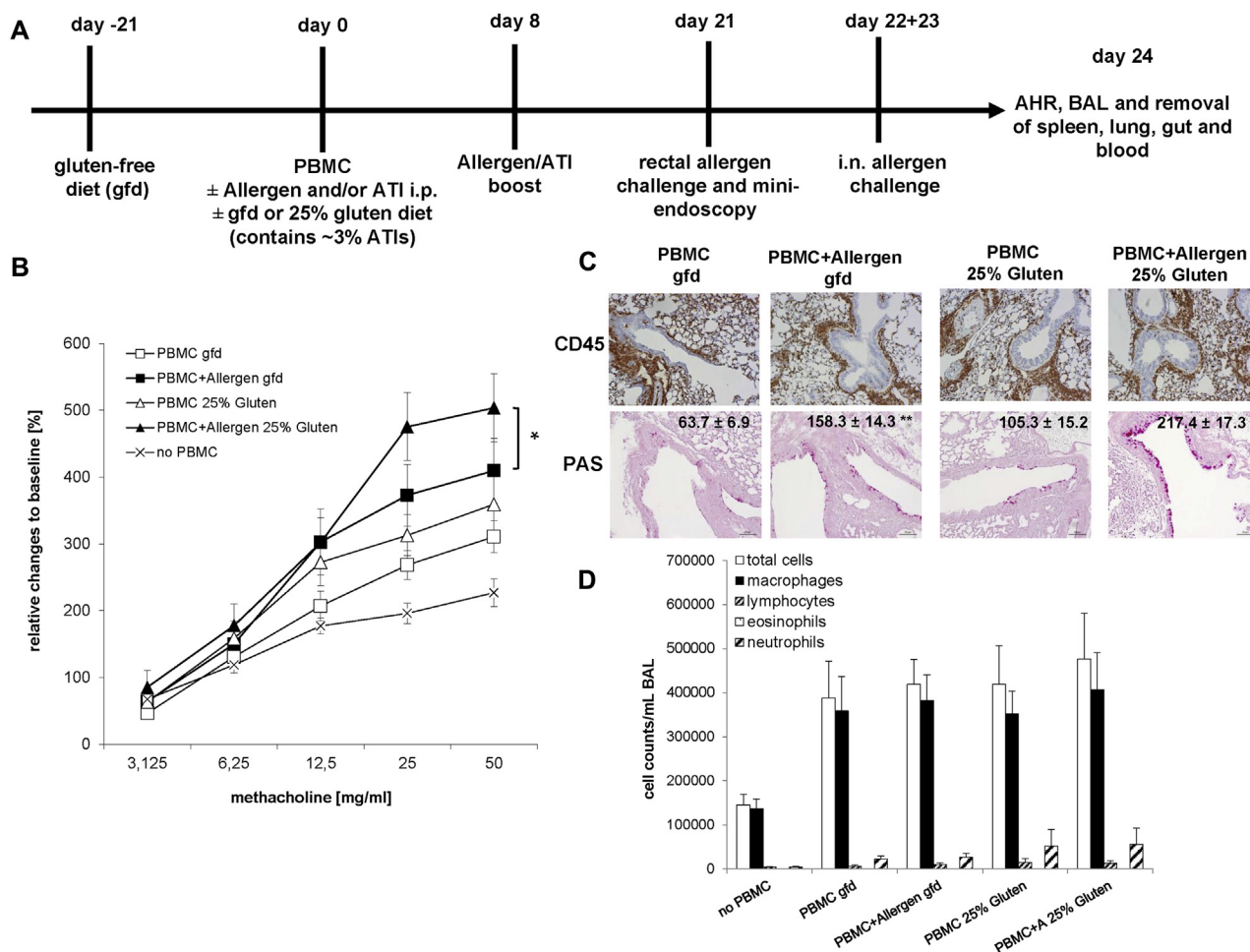


FIG 4. Increased airway hyperreactivity and lung inflammation in ATI-fed humanized mice. **A**, Experimental setup: NSG mice were treated as described in Fig 2, A. After endoscopy, mice were challenged i.n. with the respective allergen (20 μ g) on days 22 and 23, and airway resistance was measured by invasive body plethysmography 24 hours later. Shown are the means $\pm$ SEM of the relative changes to baseline value of 7 different experiments with 2 mice per group (**B**); micrographs of anti-human CD45 (magnification $\times 40$) and PAS staining (magnification $\times 200$) of a representative result; mean numbers $\pm$ SEM of mucus-producing goblet cells per millimeter basement membrane from all experiments are indicated in the upper right corner (**C**); and the means $\pm$ SEM of the number of macrophages, lymphocytes, eosinophils, and neutrophils in the BAL fluid (**D**). A, Allergen; BAL, bronchoalveolar lavage; gfd, gluten-free diet; i.n., intranasally; i.p., intraperitoneally; NSG, nonobese diabetic-severe combined immunodeficiency gamma chain knockout; PAS, periodic acid-Schiff. * $P \leq .05$, ** $P \leq .01$.

assessed by video mini endoscopy evaluating mucosal translucent structure, granularity, fibrin formation, vascularity, and stool using scores from 0 to 3, yielding a maximal score of 15.¹² Here, we could demonstrate again that no inflammation was detectable in the colon before challenge, whereas rectal allergen challenge led to a strongly enhanced endoscopic score in all mice that had been injected with PBMCs from highly sensitized birch or grass pollen-allergic donors together with the respective aeroallergen compared with mice that had received PBMCs alone or no PBMCs at all (Fig 3, A and B). The endoscopic score was significantly higher in mice fed the ATI-containing diet and treated with ATIs by intraperitoneal injection than in mice that continued with the gluten-free diet. A significantly enhanced gut inflammation was even detectable in ATI-fed mice being transplanted with PBMCs alone, but without further aggravation by additional ATI injection (Fig 3, A and B). Thus, the observed intrinsic stimulatory activity of ATIs *in vitro* could also be confirmed *in vivo*.

Histology of the colon tissue showed early inflammatory changes in the gut mucosa such as mucosal hypertrophy and enhanced wall thickening in PBMC plus allergen-treated mice, which was further augmented in ATI-treated mice. Importantly, the infiltration of the colon with human CD45⁺ cells was similar in all groups (Fig 3, A). Equal numbers of human CD45⁺, CD3⁺, CD4⁺, CD8⁺ CD19⁺, and CD4⁺ FoxP3⁺ cells were also detected in the spleen (data not shown).

Enhanced allergen-induced lung inflammation in ATI-fed humanized mice

In other mice, besides allergen-induced gut inflammation, allergen-induced lung inflammation was analyzed (Fig 4, A). Measurement of AHR after intranasal allergen challenge showed an increased airway resistance in mice engrafted with PBMCs from highly sensitized grass or birch pollen-allergic donors in

the presence of the respective allergen, whereas engraftment of mice with PBMCs alone was not sufficient to induce AHR compared with control mice receiving no human cells. Again, airway resistance was significantly higher in mice fed the ATI-containing diet (Fig 4, B), with no further rise by additional ATI injection (data not shown). Histological staining of the lungs revealed significantly enhanced mucus production and goblet cell hyperplasia in PBMC plus allergen-engrafted mice compared with mice receiving PBMCs alone (158.3 ± 14.3 vs 63.7 ± 6.9 mucus-producing goblet cells per mm basement membrane; $P < .01$), which was further elevated in ATI-fed mice (217.4 ± 17.3 vs 158.3 ± 14.3 ; $P < .05$) despite equal numbers of infiltrated human CD45⁺ cells (Fig 4, C). Finally, numbers of murine neutrophils in the bronchoalveolar lavage fluid of PBMC plus allergen-treated mice were slightly but not significantly increased in mice receiving the ATI-containing diet (Fig 4, D). As previously published, murine eosinophils and human TH2 cytokines were not detectable in the bronchoalveolar lavage of this PBMC-engrafted humanized mouse model.^{13,16}

Enhanced allergen-specific proliferation in human CD4⁺ T cells recovered from ATI-fed humanized mice

After measurement of allergen-induced gut and lung inflammation, human CD4⁺ T cells recovered from mice spleens were restimulated with autologous DCs that were pulsed with the same allergen by which the mice were challenged. Freshly isolated CD4⁺ T cells from the same donor were used as control. Fig 5 shows that grass or birch pollen-specific T-cell proliferation, which is calculated as a proliferation index related to the proliferation induced by unpulsed DCs, was significantly enhanced in ATI-fed and ATI-treated mice. Of note, allergen-specific proliferation was not detectable in CD4⁺ T cells isolated from mice receiving PBMCs alone without the respective allergen, indicating that allergen-specific T cells need the right allergen stimulation to survive in mice.

Injection of ATIs but not the control protein zein enhances gut and lung inflammation in humanized mice fed with a gluten-free diet

Finally, we compared the adjuvant function of ATIs versus the control storage protein zein from corn in mice fed a gluten-free and thus ATI-free diet during the whole experiment by injection of these proteins together with PBMCs from grass or birch pollen-allergic donors. Here, only ATIs and not zein led to an enhanced inflammation of the gut (Fig 6, A and B) and the lung (Fig 6, C) after rectal or intranasal aeroallergen challenge, which was similar to the inflammation induced by the aeroallergens themselves. A significant, but less pronounced inflammation of the gut could also be observed in ATI-treated mice engrafted with PBMCs from nonallergic donors, whereas the lungs of the same mice were not significantly affected, indicating that the adjuvant activity of ATIs is dependent on the sensitization status of the donor (Fig 6, D).

The observed effects of ATI were not due to LPS contamination because the endotoxin content of all ATI preparations was substantially below the endotoxin-free limit of 0.1 ng/mg.⁹ Furthermore, injection of 1000-fold higher amounts of endotoxin, that is, 10 ng LPS per mouse, did not lead to any inflammation

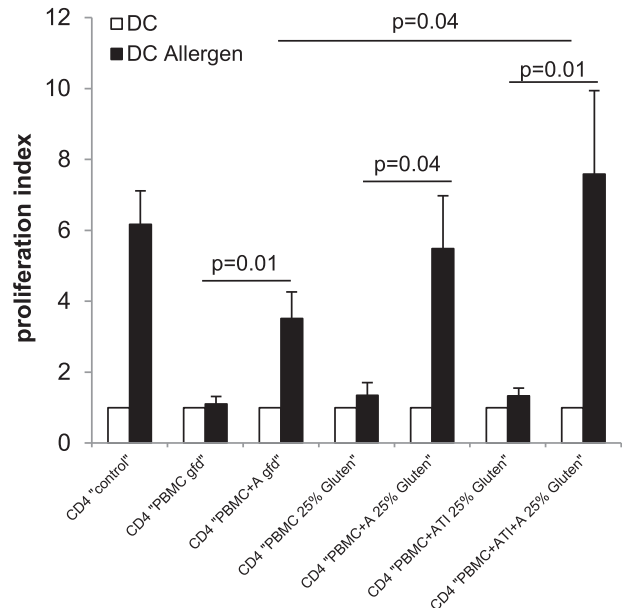


FIG 5. Enhanced allergen-specific proliferation after *in vitro* restimulation of CD4⁺ T cells recovered from humanized ATI-fed mice. NSG mice were treated as indicated in Fig 2, A, or Fig 4, A. Human CD4⁺ T cells were recovered from pooled murine spleens (at least 2 mice per group) and restimulated with autologous unpulsed DCs or allergen-pulsed DCs *in vitro*. Freshly isolated CD4⁺ T cells from the same donor were used as control (CD4⁺ "control"). The proliferation index was calculated as described in Fig 1. Shown are the means $\pm$ SEM of 10 independent experiments. A, Allergen; gfd, gluten-free diet; NSG, nonobese diabetic-severe combined immunodeficiency gamma chain knockout.

(see Fig E2 in this article's Online Repository at www.jacionline.org).

ATI-induced gut and lung inflammation is dependent on both human IgE and TLR4

We previously showed that allergen-induced gut inflammation is not mediated by murine mast cells or basophils but by human IgE and IgE-expressing effector cells, as treatment of PBMC-injected mice with the humanized anti-human IgE mAb omalizumab or depletion of human FcεR1α-expressing cells from human PBMCs before injection into mice completely decreased the endoscopic score to background levels.¹² We next investigated how far the adjuvant function of ATIs in inducing allergic intestinal and lung inflammation depends on TLR4 activation of human APCs and on human IgE expression. To achieve this aim, we injected anti-human TLR4 mAb or the respective isotype control antibody on day 0 and day 8, or administered omalizumab or IgG control to PBMC-engrafted mice 3 days and 1 hour before rectal challenge. Blockade of human TLR4 as well as inactivation of human IgE completely abolished ATI-induced gut and lung inflammation, confirming the pathogenic role both of IgE and of TLR4 activation for adjuvant activity of ATIs (Fig 7, A-F). Because all donors had no or only marginally increased ATI-specific IgE levels, ATI-specific IgE level in mouse serum was not detectable by ImmunoCAP specific IgE blood test. However, on analyzing human ATI-specific IgE level by ELISA, we could demonstrate higher OD levels in serum from ATI-treated mice being engrafted with PBMCs from those donors with the highest ATI-specific IgE levels compared with serum from ATI-

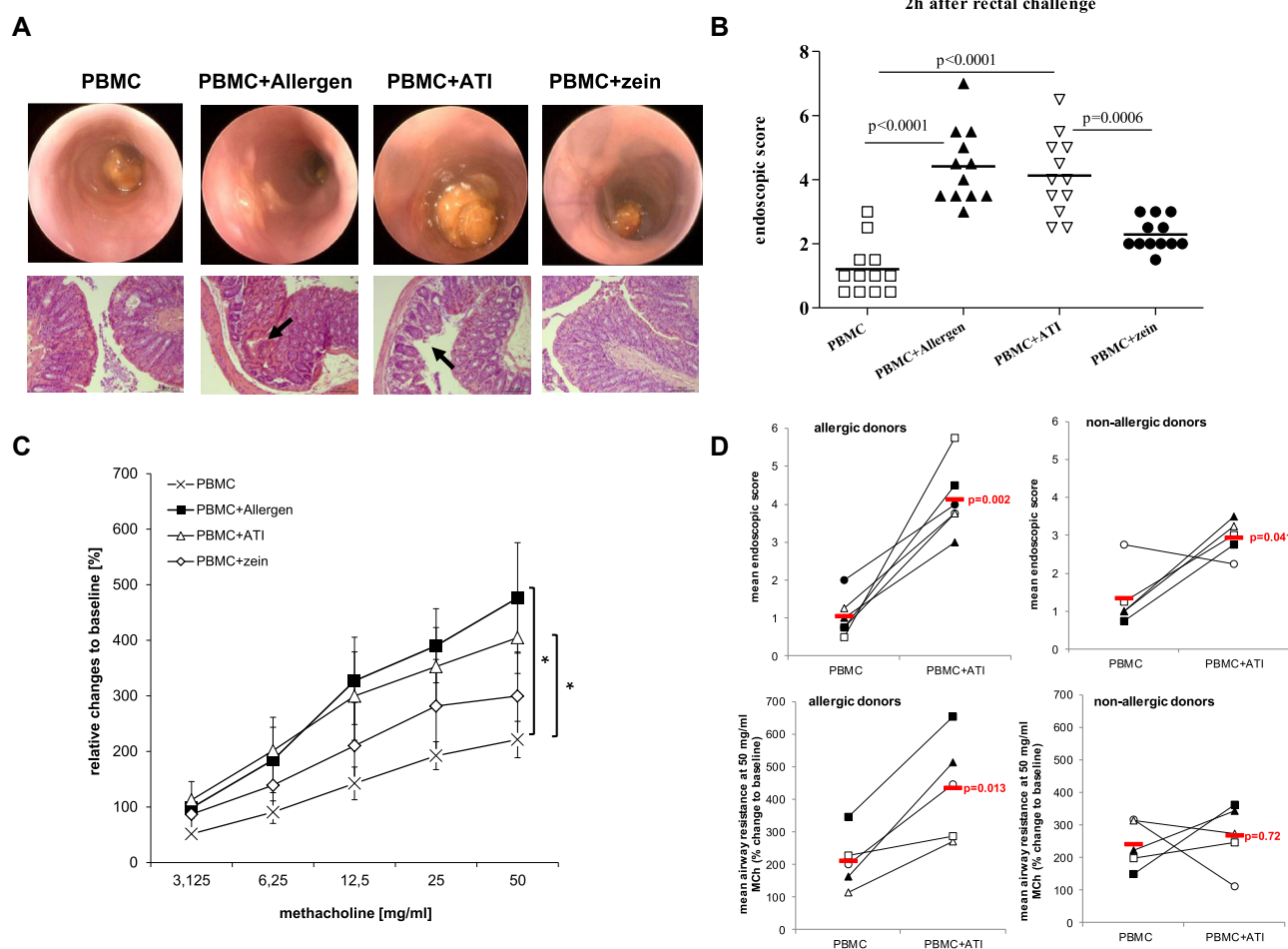


FIG 6. Injection of ATIs but not of zein leads to enhanced gut and lung inflammation in humanized mice fed with a gluten-free diet. NSG mice were treated and challenged as described in Fig 2, A, or Fig 4, A, with the exception that they continued the gluten-free diet during the whole experiment. Shown are representative endoscopic images and hematoxylin and eosin staining (magnification $\times 200$; black arrows highlighting thickening of the mucosal wall) of the colon (**A**), the single values + mean of the endoscopic score (**B**), and the means $\pm$ SEM of the airway resistance (**C**) of 6 (5 for Fig 6, C) independent experiments with 2 mice per group. **D**, The mean endoscopic scores (upper panels) and the mean airway resistance at the highest methacholine dose (lower panels) after engraftment of PBMCs from allergic versus nonallergic donors of 5 to 6 independent experiments with at least 2 mice per group. The red bar represents the mean of all experiments. MCh, Methacholine; NSG, nonobese diabetic-severe combined immunodeficiency gamma chain knockout. * $P \leq .05$.

untreated mice, which were strongly reduced after omalizumab or anti-TLR4 treatment (see Fig E3 in this article's Online Repository at www.jacionline.org).

DISCUSSION

In the present study, we have analyzed how far wheat ATIs, major components of wheat-based diets that activate the intestinal innate immune system via engagement of TLR4,^{8,9} contribute to allergen-induced allergic inflammation. We demonstrate that ATIs exacerbated grass or birch pollen-specific immune responses in a human *in vitro* setting. Importantly, nutritional ATIs as well as a parenteral ATI challenge worsened grass or birch pollen-induced gut and lung inflammation in a humanized mouse model of PBMC-engrafted immunodeficient nonobese diabetic-severe combined immunodeficiency gamma chain knockout mice *in vivo*. Interestingly, ATIs exhibited their

immunostimulatory potential in PBMC-engrafted mice even in the absence of the respective aeroallergen. This phenomenon cannot be explained by prior findings that ATIs are known major allergens of baker's asthma,^{17,18} because it was observed in all humanized mice irrespective of the donor's sensitization status against wheat because most of the donors displayed no or only very weak IgE titers against wheat or ATIs. Moreover, ATI-induced inflammation was much lower in mice receiving PBMCs from nonallergic healthy donors, further supporting the previous data that ATIs rather serve as adjuvants of preexisting T-cell immunity and here allergic sensitization than acting through their function as an allergen.

Overall, our data are in accordance with our recent findings demonstrating that mice kept on an ATI/gluten-free diet exhibited an increased expression of proinflammatory serum cytokines/chemokines (IL-8, MCP-1, IL-6) as early as 2 hours after a single oral challenge with purified ATIs (containing mainly the

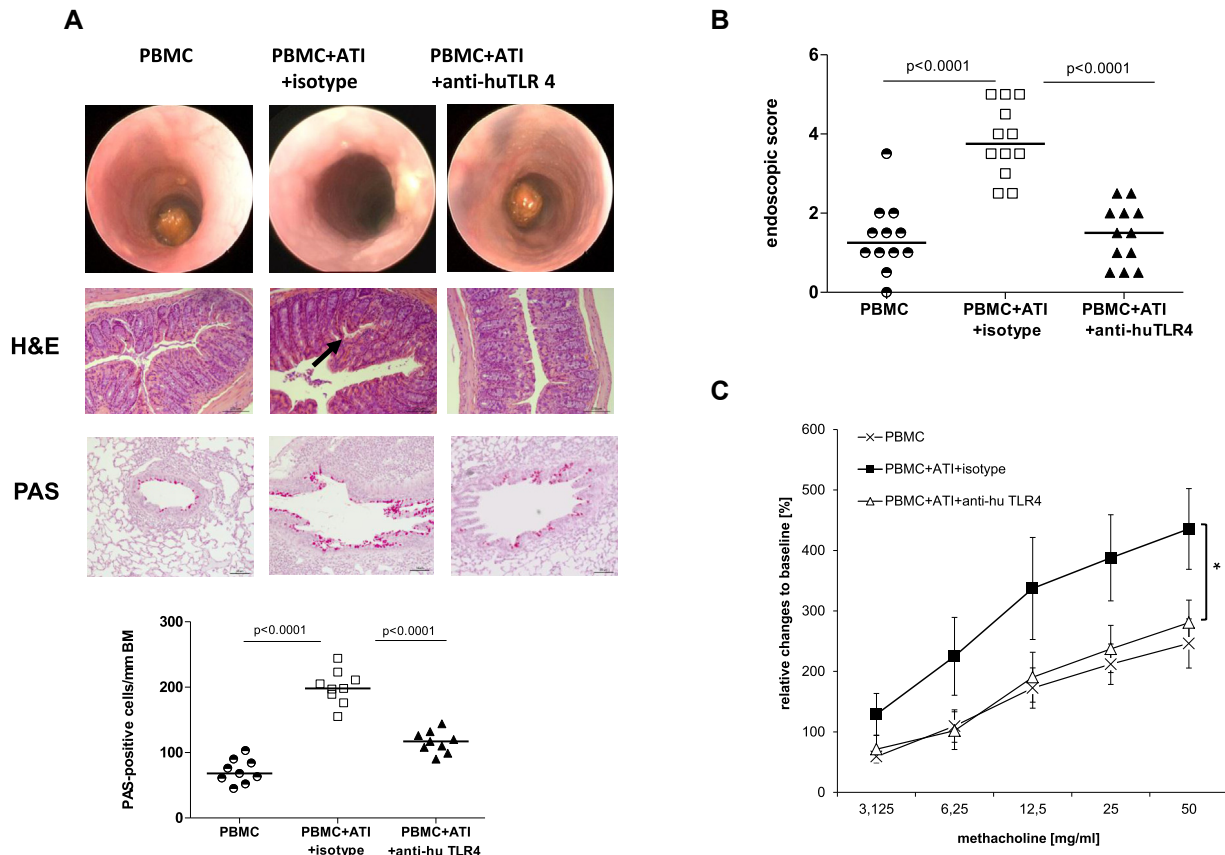


FIG 7. Inhibition of ATI-induced gut and lung inflammation by TLR4 or IgE blockade. NSG mice were treated and challenged as described in Fig 6. Anti-human TLR4 or isotype control antibodies were injected i.p. on days 0 and 8 (A-C). The humanized anti-IgE mAb omalizumab or human IgG control was administered i.p. 3 days and 1 hour before rectal allergen challenge (D-F). Shown are representative endoscopic images, hematoxylin and eosin staining (magnification $\times 200$; black arrows highlighting thickening of the mucosal wall) of the colon and PAS staining of the lung (magnification $\times 200$); and single values + mean of mucus-producing goblet cells per millimeter basement membrane (Fig 7, A and D), the single values + mean of the endoscopic score (Fig 7, B and E), and mean $\pm$ SEM of the airway resistance (Fig 7, C and F) of 5 (3 for Fig 7, C and F) independent experiments with at least 2 mice per group, leading to 10 to 12 (6-8 for Fig 7, C and F) mice per data set. i.p., Intraperitoneally; NSG, nonobese diabetic-severe combined immunodeficiency gamma chain knockout; PAS, periodic acid-Schiff. * $P \leq .05$.

prominent variants 0.19 and CM3) compared with BSA-gavaged controls. Equally, innate cytokines and chemokines (IL-1 β , IL-8, MCP-1, TNF- α , and IL-15) as well as adaptive (IFN- γ) proinflammatory genes were upregulated up to 24 hours after ATI gavage in all sections of the intestine, which was accompanied by an increase in activated F4/80 $^{+}$ MHCII $^{+}$ macrophages and CD11c $^{+}$ CD86 $^{+}$ CXCR1 $^{-}$ CD103 $^{+}$ DCs in the lamina propria.⁹ This study also showed that nutritional ATIs that are largely resistant to intestinal proteolysis had an adjuvant effect on preexisting intestinal inflammation induced by poly:IC or dextran sodium sulfate-induced colitis.⁹ Conversely, we could further show that a gluten-free (and thus ATI-free) diet prevented recruitment of eosinophils and AHR in ovalbumin-sensitized and ovalbumin-challenged mice (Zevallos V, unpublished data).

On analyzing the mechanisms of the enhanced allergic inflammation induced by ATIs in more detail, we demonstrated that this effect could be abolished by blocking human TLR4 (the ATI receptor on intestinal myeloid cells) and by inactivation of human IgE using the humanized anti-human IgE mAb omalizumab. Both treatments also inhibited human ATI-specific IgE in

mouse sera, if present, but without changing total IgE levels (data not shown). We have previously demonstrated the exclusive dependence of allergen-induced gut inflammation on human IgE and IgE-expressing effector cells in the context of aeroallergens using the same model while murine mast cells or basophils might play a role only as bystanders.¹² Patients with wheat (aero-) allergies display IgE-binding capacity also for ATIs, and blockade of human Fc ϵ RI α -expressing cells similarly abolished ATI-induced inflammation (data not shown),^{19,20} but this activity might be irrelevant in the present study, because here the adjuvant effect of ATIs on unrelated (pollen) allergies was clearly due to their TLR4-activating activity, as demonstrated by blocking human TLR4 in our humanized allergy model *in vivo*. Moreover, the fact that ATI-induced inflammation was comparably pronounced in all humanized mice, including those receiving PBMCs from aeroallergen-sensitized donors with no sensitization against ATIs, and comparably inhibited by anti-human TLR4 or omalizumab, suggests that mechanisms other than IgE binding might be involved, which are currently under investigation. Whether TLR4 from murine epithelial cells also contributes to

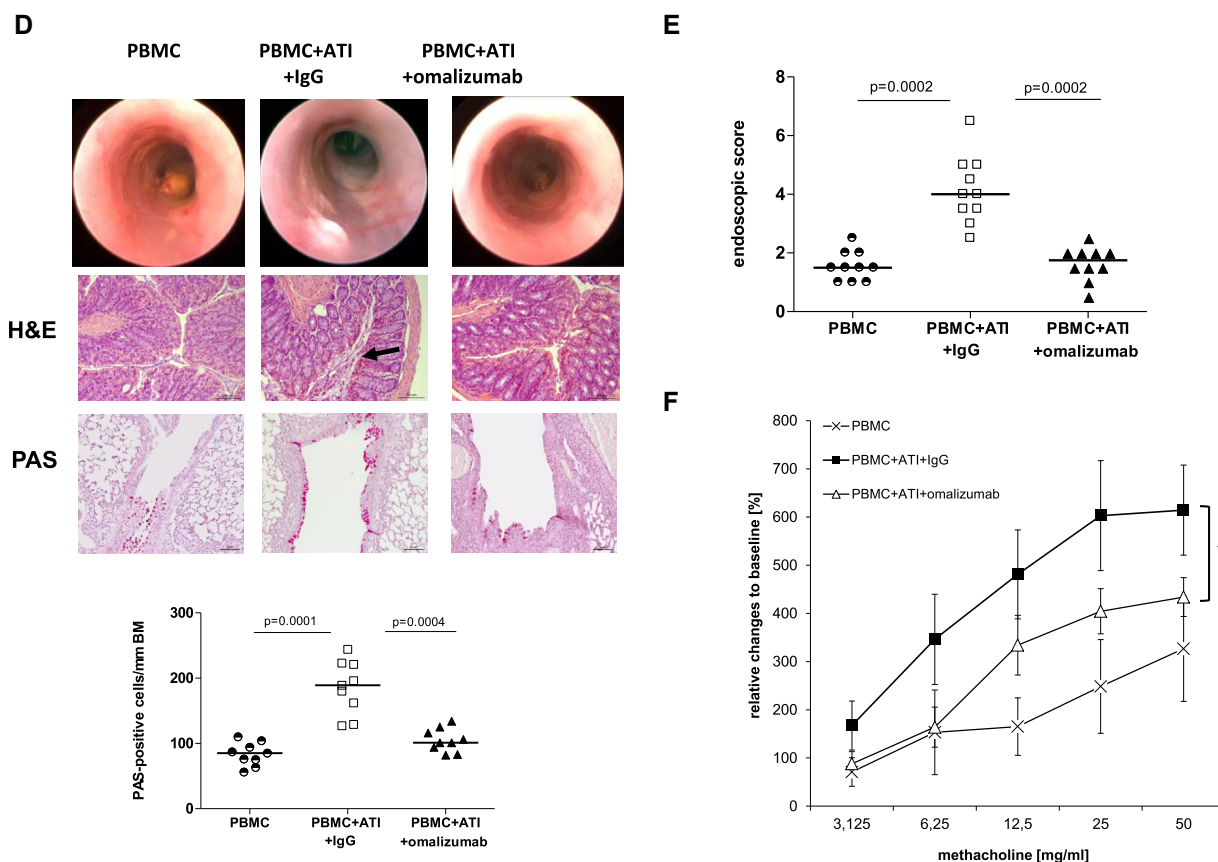


FIG 7. (Continued).

ATI-induced inflammation further remains to be investigated in future studies. However, TLR4 expression on mucosal epithelia is minimal compared with myeloid cell TLR4 expression, and if expressed mainly located in intracellular compartments.^{8,21} Omalizumab was equally effective in abrogating the *in vivo* allergic responses with or without ATI costimulation, because this humanized antibody not only binds circulating free IgE but is also associated with a marked decrease in epithelial and subepithelial cells expressing FcεRI and IL-4, as well as of submucosal B cells, and CD3⁺, CD4⁺, and CD8⁺ T lymphocytes.²² In this line, anti-IgE therapy has been reported to be even effective in intrinsic asthma,²³ suggesting that omalizumab may also modulate innate (adaptive) immunity and thus ATI-activated innate immune cells. In addition, recent clinical studies have shown that omalizumab may also contribute to favorable airway remodeling by inhibiting the release of proinflammatory cytokines.^{24,25}

Our finding about the central involvement of TLR4 in ATI-boosted allergic colitis is in agreement with our previous studies in which genetic deletion of TLR4 or the use of a TLR4-blocking mAb or of TLR4-deficient (but, eg, not TLR2-deficient) myeloid cells did not lead to ATI-induced upregulation of proinflammatory cytokines and maturation-dependent surface markers on monocytes, macrophages, and human monocyte-derived DCs.⁸ But, anti-TLR4 treatment did not completely abrogate proinflammatory cytokine production (Fig E1), which may be due to incomplete blocking of the ATI-TLR4 interaction, which is different from the classical LPS-TLR4 interaction by engaging a different interaction site in TLR4.²⁶ Furthermore, *in vitro* digestion and studies in ATI-fed wild-type mice showed

an increased production of proinflammatory cytokines in the small intestinal and colonic mucosa, confirming the resistance of ATIs to gastric and enteric proteases.⁸ Although we did not study the role of ATIs as allergens, they share an interesting property with the major house dust mite allergen Der p 2, which structurally and functionally resembles myeloid differentiation protein-2, which is needed for TLR4 and CD14 cosignaling.^{27,28} Other lipid-binding allergens such as Fel d or Can f enhance TLR2 and TLR4 signaling by formation of complexes between the bacterial lipids and the allergen.²⁹⁻³¹ Allergen-lipid complexes may also facilitate the passage through the intestinal epithelial barrier and thus enhance the allergenicity of a protein. Furthermore, some dietary lipids have been shown to act as adjuvants when used in combination with specific allergens by activating innate immunity and subsequent allergen-specific immune responses.³⁰

Activation of APCs by wheat gluten preparations was shown before, but this was exclusively reported in the context of celiac disease, a disease with a potent adaptive immune response to defined gluten peptides.³²⁻³⁸ Moreover, in regard to innate immunity these studies focused mainly on intestinal epithelial cell cultures as responding cells and did not identify a putative gluten receptor. Importantly, the authors largely used gluten or gliadin preparations, with a likely contamination by non-gluten proteins, such as ATIs. In fact, our identification of wheat ATIs but not gluten (or other nongluten proteins) as activators of classical APCs but not of intestinal epithelia via TLR4 has largely ruled out gluten as relevant innate immune stimulator. The current study extends this role of ATIs beyond

an adjuvant activity for T_H1-mediated inflammation toward an adjuvant to T_H2-mediated allergic reactions. Notably, this adjuvant effect is not limited to intestinal, for example, food allergy, but also affects extraintestinal, that is, airway allergy directed to pollen allergens. Because our study uses a humanized mouse model, with major immune cells derived from allergic patients, our results will likely be transferable to human allergies. Here, the adjuvant effect of ATIs is difficult to prove in clinical practice, because wheat is such a common staple, and patients with allergies or their doctors will not easily connect exacerbation of, for example, airway allergies to consumption of a specific common food product, such as wheat and ATIs. Thus, the identification of a defined common food component may serve as a novel paradigm of possible other food components to be identified that modulate the severity of allergies in a positive or negative way.

Finally, with the identification of the underlying molecular and immunological players, prospective, randomized, and placebo controlled clinical studies with airway or food-allergic patients and an ATI (wheat) challenge will be possible.

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Clinical implications: Allergen-induced IgE-mediated inflammation of the intestine and the lung is exacerbated by ATIs, which might be important for future therapies.

REFERENCES

- Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;133:621-31.
- Schuppan D, Junker Y, Barisani D. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology* 2009;137:1912-33.
- Schuppan D, Zimmer KP. The diagnosis and treatment of celiac disease. *Dtsch Arztebl Int* 2013;110:835-46.
- Kaukinen K, Lindfors K, Maki M. Advances in the treatment of coeliac disease: an immunopathogenic perspective. *Nat Rev Gastroenterol Hepatol* 2014;11:36-44.
- Schuppan D, Pickert G, Ashfaq-Khan M, Zevallos V. Non-celiac wheat sensitivity: differential diagnosis, triggers and implications. *Best Pract Res Clin Gastroenterol* 2015;29:469-76.
- Fasano A, Sapone A, Zevallos V, Schuppan D. Nonceliac gluten sensitivity. *Gastroenterology* 2015;148:1195-204.
- Catassi C, Bai JC, Bonaz B, Bouma G, Calabro A, Carroccio A, et al. Non-celiac gluten sensitivity: the new frontier of gluten-related disorders. *Nutrients* 2013;5:3839-53.
- Junker Y, Zeissig S, Kim SJ, Barisani D, Wieser H, Leffler DA, et al. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *J Exp Med* 2012;209:2395-408.
- Zevallos VF, Raker V, Tenzer S, Jimenez-Calvente C, Ashfaq-Khan M, Russel N, et al. Nutritional wheat amylase-trypsin inhibitors promote intestinal inflammation via activation of myeloid cells. *Gastroenterology* 2017;152:1100-13.
- Schuppan D, Zevallos V. Wheat amylase trypsin inhibitors as nutritional activators of innate immunity. *Dig Dis* 2015;33:260-3.
- Altenbach SB, Vensel WH, Dupont FM. The spectrum of low molecular weight alpha-amylase/protease inhibitor genes expressed in the US bread wheat cultivar Butte 86. *BMC Res Notes* 2011;4:242.
- Weigmann B, Schughart N, Wiebe C, Sudowe S, Lehr HA, Jonuleit H, et al. Allergen-induced IgE-dependent gut inflammation in a human PBMC-engrafted murine model of allergy. *J Allergy Clin Immunol* 2012;129:1126-35.
- Martin H, Reuter S, Dehzad N, Heinz A, Bellinghausen I, Saloga J, et al. CD4-mediated regulatory T-cell activation inhibits the development of disease in a humanized mouse model of allergic airway disease. *J Allergy Clin Immunol* 2012;129:521-8.
- Eschborn M, Weigmann B, Reissig S, Waisman A, Saloga J, Bellinghausen I. Activated glycoprotein A repetitions predominant (GARP)-expressing regulatory T cells inhibit allergen-induced intestinal inflammation in humanized mice. *J Allergy Clin Immunol* 2015;136:159-68.
- Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. *Nat Protoc* 2006;1:2900-4.
- Bellinghausen I, Reuter S, Martin H, Maxeiner J, Luxemburger U, Tureci O, et al. Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. *J Allergy Clin Immunol* 2012;130:1384-93.
- Tatham AS, Shewry PR. Allergens to wheat and related cereals. *Clin Exp Allergy* 2008;38:1712-26.
- Salcedo G, Quirce S, Diaz-Perales A. Wheat allergens associated with Baker's asthma. *Acta Allergol Clin Immunol* 2011;21:81-92.
- Simonato B, De Lazzari F, Pasini G, Polato F, Giannattasio M, Gemignani C, et al. IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion. *Clin Exp Allergy* 2001;31:1771-8.
- Theobald K, Thiel H, Kallweit C, Ulmer W, König W. Detection of proteins in wheat flour extracts that bind human IgG, IgE, and mouse monoclonal antibodies. *J Allergy Clin Immunol* 1986;78:470-7.
- McClure R, Massari P. TLR-dependent human mucosal epithelial cell responses to microbial pathogens. *Front Immunol* 2014;5:386.
- Samitas K, Delimpoura V, Zervas E, Gaga M. Anti-IgE treatment, airway inflammation and remodelling in severe allergic asthma: current knowledge and future perspectives. *Eur Respir Rev* 2015;24:594-601.
- Lommatzsch M, Korn S, Buhl R, Virchow JC. Against all odds: anti-IgE for intrinsic asthma? *Thorax* 2014;69:94-6.
- Mauri P, Riccio AM, Rossi R, Di Silvestre D, Benazzi L, De Ferrari L, et al. Proteomics of bronchial biopsies: galectin-3 as a predictive biomarker of airway remodelling modulation in omalizumab-treated severe asthma patients. *Immunol Lett* 2014;162:2-10.
- Riccio AM, Dal Negro RW, Micheletto C, De Ferrari L, Folli C, Chiappori A, et al. Omalizumab modulates bronchial reticular basement membrane thickness and eosinophil infiltration in severe persistent allergic asthma patients. *Int J Immunopathol Pharmacol* 2012;25:475-84.
- Cuccioloni M, Mozzicafreddo M, Bonfili L, Cecarini V, Giangrossi M, Falconi M, et al. Interfering with the high-affinity interaction between wheat amylase trypsin inhibitor CM3 and toll-like receptor 4: in silico and biosensor-based studies. *Sci Rep* 2017;7:13169.
- Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergen-induced functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;457:585-8.
- Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410-6.
- Herre J, Gronlund H, Brooks H, Hopkins L, Waggoner L, Murton B, et al. Allergens as immunomodulatory proteins: the cat dander protein Fel d 1 enhances TLR activation by lipid ligands. *J Immunol* 2013;191:1529-35.
- Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* 2014;134:521-9.
- Lambrecht BN, Hammad H. Allergens and the airway epithelium response: gateway to allergic sensitization. *J Allergy Clin Immunol* 2014;134:499-507.
- Nikulina M, Habich C, Flohe SB, Scott FW, Kolb H. Wheat gluten causes dendritic cell maturation and chemokine secretion. *J Immunol* 2004;173:1925-33.
- Cinova J, Palova-Jelinkova L, Smythies LE, Cerna M, Pecharova B, Dvorak M, et al. Gliadin peptides activate blood monocytes from patients with celiac disease. *J Clin Immunol* 2007;27:201-9.
- Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, et al. Association between innate response to gliadin and activation of pathogenic T cells in celiac disease. *Lancet* 2003;362:30-7.
- Kim SM, Mayassi T, Jabri B. Innate immunity: actuating the gears of celiac disease pathogenesis. *Best Pract Res Clin Gastroenterol* 2015;29:425-35.
- Abadie V, Jabri B. IL-15: a central regulator of celiac disease immunopathology. *Immunol Rev* 2014;260:221-34.
- Barone MV, Troncone R, Auricchio S. Gliadin peptides as triggers of the proliferative and stress/innate immune response of the celiac small intestinal mucosa. *Int J Mol Sci* 2014;15:20518-37.
- Rakhimova M, Esslinger B, Schulze-Krebs A, Hahn EG, Schuppan D, Dieterich W. In vitro differentiation of human monocytes into dendritic cells by peptic-tryptic digest of gliadin is independent of genetic predisposition and the presence of celiac disease. *J Clin Immunol* 2009;29:29-37.

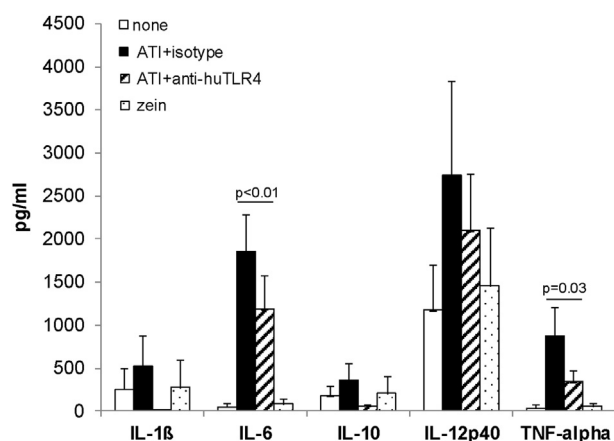


FIG E1. ATIs induce TLR4-dependent proinflammatory cytokines in immature DCs. On day 6 of DC culture, immature DCs were treated with 5 μ g/mL function-blocking anti-human TLR4 or isotype control antibodies 30 minutes before stimulation with 20 μ g/mL ATIs or zein. Supernatants were collected 48 hours later and analyzed for IL-1 β , IL-6, IL-10, IL-12p40, and TNF- α by ELISA. Shown are the means $\pm$ SEM of 7 independent experiments.

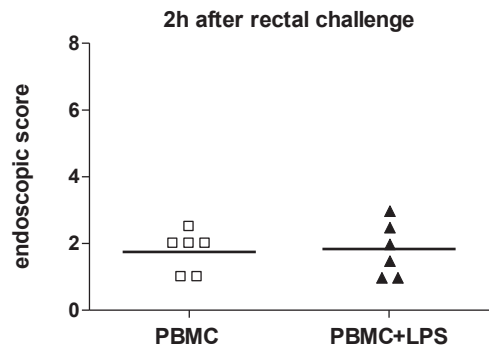


FIG E2. ATI-induced gut inflammation is not due to endotoxin contamination. NSG mice were transplanted with PBMCs as described in Fig 2, A, in the presence or absence of 10 ng LPS (equivalent to 1000-fold higher endotoxin content than measured in the ATI preparations). Shown are the single values + mean of the endoscopic score of 3 independent experiments. NSG, Nonobese diabetic-severe combined immunodeficiency gamma chain knockout.

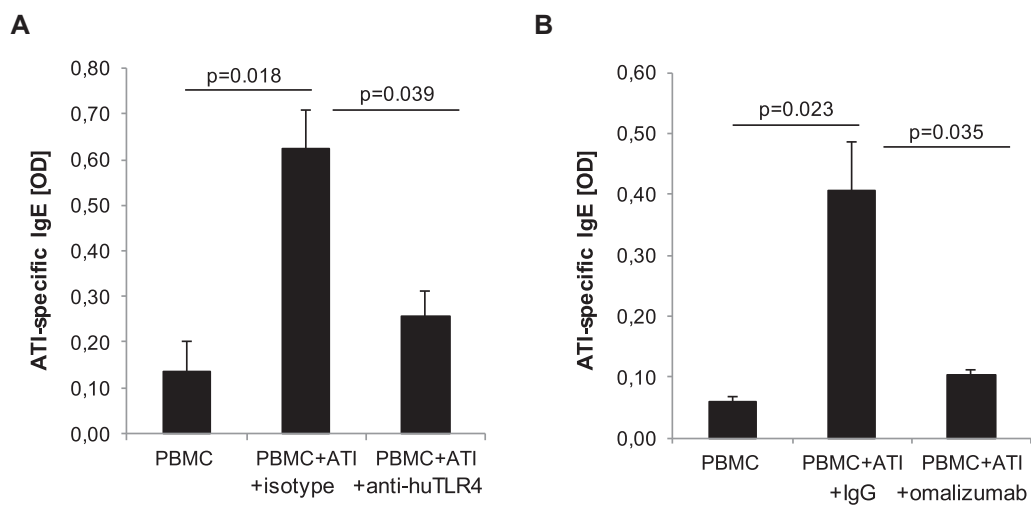


FIG E3. Inhibition of ATI-specific IgE by anti-human TLR4 or omalizumab. NSG mice were treated and challenged as described in Fig 7 in the presence of anti-human TLR4 or isotype control antibodies (**A**) or humanized anti-IgE mAb omalizumab or human IgG control (**B**). ATI-specific human IgE level was measured in mouse sera by ELISA. Shown are means $\pm$ SEM of 4 (3 for Fig E3, **B**) independent experiments. *NSG*, Non-obese diabetic-severe combined immunodeficiency gamma chain knockout.

TABLE E1. Characteristics of allergic donors

ID	Sex	Age (y)	Total IgE (kU/L)	Birch-specific IgE (kU/L)	Grass-specific IgE (kU/L)	Wheat-specific IgE (kU/L)	ATI-specific IgE (kU/L)
1	F	33	852	>100	58.5	0.6	0.3
2	M	29	268	13.6	55.5	2.85	3.02
3	F	28	910	96.3	>100	1.12	0.66
4	F	26	316	50.0	23.5	0.31	0.11
5	F	46	468	13.1	29.2	0.36	0.07
6	M	29	445	77.1	94.3	2.24	0.56
7	F	28	929	48.2	5.03	0.2	0.16
8	F	35	3268	>100	>100	1.25	0.87
9	M	31	143	86.8	0.28	0.17	0.07
10	F	26	421	63.7	>100	0.56	0.15
11	M	31	418	9.22	22.9	3.98	2.09
12	M	27	1698	14.1	>100	6.12	4.14
13	F	30	92.5	0.9	18.7	0.1	0.04
14	M	31	186	25.7	>100	0.13	0.04
15	M	29	939	>100	>100	3.05	0.66
16	F	50	112	32.4	3.71	0.05	0.01
17	F	23	242	0.86	80.2	0.09	0.04

F, Female; M, male.