

The vast majority of participants had no missing covariate data. Seven covariates had 5 or fewer missing data, which we imputed with the most common category or mean value. Organic dairy consumption was imputed for 19 children by using the categories predicted by using a regression model based on prior dietary and lifestyle information.

Our results indicate that the presence of *M. stadtmanae* is associated with a lower risk of asthma at 6 to 10 years of age (adjusted odds ratio [OR], 0.32; 95% CI, 0.08–0.98; $P = .045$; Table I). Furthermore, the risk of asthma showed a monotonic decrease over the 3 categories of *M. stadtmanae* abundance (adjusted $P = .036$ for linear trend over the *M. stadtmanae* abundance categories, Table II). No significant interaction was found between *M. stadtmanae* exposure and parental asthma history ($P = .495$), suggesting that the association does not significantly differ by parental asthma status.

For secondary outcomes, *M. stadtmanae* also appeared to be associated with a slightly lower likelihood of eczema, aeroallergen sensitization, and food allergen sensitization, but these associations did not reach statistical significance (Table I). *M. stadtmanae* was not associated with either of the continuous outcomes of total serum IgE or FEV₁/forced vital capacity z score (see Table E4 in this article's Online Repository at www.jacionline.org). We found no significant associations between *M. smithii* and any outcome (Tables I and see Table E4). We also conducted a complete case analysis that produced similar results (see Table E5 in this article's Online Repository at www.jacionline.org).

Additionally, to check whether the oversampling of asthma cases in the second sampling round had introduced any bias, we repeated the analyses with only the 372 children sampled in the first round (see Table E6 in this article's Online Repository at www.jacionline.org). Similar ORs were observed in this subgroup (eg, *M. stadtmanae* presence and asthma: OR, 0.39; 95% CI, 0.06–1.60). The lower precision observed was expected from the smaller sample size and fewer asthma cases, and this sensitivity analysis otherwise showed no evidence of any bias introduced by the oversampling.

Because of the low prevalence of *M. stadtmanae* and cross-sectional study design, our results should be interpreted with caution. Although bacterial species not examined in our analysis might confound this association, we speculate that a mechanistic link between *M. stadtmanae* and allergic disease appears plausible. Prior evidence demonstrates the airway and gut immunogenicity of *M. stadtmanae* in mouse models and human cells.^{3,7} Epidemiologic evidence also indicates a timing-dependent relationship between asthma risk and exposure to farm environments⁸ where *M. stadtmanae* is present.⁹ However, farm exposure has also been associated with risk of atopy, which our study does not show.

Exposure to immunogenic bacterial species in infancy has been shown to be tolerogenic and reduce asthma risk.¹ Our study identifies an association between the presence of *M. stadtmanae* at school age and lower asthma risk, leading us to speculate that early-life archaeal exposures might also be tolerogenic. Therefore we encourage further research into the immunologic roles of archaea.

We would like to thank Christel Driessen and Erik Beuken for their technical assistance with quantitative PCR analyses.

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The microbiological analysis was partly supported by TI Food and Nutrition. Funding for data collection was received from the Netherlands Organisation for Health Research and Development (grant 2100.0090), the Netherlands-America Foundation (grants 3.2.03.48, 3.2.07.022), Stichting Astma Bestrijding, Netherlands Heart Foundation (grant 2008B112), Royal Friesland Foods (currently FrieslandCampina), the Netherlands Sugar Foundation, and the Netherlands Ministry of Economic Affairs.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Available online February 20, 2019.
<http://dx.doi.org/10.1016/j.jaci.2019.02.009>

Prophylactic allergen immunotherapy with Der p 2 prevents murine asthma by regulating lung GM-CSF



To the Editor:

The prevalence of allergic diseases is increasing, urging new ways of prevention. Allergen immunotherapy (AIT) is currently the only clinical intervention that can alter the natural course of allergy and can offer long-term clinical benefit. Although AIT is traditionally used as a treatment in patients with established disease, the National Institutes of Health's Immune Tolerance Network has proposed that prophylactic AIT could also be used as a primary prevention for new sensitizations and allergic disease in high-risk children born to atopic parents and with a personal

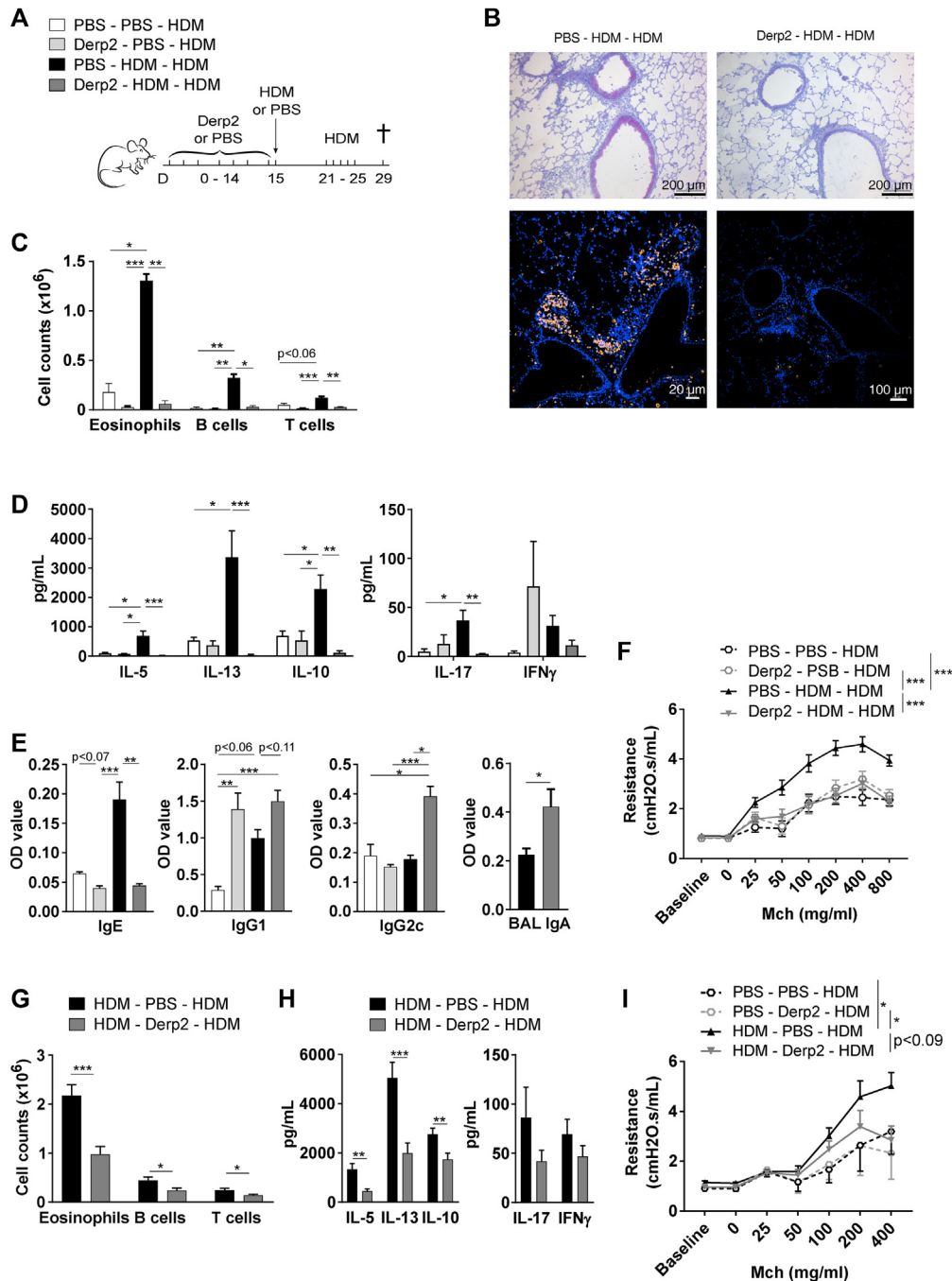


FIG 1. Prophylactic Der p 2 inhalation prevents HDM-induced allergic asthma development. **A**, Experimental setup for Fig 1, B-F. **B**, Mucus (purple, upper panel) and eosinophil (yellow, lower panel) production in the lungs. **C**, Immune cells in BAL fluid. **D**, Cytokine production by HDM-restimulated LDLN cells. **E**, Immunoglobulins in serum (IgE, IgG $_1$, and IgG $_{2c}$) or BAL fluid (IgA). **F**, Airway resistance in response to methacholine (Mch). **G-I**, Secondary prevention setting. Fig 1, G, Immune cells in BAL fluid. Fig 1, H, Cytokine production by HDM-restimulated LDLN cells. Fig 1, I, Airway resistance in response to Mch. Results are representative of 1 (Fig 1, B), 2 (Fig 1, E), 4 (Fig 1, D), or 6 (Fig 1, C) independent experiments or pooled from 2 (Fig 1, F and I) or 3 (Fig 1, G and H) independent experiments, with 4 to 7 (Fig 1, B-F) or 3 to 6 (Fig 1, G-I) mice per group for single experiments. Data are shown as means $\pm$ SEMs or in Fig 1, F and I, as predictions of means $\pm$ SEM obtained from repeated measurement analysis by using residual maximum likelihood. * $P < .05$, ** $P < .01$, and *** $P < .001$. OD, Optical density at 650 nm/850 nm.

history of atopic dermatitis, food allergen sensitization, or both in early life.¹

AIT involves repeated administration of allergen extracts, leading to induction of an ill-defined state of systemic

allergen-specific immunologic tolerance associated with decreased symptom scores, particularly in patients with allergic rhinitis but less so in asthmatic patients.² Although current AIT involves subcutaneous or sublingual administration of crude

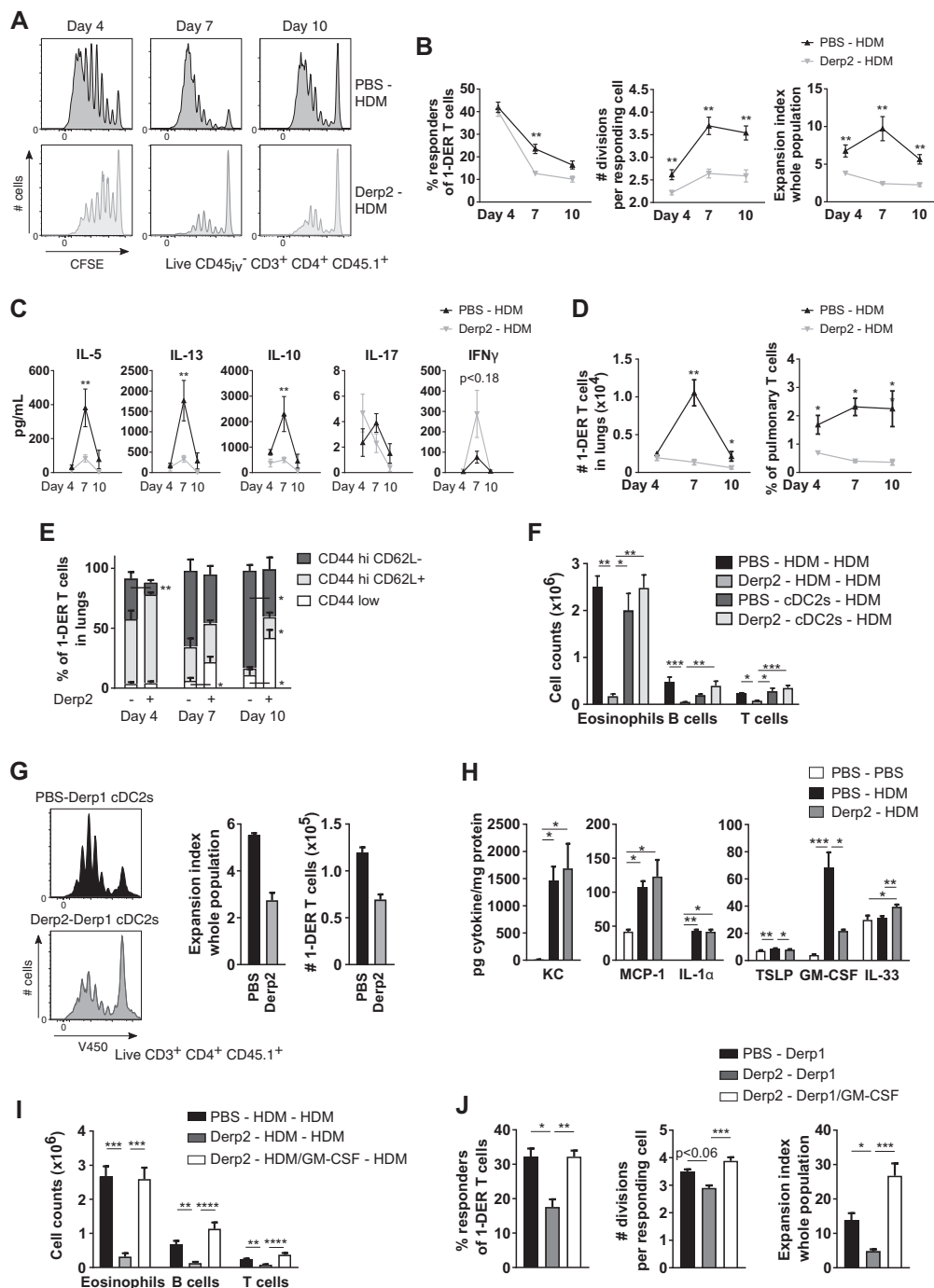


FIG 2. Prophylactic Der p 2 inhalation suppresses cDC2-mediated T_H2 responses by blocking GM-CSF release. **A-E**, Der p 2 or sham PBS mice received carboxyfluorescein succinimidyl ester-labeled 1-DER T cells intravenously and HDM intratracheally and were analyzed 4, 7, or 10 days later. Fig 2, A and B, Cell division profiles (Fig 2, A) and proliferation parameters (Fig 2, B) of tissue-resident (CD45^{intravenously injected}) 1-DER T cells in LDLNs. Fig 2, C, Cytokine production by HDM-restimulated LDLN cells. Fig 2, D and E, Numbers (Fig 2, D) and phenotype (Fig 2, E) of tissue-resident lung 1-DER T cells. **F**, Treatment as in Fig 1, A, with 2 groups of mice sensitized intratracheally with donor HDM-primed cDC2s instead of HDM. Immune cells in BAL fluid are shown. **G**, V450-labeled 1-DER T cells cocultured with LDLN cDC2s of Der p 2 or sham PBS mice instilled intratracheally with Der p 1. Cell division profiles, expansion indexes, and counts of 1-DER T cells are shown. **H**, Cytokines and chemokines in lung tissue of Der p 2 or sham PBS mice 2 hours after HDM instillation. **I**, Treatment as in Fig 1, A, with 1 group of mice sensitized intratracheally with HDM plus GM-CSF. BAL immune cells are shown. **J**, Der p 2 or sham PBS mice received carboxyfluorescein succinimidyl ester-labeled 1-DER T cells intravenously and Der p 1 with or without GM-CSF intratracheally. Proliferation parameters of LDLN 1-DER T cells are shown. Results are representative of 2 (Fig 2, F-H and J) or 3 (Fig 2, A-E) independent experiments, with 4 to 8 (Fig 2, A-F and H-J) mice per group or 2 to 3 (Fig 2, G) replicates per group for single experiments. Results are shown as means $\pm$ SEMs (or $\pm$ SDs in Fig 2, G). * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

allergen extracts, regimens based on natural routes of mucosal allergen administration and defined single allergens might improve the success rate. Several studies have suggested that the natural route of allergen exposure (eg, ingestion of peanut allergen in infants, bee stings in beekeepers, and inhalation of high doses of cat allergens in pet owners) can be very successful in preventing the onset of clinical allergies.³⁻⁵ Exposure to immunodominant allergens in these studies, such as Ara h 6 peanut allergen in breast milk, phospholipase A₂ in bee venom, and Fel d 1 cat allergen in house dust, suggests that the same allergens that can cause disease are also best at inducing tolerance through the natural route of exposure.

To improve the success rate of prophylactic AIT in asthmatic patients, we hypothesized that inhalation of Der p 2, a major immunodominant house dust mite (HDM) allergen, would prevent the onset of HDM sensitization and HDM-induced asthma. Recombinant Der p 2 was produced in the yeast *Pichia pastoris* and was administered as prophylactic AIT intranasally to naive C57Bl/6J mice every other day for a period of 15 days before intratracheal sensitization to the full HDM extract and HDM extract airway challenges (Fig 1, A). In mice treated with sham PBS AIT, HDM sensitization and challenge induced robust asthma features, including airway and tissue eosinophilia (Fig 1, B and C), goblet cell metaplasia (Fig 1, B), T- and B-cell influx in bronchoalveolar lavage (BAL) fluid (Fig 1, C), T_H2 cytokine production by lung-draining lymph node (LDLN) cells (Fig 1, D), IgE synthesis (Fig 1, E), and bronchial hyperreactivity (BHR) to methacholine (Fig 1, F). These allergic asthma features were almost completely abolished in mice that received active Der p 2 AIT (Fig 1, B-F).

To address whether intranasal Der p 2 AIT would also be effective in a more therapeutic secondary prevention setting, mice were first sensitized to HDM extract, and after 1 week, they were treated for 17 days with Der p 2 AIT, followed by HDM airway challenges. In this setting Der p 2 AIT prevented development of the salient features of asthma, among which are eosinophilia, T_H2 cytokine production in LDLNs, and BHR ($P < .09$; Fig 1, G-I).

We next attempted to determine the immunologic mechanism or mechanisms of prophylactic AIT. In the active Der p 2 AIT group, we observed increased concentrations of HDM-specific IgG_{2c} and IgG₁ in serum and of HDM-specific IgA in BAL fluid (Fig 1, E). In human subjects successful AIT is often accompanied by increased titers of IgG₁, IgG₄, and/or IgA.⁶ However, despite the increase in levels of several immunoglobulins, our prophylactic Der p 2 AIT did not require B cells because the effects of AIT on lung eosinophilia and T_H2 cytokine production were preserved in *Mbl*^{Cre} × *Rosa26-Lox-Stop-Lox*^{DTA} mice genetically lacking B cells (see Fig E1 in this article's Online Repository at www.jacionline.org).

Generation of adaptive type 2 immunity to HDM depends on allergen presentation by type 2 conventional dendritic cells (cDC2s), which bridge innate and adaptive immunity. The capacity of lung dendritic cells (DCs) to take up HDM allergen in the lungs and transport it to the LDLNs was not reduced by prophylactic Der p 2 AIT (see Fig E2, A, in this article's Online Repository at www.jacionline.org), and there were only small effects of Der p 2 AIT on expression of the costimulatory molecules CD80 and CD86 on cDC1s and cDC2s (see Fig E2, B).

To study the effect of Der p 2 AIT on functional allergen presentation by DCs, we used T-cell receptor–transgenic 1-DER

mice in which all CD4⁺ T cells react to an immunodominant peptide of Der p 1.⁷ CD4⁺ 1-DER T cells were transferred to mice previously treated with Der p 2 or sham PBS AIT, and their active division was induced by HDM extract inhalation. Four, 7, and 10 days after HDM inhalation, the LDLNs of sham-treated mice contained highly proliferating 1-DER T cells, yet proliferation was strongly reduced in mice receiving Der p 2 AIT (Fig 2, A and B). Der p 2 AIT also suppressed IL-5, IL-13, and IL-10 secretion (Fig 2, C) and *Gata3* mRNA expression (see Fig E2, C) by LDLN cells and reduced numbers of total and effector CD44⁺CD62L[−] 1-DER T cell numbers in lung tissue (Fig 2, D and E). Similar effects of Der p 2 AIT on 1-DER T-cell activity were observed when intratracheal Der p 1 was administered instead of HDM to trigger 1-DER T-cell responses (data not shown). In support of a role for Der p 2 AIT-induced suppression of cDC2 functions, we found that the adoptive transfer of *in vivo* HDM-primed cDC2s obtained from LDLNs of mice that had never been exposed to Der p 2 AIT was sufficient to break the tolerant state induced by prophylactic Der p 2 AIT (Fig 2, F). Furthermore, *vice versa*, LDLN cDC2s from mice undergoing prophylactic Der p 2 AIT and then exposed to a single HDM extract inhalation were less efficient in priming type 2 immunity on adoptive transfer to naive hosts (see Fig E2, D). In another set of experiments, cDCs were sorted from the LDLNs of sham or Der p 2 AIT mice that were primed with a single Der p 1 protein inhalation and cocultured with 1-DER T cells *ex vivo*. cDC2s from the Der p 2 AIT group induced less 1-DER T-cell proliferation than cDC2s from the sham AIT group (Fig 2, G). T-cell proliferation induced by cDC1s was not influenced by Der p 2 AIT (data not shown).

Finally, we addressed the mechanism of the reduced cDC2-mediated antigen presentation in mice treated with prophylactic Der p 2 AIT. In response to HDM inhalation, cDC2s are activated to perform this function through epithelial release of DC-instructing cytokines.⁸ Therefore we measured whether prophylaxis with Der p 2 affected release of proallergic cytokines and chemokines after a first inhalation of whole HDM allergen extract. In the sham AIT group a single dose of intratracheally instilled HDM extract led to increased production of monocyte chemoattractant protein 1, keratinocyte chemoattractant, IL-1 α , and GM-CSF in lungs compared with intratracheally instilled PBS (Fig 2, H). Prophylactic Der p 2 AIT only significantly reduced the production of GM-CSF, a cytokine that has been shown to break inhalation tolerance by activating DCs.⁹

To study the functional importance of reduced GM-CSF release in the tolerance mediated by Der p 2, mice treated with sham or Der p 2 AIT and sensitized and challenged with HDM extract were supplemented intratracheally with recombinant GM-CSF at the time of sensitization. Strikingly, Der p 2 AIT mice that received GM-CSF at the time of HDM sensitization were no longer protected from allergy development, as assessed by their development of a robust BAL eosinophilia (Fig 2, I). We found comparable results in a secondary prevention setting (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Similarly, the effect of prophylactic Der p 2 AIT on 1-DER T-cell division was also rescued by GM-CSF supplementation (Fig 2, J, and see Fig E3, B).

In conclusion, our findings in mice show that prophylactic exposure to the single immunodominant HDM allergen Der p 2 through the airways offers an alternative way to prevent

respiratory allergy to the complex allergen HDM by suppressing GM-CSF–driven activation of lung cDC2s.

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Supported by the Flanders Research Foundation (grant no. FWO13/ASP/138 to E.H.), an ERC advanced grant (AsthmaCrystalClear to B.N.L.), several FWO project grants (to B.N.L. and H.H.), and a Ghent University grant (GOA 01G02817).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Available online February 20, 2019.
<http://dx.doi.org/10.1016/j.jaci.2019.02.007>

Lymphoproliferative disease in patients with Wiskott-Aldrich syndrome: Analysis of the French Registry of Primary Immunodeficiencies



To the Editor:

Wiskott-Aldrich syndrome (WAS) is a rare X-linked primary immune deficiency caused by mutations in the WAS gene. The clinical triad of microthrombocytopenia, eczema, and immunodeficiency defines the classical WAS phenotype, whereas the so-called “attenuated form,” X-linked thrombocytopenia (XLT), manifests with thrombocytopenia alone with or without

mild eczema. An increased risk of lymphoproliferative disease (LPD) in patients with WAS/XLT has been described as early as 1961¹ and confers a poor prognosis to patients.²⁻⁵ However, the precise incidence of LPD in patients with WAS remains unknown.

Through a retrospective study within the national registry of CEREDIH, the French National Reference Center for primary immune deficiency (see the [Methods](#) section in this article's Online Repository at www.jacionline.org for more details), we identified 13 patients with WAS/XLT who had LPD before allogeneic stem cell transplantation (alloSCT) among the 189 patients with WAS and 46 patients with XLT treated between 1988 and October 2017. Considering that patients with WAS/XLT were at risk before undergoing alloSCT, the crude rate and incidence ratio of LPD in untransplanted patients were 8.4% and 511 per 1 × 10⁵ patient-years (95% CI, 272–873 per 1 × 10⁵ patient-years), respectively, which is greater than in the general population (7.5 per 1 × 10⁵ patient-years [95% CI 7.1–7.8]; *P* < .001). Crude rates (4.3% [95% CI, 0.5% to 14%] for the XLT cohort and 5.8% [95% CI, 2.9% to 10.2%] for the WAS cohort) and incidence ratios (334 [95% CI, 40–1200] and 531 per 1 × 10⁵ patient-years [95% CI, 265–943], respectively) of LPD were similar in patients with XLT and those with WAS. The cumulative incidence of LPD was 1.9% at age 10 years and 9.9% at age 30 years, as shown by competing risk analysis. After age 40 years, the risk of LPD persists in untransplanted patients with WAS/XLT as 3 cases occurred at age 43.2, 50.3, and 50.6 years ([Fig 1, A](#)). The median age at diagnosis of WAS/XLT was similar in the entire cohort and in the subgroup of patients who developed LPD (1 year; interquartile range, 0–3 years).

Among the 13 patients who had LPD, 2 were given a previous diagnosis of XLT. WAS scores before LPD onset were 3 (*n* = 2), 4 (*n* = 3), 5 (*n* = 5) and 5–severe refractory thrombocytopenia (5-SRT) (*n* = 1). Four patients with a score of 5 had autoimmune cytopenia, and 2 had Kaposi sarcoma. In contrast, no patients were given a diagnosis of Kaposi sarcoma among the patients with WAS/XLT without LPD. Four patients had received immunosuppressive therapy, all before the diagnosis of LPD. Nine patients were receiving immunoglobulin replacement therapy, and 6 had been splenectomized (see [Table E1](#) in this article's Online Repository at www.jacionline.org).

Genetic diagnosis of WAS/XLT was available for 195 (83%) patients, including 10 who had LPD. Five splice-site mutations, 1 nonsense mutation, 1 missense mutation, 2 insertions, and 1 deletion were identified ([Fig 1, B](#)). All these mutations, except the missense mutation, are predictive of a severe WAS phenotype. Patients with LPDs were less likely to have missense mutations than patients without LPD (10% vs 60.3%, respectively; *P* = .002). Wiskott-Aldrich syndrome protein (WASP) expression was evaluated by means of immunohistochemistry on tumor samples of 11 patients. In 6 samples WASP expression was absent in both T and B cells. In the 5 remaining patients WASP was expressed on T cells of the microenvironment but was negative on tumor large B cells ([Fig 1, C](#), and see [Table E2](#) in this article's Online Repository at www.jacionline.org), suggesting the presence of revertant T cells. We were able to further assess WASP expression using flow cytometry and Western blotting in one of them, patient 10, that we found bimodal. These results suggest that a corrective mutation has occurred in T-cell

METHODS

Mice

Mb1^{iCre} mice were obtained from Michael Reth,^{E1} ROSA^{DTA} mice were from the Jackson laboratory (Bar Harbor, Me), and female C57Bl/6J mice were from Janvier Labs (Le Genest St Isle, France). 1-DER mice have been described previously.^{E2} Mice were bred and housed at the SPF facility of VIB-University of Ghent and used at 6 to 10 weeks of age. Experiments were approved by the Animal Ethical Committees of the University of Ghent and the Center of Inflammation Research.

Models of airway allergy and Der p 2 treatment

To establish allergic asthma, mice were sensitized intratracheally with 1 µg of HDM extract (Greer Laboratories, Lenoir, NC) or sham PBS, followed by 5 consecutive intranasal challenges with 10 µg of HDM (days 6–11 in primary prevention or days 27–31 after sensitization in secondary prevention). Asthma parameters were investigated 4 days after the final HDM challenge.

Pretreatment comprised 7 intranasal instillations in a 15-day period (every other day) and treatment for secondary prevention comprised 8 intranasal instillations in a 17-day period of 65 µg of Der p 2 (supplemented with 0.59 EU LPS; LPS-EB Ultrapure, InvivoGen, San Diego, Calif) or sham PBS.

All intratracheal and intranasal treatments were administered in 80 and 40 µL of PBS, respectively, after achievement of light isoflurane anesthesia. Der p 2, Der p 1, and GM-CSF were generated in the *Pichia pastoris* expression system, and HDM was labeled with Alexa Fluor 647 succinimidyl ester (Invitrogen, Carlsbad, Calif), all by the VIB Protein Service Facility.

Adoptive DC, T-cell, and B-cell transfers and DC–T-cell cocultures

Mediastinal lymph nodes (MLNs) were cut with dissection scissors and shaken at 37°C for 20 minutes in dissociation medium (RPMI-1640 medium; Gibco, Grand Island, NY) with 20 µg/mL Liberase TM (Roche, Mannheim, Germany) and 10 U/mL DNase I recombinant grade I (Roche) to isolate DCs. Resulting single-cell suspensions were filtered, resuspended in PBS with 1% BSA (Sigma-Aldrich, St Louis, Mo) and 5 mmol/L EDTA, and enriched for DCs by using a density gradient (diluted 5 times in PBS with separation at 600g for 20 minutes; OptiPrep; Axis-shield, Dundee, Scotland). DCs were fluorescence-activated cell sorted from the enriched middle layer, and 3×10^4 cDC2s were administered intratracheally in acceptor mice or 1×10^4 DCs were cocultured with 1-DER T cells.

For 1-DER T-cell isolation, spleens and MLNs were dissected from CD45.1 congenic 1-DER mice, pressed through a 40-µm sieve in PBS, treated with osmotic lysis buffer, and enriched for CD4⁺ T cells with the MagniSort mouse CD4 T cell Enrichment Kit from Thermo Fisher (Waltham, Mass), according to the manufacturer's instructions. CD4⁺ T cells (>97% purity, 1-DER T cells) were then incubated at 37°C with CellTrace CFSE (Invitrogen) or the cell proliferation dye eFluor 450 (both 1:1000 in PBS; eBioscience, San Diego, Calif) for 7 minutes. 1-DER T cells were intravenously injected (3×10^6) or cocultured with DCs in a 1:5 (DC/T cell) ratio for 4 days in RPMI-1640 supplemented with 5% FCS (Bodinco, Alkmaar, The Netherlands), 0.1% β-mercaptoethanol, GlutaMAX (Gibco) and gentamicin (Gibco).

Flow cytometry

Cells were stained with anti-CD3e labeled with phycoerythrin (PE)–Cy5 (clone 145-2C11; Tonbo Biosciences, San Diego, Calif), CD11b–V450 (M1/70; BD, Franklin Lakes, NJ), CD19–PE–Cy5 (1D3; eBioscience), CD11c–PE–Cy7 (N418; eBioscience), MHC class II–allophycocyanin (APC)–eFluor 780 (M5/114.15.2; eBioscience), and Siglec-F–PE (E50-2440; BD) to determine the cellular composition of BAL fluid. To study 1-DER T-cell responses *in vivo*, 0.8 µg of CD45-APC (30-F11; Invitrogen) was intravenously injected into mice 2 minutes before death to label circulating leukocytes. Cells were additionally stained with CD44–PE–Cy7 (IM7; eBioscience), CD3–eFluor 450 (17A2; eBioscience), CD4–APC–Cy7 (GK1.5; BD), CD45.1–V605 (A20; BioLegend, San Diego, Calif), and CD62L–PE (MEL-14; BD).

1-DER T cells in cocultures were stained with CD45.2–peridinin-chlorophyll-protein complex (PerCP)–Cy5.5 (104; BD), CD3e–APC–Cy7

(145-2C11; BD), CD4–fluorescein isothiocyanate (FITC; RM4-5; eBioscience), and CD44–redFluor 710 (IM7; Tonbo Biosciences).

Cells were stained with CD197 (CCR7)–biotin (4B12; eBioscience) and CD64–Alexa Fluor 647 (X54-5/7.1; BD), followed by CD172a (signal-regulatory protein α)–PerCP–eFluor 710 (P84; eBioscience), XCR1–PE (ZET; BioLegend), CD26–FITC (H194-112; BD), MHC class II–APC–eFluor 780, CD11c–PE–Cy7, CD3–Alexa Fluor 700 (17A1; eBioscience), CD19–Alexa Fluor 700 (eBio1D3; eBioscience), and streptavidin–PE–CF594 (BD) to sort DCs by using fluorescence-activated cell sorting. Lung cells were stained with CD3–eFluor 450, CD19–Alexa Fluor 700, and MHC II–APC–eFluor 780 to confirm the Mb1^{iCre/+} ROSA^{DTA/+} phenotype.

For HDM uptake studies, cells were stained with CD26–FITC, CD64–BV711 (X54-5/7.1; BioLegend), XCR1–PE, CD11c–PE–Cy7, CD3e–PE–Cy5, CD19–PE–Cy5, CD172a–PerCP–eFluor 710, MHC class II–APC–eFluor 780, and CD45–Alexa Fluor 700 (30-F11; eBioscience). For activation marker studies on MLN cDCs, cells were stained with the same antibodies, except for CD11c–eFluor 450 (N418; eBioscience) and XCR1–BV650 (ZET; BioLegend) and additionally with CD40–APC (5C3; BD), CD86–PE–Cy7 (PO3; BioLegend), or CCR7–PE–Cy7 (4B12; eBioscience) and CD80–PE (16-10A1; eBioscience).

In all stainings unspecific antibody binding was prevented by adding 2.4G2 (antibody to the Fcγ receptor II/III; Bioceros, Utrecht, The Netherlands). Dead cells were excluded by adding fixable viability dye conjugated to eFluor 506 or eFluor 780 (eBioscience). Samples were acquired on an LSRFortessa or sorted on a FACSAria II or III cytometer (all from BD) and analyzed with FlowJo Software (TreeStar, Ashland, Ore). Proliferation statistics were calculated by using the Proliferation tool of FlowJo (version 9).

Microscopy on lung tissue sections

Lungs were injected with 1 mL of PBS/OCT (1:1) solution (Tissue-Tek) through a cannula in the trachea, snap-frozen in liquid nitrogen, and cut in slices (10 µm) with the HM560 microtome (Thermo Scientific). Mucus was visualized by using periodic acid–Schiff staining, and images were acquired on an Olympus BX53 light microscope with analysis getIT software (Olympus Soft Imaging Solutions; Olympus, Center Valley, Pa). Slides for immunohistology were first fixed in 2% paraformaldehyde for 2 minutes, rinsed in PBS for 10 minutes, and then incubated for 1 hour at room temperature with 1:100 Siglec-F–PE antibody (E50-2440; BD) to stain eosinophils. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride. Images were acquired with a Zeiss LSM710 microscope (Carl Zeiss, Oberkochen, Germany) and analyzed with Imaris software (Bitplane, Belfast, United Kingdom).

BHR measurements

For BHR measurements, mice received 1 additional HDM challenge 24 hours before measurement. Airway resistance was assessed with a flexiVent apparatus (SCIREQ, Montreal, Quebec, Canada). Mice were anesthetized with urethane and paralyzed with D-tubocurarine, followed by mechanical ventilation through a cannula in the trachea. Methacholine was nebulized in increasing doses (0–400 mg/mL), with baseline restoration between subsequent doses. Per dose, a standardized inhalation maneuver was administered every 10 seconds for 2 minutes (respiratory frequency of 150 breaths/min, tidal volume of 10 mL/kg, and positive end-expiratory pressure of 3 cm H₂O), and airway resistance was recorded.

Analysis of immunoglobulin, cytokine, chemokine, and mRNA levels

Blood was collected after achievement of terminal anesthesia through the eye cavity, and serum was obtained by means of centrifugal phase separation (400g). Immunoglobulin levels in blood and BAL fluid were assessed with the respective BD Bioscience ELISA sets. Coating antibodies for HDM-specific IgG₁, IgG_{2a}, and IgA ELISAs were interchanged for 100 µg/mL HDM. Detection antibody for HDM-specific IgE was interchanged for biotin-labeled HDM (100 µg/mL) in PBS plus 10% FCS.

Total lungs were snap-frozen and homogenized in a TissueLyser II device (Qiagen, Hilden, Germany) for 4 minutes at 20 Hz in 20% glycerol in dH₂O with 40 mmol/L Tris-HCl, 275 mmol/L NaCl, and an EasyPack complete ULTRA-tablet mini (Roche). Igepal CA-630 (2%; US Biologicals, Salem, Mass) was added, and homogenates were rotated for 30 minutes and then centrifuged. Cleared lung lysates were quantified for total protein content with NanoOrange reagent (Invitrogen). MLN single-cell suspensions (2×10^6 cells/mL) were restimulated for 3 days with 15 μ g/mL HDM in 96-well plates in RPMI-1640 supplemented with 5% FCS (Bodinco), 0.1% β -mercaptoethanol, GlutaMAX (Gibco), and gentamicin (Gibco). Cytokine and chemokine levels were determined with Ready-Set-Go ELISA sets from eBioscience.

mRNA was isolated by using TriPure Isolation Reagent (Roche) and chloroform/isopropanol, according to the manufacturer's instructions. RNA was reverse transcribed with the SensiFAST cDNA Synthesis Kit (BioLine, Modi'in, Israel) and amplified in a quantitative PCR reaction by using SensiFAST Sybr No-ROX Kit (BioLine) and the following primer pairs: *Hprt* forward 5'-TGAAGAGCTACTGTAATGATCAGTCAAC-3'; *Hprt* reverse 5'-AGCAAGCTTGCAACCTTAACCA-3'; T-bet forward 5'-AGCAAGGACGGCGAATGTT-3'; T-Bet reverse 5'-GGGTGGACATATAAGCGGTTC-3'; *Gata3* forward 5'-TTATCAAGCCCAAGCGAAG-3'; *Gata3* reverse 5'-TGGTGGTCTGACAGTTC-3'; *Rorc* forward 5'-TCACCTGTGAGGGGTGCAAG-3'; and *Rorc* reverse 5'-GTTCGGTCAATGGGGCAGTT-3'.

Statistics

Differences between 2 or more groups were calculated by using the Mann-Whitney *U* test for unpaired data or the Kruskal-Wallis test for unpaired data,

respectively, without multiple-comparison correction (GraphPad Prism, version 7.0; GraphPad Software, San Diego, Calif). *P* values of less than .05 were considered significant. Lung resistance data measured at various doses were analyzed as repeated measurements (also called longitudinal data) by using the residual maximum likelihood as implemented in Genstat (version 19).^{E3} Briefly, a linear mixed model (random terms underlined) of the form $y = \mu + \text{Treatment} + \text{dose} + \text{Treatment}*\text{dose} + \text{Replicate} + \text{Mouse}*\text{dose}$ was fitted to longitudinal data. The term *mouse*dose* represents the residual error term with dependent errors. Times of measurement were set as equally spaced, and the best model for correlation on the longitudinal data (eg, uniform, autoregressive, antedependence, and unstructured) was selected based on the Aikake information coefficient. Significance of treatment effects across doses (ie, treatment*dose) and changes in differences between treatment effects across doses were assessed by using an approximate *F* test, of which the denominator *df* values were calculated by using algebraic derivatives, as implemented in Genstat (version 19). Results are represented as predictions of means $\pm$ SEMs with *F* test probability (**P* < .05 and ****P* < .001) for treatment*dose effects.

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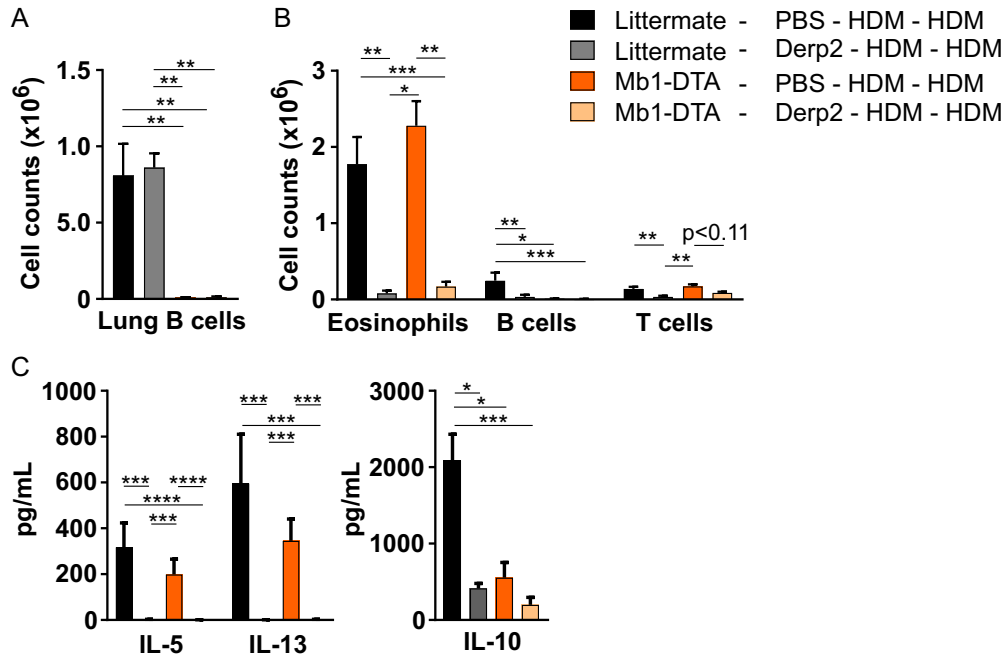


FIG E1. B cells are dispensable for Der p 2-mediated asthma protection. Mb1^{Cre/+}Rosa^{DTA/+} (Mb1-DTA) or Mb1^{Cre/+}Rosa^{+/+} (littermate) mice were treated as in Fig 1, A. **A**, B-cell counts in lung tissue. **B**, Inflammatory cell counts in BAL fluid. **C**, Cytokine production by HDM-restimulated LDLN single-cell suspensions. Results are representative of 2 (Fig E1, A and B) independent experiments or pooled from 2 (Fig E1, C) independent experiments with 5 to 6 mice per group for single experiments. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

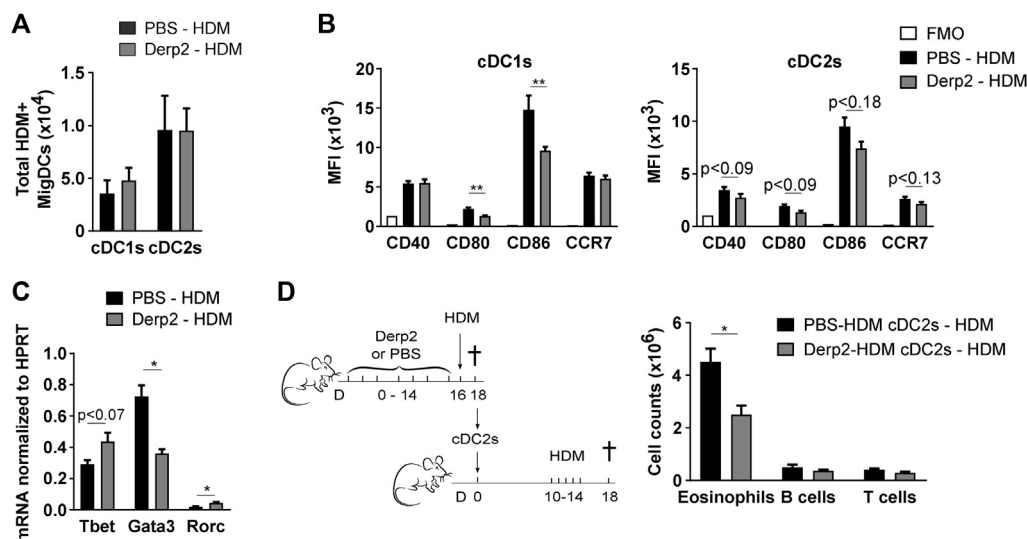


FIG E2. Repetitive Der p 2 inhalation suppresses cDC2-mediated T_H2 responses. **A** and **B**, Mice were treated with Der p 2 or sham PBS AIT and after 24 hours were intratracheally instilled with 100 μ g (high LPS content) of HDM (Alexa Fluor 647 labeled in Fig E2, A). Fig E2, A, Numbers of migratory HDM⁺ cDC1s and cDC2s in LDLNs 24 hours after HDM instillation. Fig E2, B, Activation marker expression on LDLN cDC1s and cDC2s 48 hours after instillation. **C**, Mice were treated as in Fig 2, A-E, and killed at day 7. LDLN single-cell suspensions were HDM restimulated for 3 days *in vitro* and analyzed for mRNA levels of transcription factors. **D**, cDC2 donor mice were treated with Der p 2 or sham PBS AIT and intratracheally received 100 μ g of HDM, followed after 2 days by sorting of LDLN cDC2s. Sorted cDC2s were then intratracheally transferred to acceptor mice, which were subsequently HDM challenged. Inflammatory cell counts in BAL fluid of acceptor mice 4 days after the last HDM challenge are shown. Results are representative of 1 (Fig E2, C) or 2 (Fig E2, B and D) independent experiments or pooled from 3 (Fig E2, A) independent experiments, with 4 to 7 mice per group for single experiments and shown as means $\pm$ SEMs. * P < .05 and ** P < .01. *MFI*, Mean fluorescence intensity.

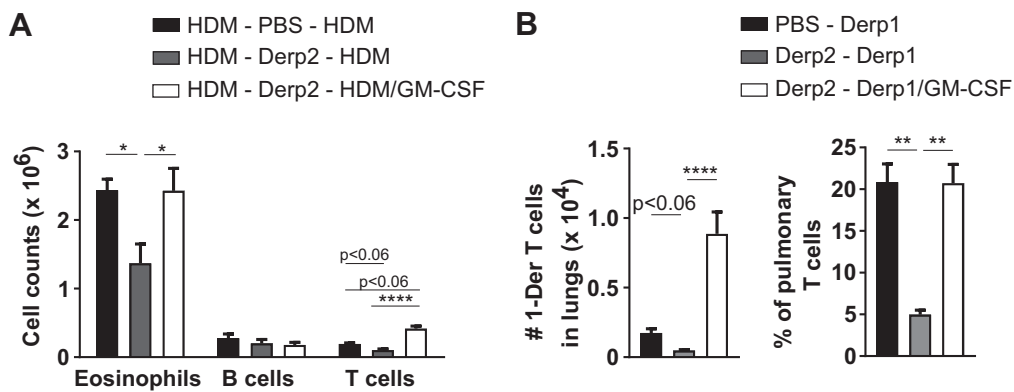


FIG E3. GM-CSF supplementation rescues primary T-cell responses and secondary eosinophilia. **A**, Cell counts in BAL fluid of mice treated as in Fig 1, G-I, with one group of mice challenged 5 times with 10 μ g of HDM supplemented with 1 μ g of GM-CSF. **B**, Tissue-resident 1-DER T-cell numbers in lung tissue of mice treated as in Fig 2, J. Results are representative of 2 independent experiments, with 4 to 8 mice per group for single experiments. Data are shown as means $\pm$ SEMs. * P < .05, ** P < .01, and **** P < .0001.