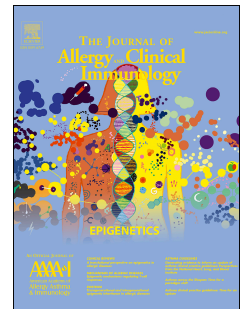


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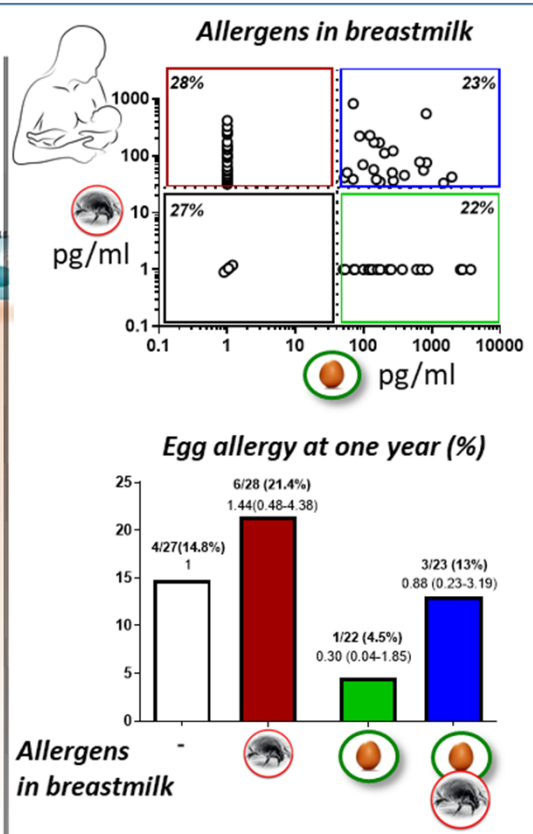
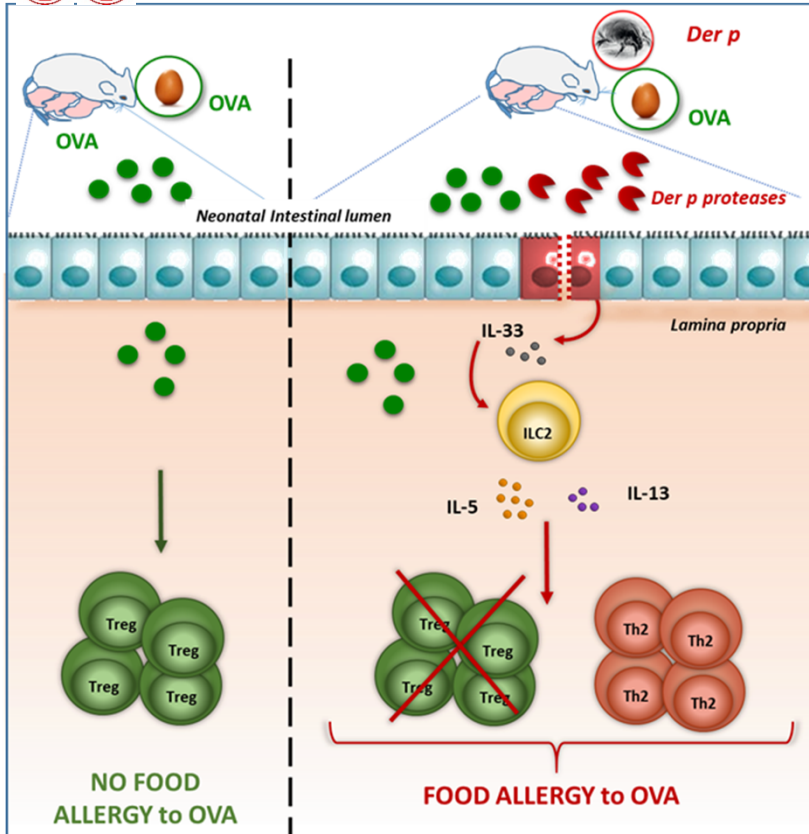
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House dust mite allergens in breastmilk interfere with oral tolerance to food antigens



A role for early oral exposure to house dust mite allergens through breastmilk in IgE-mediated food allergy susceptibility

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ABSTRACT

Background: Successful prevention of food allergy requires the identification of the factors adversely affecting the capacity to develop oral tolerance to food antigen in early life.

Objectives: To determine whether oral exposure to *Der p* through breastmilk affects gut mucosal immunity with long-term effects on IgE-mediated food allergy susceptibility.

Methods: Gut immunity was explored in 2 weeks-old mice breastfed by mothers exposed to *Der p*, protease-inactivated *Der p* (*iDer p*) or to PBS during lactation. We further analysed oral tolerance to a bystander food allergen, Ovalbumin (OVA). In a proof of concept study, *Der p* 1 and OVA levels were determined in 100 human breastmilk samples and the association with prevalence of IgE-mediated egg allergy at one year assessed.

Results: Increased permeability, IL-33 levels, ILC2 activation and Th2 cell differentiation were found in gut mucosa of mice nursed by mothers exposed to *Der p* as compared to PBS. This pro-Th2 gut mucosal environment inhibited the induction of antigen-specific FoxP3 regulatory T cells and the prevention of food allergy by OVA exposure through breastmilk. In contrast, *iDer p* had no effect on offspring gut mucosal immunity. Based on the presence of *Der p* and/or OVA in human breastmilk, we identified groups of lactating mothers, which mirror the ones found in mice to be responsible for different egg allergy risk.

Conclusion: This study highlights an unpredicted potential risk factor for the development of food allergy, i.e. *Der p* in breastmilk, which disrupts gut immune homeostasis and prevents oral tolerance induction to bystander food antigen through their protease activity.

KEY MESSAGES

- *Der p* allergens in breastmilk trigger critical immunological events in the neonatal small intestine that will induce higher susceptibility to food allergy in the long term.
- Protease from *Der p* allergens are key players for the induction of gut Th2 mucosal immune imbalance in mice breastfed by mothers inhaling *Der p*
- The evidence that *Der p* allergens and their proteases in breastmilk may be a risk factor for food allergy will stimulate research aimed at modulating their levels and enzymatic activity. Ultimately, this should contribute to ensure food allergy prevention in breastfed children.

CAPSULE SUMMARY

Breastmilk is a source of oral exposure to *Der p* allergens that may interfere with the setup of oral tolerance to food antigen in early life and increase food allergy risk

KEY WORDS

House dust mite; Food Allergy; breastfeeding; protease

ABBREVIATIONS

OVA, Ovalbumin

Der p, *Dermatophagoides Pteronyssinus*

iDer p, *Dermatophagoides Pteronyssinus*, which protease have been inactivated

HDM, house dust mite

ILC, innate lymphoid cells

INTRODUCTION

Food allergies represent a major public health issue, particularly in westernized countries where the prevalence is as high as 10% in some regions (1, 2). IgE-mediated food allergy results from inappropriate Th2 lymphocyte differentiation towards an innocuous dietary antigen, leading to a symptomatic inflammatory response upon exposure instead of the silent non-inflammatory regulatory response observed in tolerant individuals (3). One highly investigated hypothesis for the risk of developing food allergy is that the delayed oral exposure to potential dietary allergens, and hence the lack of oral tolerance induction, may allow sensitization through an impaired skin barrier (4). Recent data have shown that a significant proportion of infants were already sensitized to egg by 4 months of age and at risk of anaphylactic reaction upon ingestion of egg (5, 6). This suggests that a critical period to induce oral tolerance may be prior to the introduction of solid foods. Accordingly, a possible strategy for early intervention to reduce the risk for food allergy may be to induce oral tolerance to dietary allergens through breastmilk (7). Studies in mice have examined conditions that are required to induce oral tolerance towards the food antigen ovalbumin (OVA) upon its transfer through breastmilk and showed that maternal milk factors such as TGF- β , vitamin A and maternal OVA-specific IgG were required for oral tolerance induction and prevention of respiratory or food allergy (8-11). Besides food allergens derived from the maternal diet, human milk is also a source of respiratory allergens such as *Der p 1* and *Blo t 5* from house dust mite (HDM) (12, 13), which are found in breastmilk in the same range of concentration as dietary antigens such as OVA (13). This observation is most probably related to the fact that most inhaled protein are actually ingested because of respiratory tract muco-ciliary clearance (14-16) and will then follow the same route to the mammary gland as dietary antigens (7, 17, 18). This hypothesis is supported by recent finding demonstrating the

presence of *Der p* allergen in human digestive fluids (17). In contrast to the observation made with OVA antigen, the exposure of lactating mice to *Dermatophagoides pteronyssinus* (*Der p*) allergens strongly primed the allergic immune response to *Der p* in adulthood (13). A role for early oral exposure to *Der p* allergens through breastmilk in sensitization to *Der p*, was further supported by observations in a human cohort, which demonstrated that the presence of *Der p* 1 allergen in human breastmilk was associated with an increased risk for the development of allergic sensitization, asthma and allergic rhinitis in children (19). Compared to OVA, *Der p* allergens display strong adjuvant properties related to their proteolytic activity and the presence of endotoxin (20). As a result, upon respiratory exposure to *Der p*, lung epithelial cells release inflammatory mediators that will activate type 2 innate lymphoid cells, prime dendritic cells and induce Th2 differentiation (21). Data gathered both in adult human and mice further showed that *Der p* allergens could affect the gut barrier (17). This effect was dependent on *Der p* proteolytic activity (17). Here, we examined whether the presence of *Der p* allergen in breastmilk could affect the small intestine gut mucosal immune ontogeny and disturb the long-term gut immune homeostasis to food antigens.

MATERIALS AND METHODS

Mice

Synchronous pregnant and non-pregnant BALB/c mice were purchased from Janvier Labs (France) or from Animal Resources Centre (Australia). RAG-2 deficient- DO11.10 TCR transgenic mice were obtained from Fiona Powrie (Oxford, UK). Mice were housed under SPF conditions at “Centre Méditerranéen de Médecine Moléculaire (C3M)” or at the Harry Perkins Institute of medical Research (Perth). Experiments were performed according to the CIEPAL animal ethics agreement NCE/81, NCE/186 and APAFIS 268 (France) and in accordance with National Health and Medical Research Council of Australia guidelines for the care and use of animals in research and were approved by the Harry Perkins Institute of medical Research Animal Ethics Committee (AEC permit number AE111) (Australia).

Mouse model of Der p and OVA allergens transfer through breast milk

Der p and OVA allergen transfer to neonates through breastmilk of immunized mothers was induced as previously described (10, 13) with some adaptations as detailed in the Online Repository and in Fig.1. *Der p* extract was generously provided by Stallergenes (Anthony, France) and had a very low content in endotoxin (4.48EU +/- 0.19 EU/mg of *Der p* 1 in HDM extract, mean +/- sem for 5 different batches).

Der p proteases activity measurement and inactivation

Protease activity in *Der p* extract and in milk collected from neonates’stomach, was quantified using the preferential *Der p* substrate, Boc-Gln-Ala-Arg-AMC (100 µM) in an enzymatic assay as previously described (22, 23). Irreversible inactivation of cysteine and serine protease from

Der p extract was performed by the use of irreversible serine protease inhibitor AEBSF (Santa Cruz, 1 mM) and cysteine protease inhibitor E-64 (Sigma-Aldrich, 100 μ M) (23). Protease inhibitor treatment reduced the protease activity by 95% (Fig. E1 in the Online Repository).

In vivo gut permeability

Two week-old pups were given Fluorescein isothiocyanate–dextran (MW 4.4 kDa) (Sigma-Aldrich, Lyon, France) by intra-gastric administration at a dose of 0.4 mg/g body weight. After 1 or 24 hours, 50 μ l blood was collected from the facial vein and the fluorescence of a 2-fold diluted serum measured at 488nm using a fluorimeter (Tecan Infinite, Switzerland)). Concentration of dextran was calculated using a standard curve; detection limit was 0.1 μ g/mL.

IL-33 levels in small intestine tissue

Frozen intestinal tissue samples from 2 week-old pups were homogenized in PBS containing a cocktail of protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Meylan, France) using the universal tissue homogenizers (Precellys, Thermofisher, Courtaboeuf France). After centrifugation at 10,000 *g* to pellet debris, concentrations of IL-33 in supernatants was measured using ELISA assay (ebioscience, Paris, France). Cytokine levels were normalized to the total protein level in each sample, as measured using the colorimetric assay (DC protein Assay; Bio Rad, Marnes-la-Coquette, France)

Small intestine ILC and CD4 T cells isolation and characterization

Two week-old pups were sacrificed by lethal IP administration of Pentothal and the small intestine was harvested for Lamina Propria (LP) cells analysis using a protocol adapted to neonates that is available as online repository material. To analyze cytokine production, cells were stimulated with Phorbol myristate acetate (Sigma-aldrich, Lyon, France, 50 ng/m) and Ionomycin (Sigma-aldrich, Lyon, France, 500 ng/mL) in the presence of Brefeldin A (2.5µg/mL, Sigma-aldrich, Lyon, France) for 3.5 hours at 37°. The phenotype and cytokine production by innate lymphoid cells and T lymphocytes were characterized by FACS Canto flow cytometer using specific monoclonal antibodies, which are detailed in the Online Repository.

Immunofluorescence staining in neonatal small intestine

ZO-1, IL-33 and E-cadherin immunofluorescence staining was performed on 5 µm thick paraffin sections of jejunum section from 2 weeks old mice as detailed in the online repository.

In vivo induction of FOXP3 expressing OVA specific T cells

We followed the induction of FOXP3 expression in OVA specific Tcells injected in 1 day old mice following a previously published protocol (9) and described in the Online Repository. One week after the last OVA and/or Der p administration to mothers, mesenteric lymph nodes were harvested and cell suspension analyzed by flow cytometry. Injected DO-11.10 CD4 T cells were identified using anti-CD3, anti-CD4 and KJ1.26 mAb (ebioscience, Paris, France). OVA specific CD4 T cells were analyzed for FoxP3 expression after cell fixation and permeabilization using transcription factor buffer set (ebioscience, Paris, France).

OVA-induced allergic diarrhea in the offspring

Mice were tested for their susceptibility to OVA-induced allergic diarrhea when 6-8 week old using a protocol previously published (24) and described in the Online Repository. One hour after the last OVA challenge, mice were euthanized and serum collected. MMCP1 levels in serum were quantified by ELISA (ebiosciences, Paris, France) to evaluate mast cells degranulation (24). Allergic sensitization to OVA was evaluated by measuring the levels of OVA-specific IgE as previously described (9).

Human cohort study population

The 100 mothers and infants included were control participants of a larger double-blind randomized controlled trial (RCT) of healthy term infants, born to allergic mothers, the Infant Fish Oil Supplementation Study(25). This RCT was registered with the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au; ACTRN12606000281594). IgE-mediated egg allergy was defined at 1 and 2.5 years of age as a history of immediate symptoms following ingestion of egg and a positive skin prick test (≥ 3 mm) to egg white (25).

Mouse and human breastmilk collection

Mouse breastmilk was collected from the stomach of 2-week-old pups and diluted in 1 volume of PBS. Samples were spun down at 10,000 g for 10 min and the aqueous phase was collected and stored at -20°C until analysis

Human breastmilk samples were collected at 6 months of lactation. Samples were divided into 2ml aliquots of whole milk and frozen at -80°C prior to analysis.

Der p 1 and OVA levels in milk

Der p 1 and OVA levels were determined in the aqueous fraction of breastmilk by ELISA as previously described (13, 17, 19). Detection level for *Der p* was 30pg/mL and for OVA 50pg/mL.

Statistical analysis

We used two-sided tests to test for differences in potential confounding variables across all four comparison groups (Table 1); for continuous variables we used one-way ANOVA (3 degree of freedom (DF) F-test) and the t-test, for categorical variables we used Chi-Square tests of Fisher's exact test where appropriate. To estimate effect size, we computed the relative risk (RR) with its 95% confidence interval, probability values of less than 5% were considered significant (p -value < 0.05). Analyses were performed with GraphPad Prism 6 (GraphPad Software, San Diego CA, USA) and SAS 9.4 (The SAS Institute, Cary, NC, USA).

RESULTS*Breastmilk of mice inhaling Der p contains Der p 1 allergen and displays increased protease activity*

We previously published a mouse model that mimics the exposure of human infants to *Der p* allergen through breastmilk (13). Briefly, mice are nursed by mothers that have been sensitized to *Der p* by respiratory exposure before mating and that continue to be exposed during lactation (Fig.1A). To specifically address the impact of maternal exposure to *Der p* during lactation period, and to avoid maternal *in utero* effect, neonates from naïve mothers are adopted and fostered at birth by *Der p*-exposed mothers (Fig.2A). Here, we confirmed the presence of *Der p 1* allergen in milk collected from stomach of pups nursed by mothers exposed to *Der p* by intra-nasal route (Fig.2A). *Der p 1* was detected at the level of 538 +/- 77 pg/ml (mean +/- sem) while it was undetectable in milk from PBS-exposed mothers (Fig. 2A). *Der p* allergens are known to exhibit proteolytic activity, which plays a key role in the initiation of the cascade leading to Th2 priming (20). We therefore addressed whether *Der p*-containing milk displayed increased protease activity using the preferential substrate of *Der p 1*. We found that milk from *Der p*-exposed mothers had an increased proteolytic activity as compared to milk from PBS-exposed mothers (Fig. 2B, and Fig E2 in online repository). Altogether, these data indicate that neonates nursed by mothers that inhale *Der p*, are orally exposed to milk that contains *Der p 1* allergen and shows increased protease activity.

Leaky and pro-inflammatory gut epithelium in neonatal mice exposed to Der p protease through breastmilk

There is clinical and experimental evidence that a leaky epithelium and the secretion of alarmins by damaged epithelium are involved in food antigen sensitization and food allergy development (26, 27). We assessed gut barrier function in 2 week-old mice orally exposed to *Der p* allergens through breastmilk. We first verified that *Der p* and increased proteolytic activity were also found in the small intestine of mice nursed by *Der p*-exposed mothers (Fig. E3 in the Online Repository). We observed a 2-fold increase in the transfer of FITC-Dextran across the intestinal barrier of mice breastfed by mothers exposed to *Der p* allergens compared to PBS-exposed mothers (Fig. 3A). This increase in gut permeability upon *Der p* exposure through breastmilk was transient as it was observed 2 hours after maternal exposure but not after 24 hours (Fig. 2A). To conclude whether the increased permeability was due to damage of epithelial tight junction proteins, we have examined Zona Occludens 1 (ZO-1) expression by immunofluorescence on neonatal gut tissue. We found a disrupted and reduced expression of ZO-1 in small intestine epithelial cells from neonates exposed to *Der p* through breastmilk (Fig. 3B, C). Alarmins are danger signals produced by injured epithelium and the secretion of IL-33 by lung epithelial cells upon *Der p* exposure has been shown to be critical for initiation of allergic response both in adults and neonates (28, 29). We detected a 10-fold increase in IL-33 levels in the small intestine tissue from mice breastfed by mothers exposed to *Der p* compared to PBS (Fig. 4A). Its kinetics mirrored the change in permeability (Fig. 4A). Immunofluorescence staining of IL-33 in gut tissue, showed that intestinal epithelial cells were a source of increased IL-33 production in neonates exposed to *Der p* through breastmilk (Fig. 4 B,C).

The cysteine and serine protease activity of *Der p* allergens are known to affect epithelial cell barrier by cleaving their tight junctions and/or by activating protease activated receptors (17, 20). We examined here whether proteases from *Der p* were involved in neonatal gut barrier

dysfunction by analyzing the gut barrier in neonates exposed to *Der p* allergens with no cysteine and serine protease activity (*iDer p*) through breastmilk. To address specifically this question, we sensitized mothers to *Der p* before mating and exposed them during lactation to *Der p*, which cysteine and serine proteases had been irreversibly inactivated by the use of E-64 and AEBSF compounds (*iDer p*)(17, 23) (Fig E1 in online repository). We observed that, in contrast to breastmilk from *Der p* exposed mothers, breastmilk from mice exposed to *iDer p* had no increase in protease activity as compared to mice exposed to PBS (Fig. 2B and Fig E2 in online repository). We further verified that *Der p* and *iDerp* - exposed mothers exhibited similar amounts of *Der p 1* allergen in breastmilk (Fig. 2A) and were similarly sensitized to *Der p* (Fig. E4 in the online repository). We then analysed the transfer of FITC Dextran across the gut barrier as well as IL-33 levels in small intestine of mice exposed to *iDer p* through breastmilk. We found that the *Der p*-induced increase in gut permeability (Fig. 3) and IL-33 production (Fig.4) were completely abolished upon inhibition of serine and cysteine protease activity of *Der p* extracts. These data show that the neonatal exposure to *Der p* allergens through breastmilk is triggering neonatal gut barrier dysfunction through its proteolytic activity.

Oral exposure to Der p protease through breastmilk activates small intestine group 2 innate lymphoid cells in neonate mice

We next questioned if *Der p*-induced IL-33 in the neonatal gut would promote the activation of the group 2 innate lymphoid cells (ILC2). There is accumulating evidence that lung ILC2 represent a crucial link between allergen-induced epithelium alarmin secretion and the initiation of Th2 cell-mediated allergic airway inflammation (30). To analyze the representation of ILC2 in the *lamina propria* of 2 week-old mice with or without *Der p* exposure through breastmilk, we

gated on lineage negative CD45⁺CD90⁺GATA3⁺ cells as previously published (31, 32) (Fig.5A). Neonates' oral exposure to *Der p* neither affected the number of GATA3⁺ILC nor their frequency (Fig.5B and Fig. E5 in online repository). However, ILC were clearly activated by oral exposure to *Der p* through breastmilk as we observed a 2-fold increase in the number and frequency of ILC producing IL-13 and IL-5 compared to mice breastfed by mothers exposed to PBS (Fig.5C and Fig.E4 in online repository). IL-5 mostly originated from GATA3⁺ ILC (Fig.5D and Fig.E5 in online repository). We further examined whether increased levels of IL-33 in small intestine from neonates exposed to *Der p* through breastmilk were responsible for the stimulation of IL5 secretion by ILC. IL-5 secretion by ILC was totally abolished in mice breastfed by mothers exposed to *Der p* and treated with an IL-33 neutralizing antibody (Fig.E6 in online repository).

Proteolytic activity of *Der p* 1 was shown to be involved in allergic sensitization upon systemic (33, 34) or respiratory (35) administration of *Der p* 1 but evidence is lacking for a proteases-mediated effect of *Der p* on ILC2 activation and its role in the intestine. To assess whether *Der p* enzymatic activity was involved in ILC2 activation in the neonatal gut, we compared ILC2 activation in the *lamina propria* from 2 week-old mice breastfed by mothers exposed to intact *Der p* extracts with mice breastfed by mothers exposed to *Der p* extract where cysteine and serine protease activity had been neutralized. Neutralization of protease activity of *Der p* completely abolished the induction of IL-13 and IL-5 production by ILC (Fig. 5C, D and Fig.E5 in online repository). Altogether, our data indicate that *Der p* protease-induced injury to gut epithelial cells leads to IL-33 secretion, which in turns activate ILC2.

Oral exposure to *Der p* proteases through breastmilk expands small intestine Th2 lymphocytes and prevents oral tolerance induction to bystander food antigen in neonatal mice

We next investigated the impact of *Der p*-induced ILC2 activation on small intestine immune homeostasis in the neonate. We found an increased frequency and number of GATA3 expressing CD4⁺ T lymphocytes in the *lamina propria* of 2 week-old mice breastfed by mothers exposed to *Der p* but not to *iDer p* (Fig. 5E and Fig.E5 in online repository). However, oral exposure to *Der p* through breastmilk did not increase their IL-5 secretion (Fig.5E and Fig.E5 in online repository) and IL-13 was undetectable. The frequency and number of FoxP3-expressing Tregs remained unaffected by oral exposure to *Der p* and was slightly decreased upon exposure to *iDer p* (Fig.5E)

We then examined whether the gut barrier dysfunction, the ILC2 activation and the Th2 differentiation induced in neonates exposed to *Der p* through breastmilk, would affect their capacity to mount oral tolerance to an unrelated dietary antigen also transferred through breastmilk (Fig. 1B). Therefore, we took advantage of our previous observations showing the induction of OVA specific FoxP3 Tregs and oral tolerance to OVA in mice nursed by OVA-exposed immunized mothers (9, 36). We first verified that maternal exposure to *Der p* did not affect OVA transfer to neonates through milk. Therefore, we measured OVA levels in breastmilk and found similar levels whether mothers exposed to OVA only or OVA and *Der p* (Fig.E6 in online repository). Furthermore, we evaluated whether *Der p* and its protease activity would affect the transfer of the immunogenic form of OVA through breastmilk. We injected 1-day-old neonates with RAG2 deficient DO11.10 TCR transgenic OVA-specific CD4⁺T cells in and we monitored their expansion in mice nursed by mothers exposed to OVA only or to both OVA and *Der p* (Fig.6A). In both groups, we found a 6-fold expansion of OVA-specific T cells as

339 compared to mice breastfed by naïve mothers exposed to PBS (Fig.6A). This indicates that
340 neonates nursed by mothers exposed to OVA only or OVA and Der p are exposed to similar
341 amount of immunogenic forms of OVA in breastmilk. As previously shown (9), oral exposure to
342 OVA through breastmilk resulted in the induction of FoxP3 expression in OVA specific CD4 T
343 cells. There was a 2.5-fold increase in the frequency of FoxP3 expressing OVA specific T cells
344 and a 13-fold increase in their number as compared to mice nursed by PBS exposed mothers
345 (Fig.6A and Fig.E4 in online repository). In contrast, when mice were breastfed by mothers
346 exposed to both OVA and *Der p*, there was no increase in the frequency of FoxP3 expressing
347 OVA specific T cells and the population of FoxP3⁺OVA-specific T cells only increased by a
348 factor 8 as compared to mice nursed by naive mothers (Fig.6A and Fig.E4 in online repository).
349 There was a 40% decrease in the number of OVA specific FoxP3 Tregs in mice breastfed by
350 mothers exposed to OVA and Der p as compared to mice nursed by mothers exposed to OVA
351 only. In the absence of OVA administration, *Der p* neither affected the expansion of OVA-
352 specific T cells nor their FoxP3 expression (Fig.6A). In line with a role for *Der p* protease in the
353 induction of gut immune imbalance, the neutralization of *Der p* protease (*iDer p*) prior to
354 administration of Der p extract to lactating mothers, restored the capacity to induce FoxP3 Tregs
355 to a similar extend as observed in mice breastfed by OVA only- exposed mothers (Fig.6A and
356 Fig.E5 in online repository). Altogether, these data show that neonates exposed to *Der p*
357 protease through breastmilk have a dysregulated gut immunity, which includes lack of oral
358 tolerance induction to bystander OVA dietary antigen.

360 *Der p* allergens in breast milk are a risk factor for developing food allergy in young adult mice

Epidemiologic studies suggest an early life window of susceptibility for later risk of allergic disease development (37). This prompted us to assess the impact of mucosal immune imbalance induced by early oral exposure to *Der p* on food allergy susceptibility in the long term. Therefore, 6-8 week-old mice that had been exposed to *Der p*, OVA, or OVA and *Der p* through breastmilk, were submitted to a protocol of food allergy to egg (24, 36). As recently described (10), we found that mice breastfed by immunized-mothers exposed to OVA during lactation were significantly protected from diarrheic symptoms of food allergy as compared to mice breastfed by naïve mothers (Fig. 6B). Decrease in diarrhea was accompanied by a decrease in the serum levels of biological markers of the allergic reaction. Both mucosal mast cell protease 1 (MMCP1), a mast-cell derived mediator of allergic reaction, and serum levels of OVA-specific IgE were decreased by a factor 3 in mice breastfed by OVA-exposed mothers compared to naïve mothers (Fig. 6B). Mice exposed only to *Der p* through breastmilk showed a similar susceptibility to food allergy as compared to mice breastfed by naïve mothers (Fig. 6B). In agreement with the observation of defective induction of OVA-specific Tregs in the presence of both OVA and *Der p* in breastmilk, we found a lack of protection from food allergy in mice breastfed by mothers exposed to both OVA and *Der p* as compared to mice breastfed by naïve mothers (Fig. 6B). Inactivation of *Der p* proteases activity restored the capacity of OVA in breastmilk to protect offspring from allergic diarrhea and to decrease OVA specific IgE as compared to mice nursed by naïve mothers (Fig. 6B).

Heterogeneity of human Breastmilk content in Der p 1 and OVA may influence food allergy risk in infants

Finally, we aimed at evaluating if data obtained in the mice could be translated to the human scenario. Therefore, we first addressed whether we could find heterogeneity in the levels of *Der p 1* and OVA in human breastmilk. We measured OVA and *Der p 1* in 100 breastmilk samples from a human cohort in which all the mothers had a history of allergic disease (Table 1). *Der p 1* was detected in 51% of samples and median value was 64.5 pg/mL among samples with concentrations above the detection limit (undetectable – 831.5 pg/mL; min-max levels; detection limit 30 pg/mL) (Fig.7A). OVA was detected in 45% of samples with a median value of 171.1 pg/mL among positive samples (undetectable - 3729 pg/mL; min-max levels; detection level 50 pg/mL) (Fig.7A). There was no correlation between OVA and *Der p 1* levels in maternal milk (Spearman r 0.002, p 0.97). We first analyzed whether the presence of *Der p 1* in breast milk was associated with a higher risk of IgE-mediated food allergy in infants at 1 year of age. Although the observation did not reach statistical significance, we found a trend to increased prevalence of food allergy to egg and cow milk in 1-year-old infants who had consumed breast milk with detectable levels of *Der p 1* compared to undetectable levels (Fig.E8 in online repository and Table E1 in online repository). Prevalence of atopic eczema was similar whether *Der p 1* was detectable or not in breast milk. (Fig.E8 in online repository and Table E1 in online repository). We then analyzed whether the 4 groups we had identified in the mice to have different egg allergy risk (Fig. 6 B) were represented in this birth cohort. We found that 27% of infants were exposed to breastmilk with undetectable levels of OVA and *Der p*, 28 % to breastmilk with undetectable levels of OVA but detectable levels of *Der p 1*, 22 % to breastmilk with detectable levels of OVA but undetectable *Der p 1* and 23 % to breastmilk with detectable levels of both *Der p 1* and OVA (Fig.7C and Table 1). In this proof of concept study of 100 infants, we further performed a preliminary analysis of egg allergy prevalence in children from these 4 groups (Fig.

7D and Table 1). Population characteristics were similar (Table 1). In particular, in this birth cohort from 2011, none of the children had introduced egg before 6 months and there was a similar proportion of egg introduction before 1 year in the 4 groups (Table 1). The IgE-mediated egg allergy prevalence was 14.8% in one year- old infant who had been exposed to breastmilk with undetectable OVA and *Der p 1* (Table 1 and Fig.7D). We found the lowest prevalence of egg allergy in infants exposed to milk containing OVA but no *Der p 1* (4.5%). Relative Risk (RR) and confidence Interval, (CI) was 0.30 (0.04-1.85) in infants exposed to breastmilk containing detectable OVA versus undetectable. In the presence of both OVA and *Der p1* in breastmilk, the prevalence of egg allergy raised to 13%; RR (CI) was 2.87 (0.44-19.33) in infants exposed to breastmilk containing OVA and *Der p* versus only OVA. The highest prevalence of egg allergy was found in infants exposed to breastmilk containing *Der p 1* but no OVA (21.4%). The relative risk of developing egg allergy was 3.4 (CI: 0.5-21.5;) when comparing the 2 extreme groups (*Der p* but no OVA versus OVA but no *Der p* detected in breast milk). No statistical difference were found upon analysis of the prevalence of egg allergy in the 4 different groups.

DISCUSSION

There is increasing evidence that food allergy may result from lack of oral tolerance induction due to an inappropriate route of allergen exposure such as exposure via the skin instead of the oral route (4). A less explored hypothesis is that food allergy may also result from defective mechanism of oral tolerance in the gut itself. Gut colonization by microbiota (38) and exposure to dietary-derived antigens are necessary for efficient oral tolerance induction in the adult mice (39). In the breastfed mice, maternal milk derived-factors such as TGF- β , antigen-specific IgG and Vitamin A were shown to be required for oral tolerance induction to breastmilk transferred dietary egg antigen (7-9, 11, 18). Here, we identify in a mouse model that the presence of *Der p* allergen in maternal milk abolishes the capacity of the neonate to mount oral tolerance to OVA egg antigen. Our data in a birth cohort further show that *Der p* 1 and OVA are found in breastmilk at various levels. The groups of lactating mice we identified to induce different risks of egg allergy in offspring were also found in the human with about 25% in each category, i.e *Der p* 1 and OVA both undetectable, *Der p* 1 and OVA both detectable, OVA but no *Der p* 1 detectable and *Der p* 1 but no OVA detectable. The preliminary analysis of egg allergy risk in children exposed to breastmilk from these 4 categories did not show statistical significance between the groups. However, a clear drawback of this proof of concept study in a human birth cohort of 100 infants is the limited sample size with small numbers of food allergy cases per comparison group. The results presented here should enable planning of a properly powered human study: distribution of OVA and *Der p*1 in breastmilk and expected disease frequencies in the 4 groups.

Previous studies showed that maternal systemic immunization to *Der p* resulted in decreased IgE responses in offspring in early post-natal life (40, 41). This observation goes along

with other studies in rodents showing inhibition of Th2 immune responses in rodents fostered by antigen-sensitized mothers (42-44). Maternal antigen-specific IgG transferred to the neonates via breastmilk were described to be responsible for this protection (45-47). In contrast, we found that lactating mothers, which are sensitized to *Der p* before mating by physiological respiratory route, and continued to be exposed to *Der p* during lactation, primed the offspring for allergic responses to *Der p* (13) and broke oral tolerance induction to bystander antigen. The data we are showing here further suggest that maternal antibodies are not key players in allergic priming observed in mice nursed by *Der p* exposed mothers. Indeed, while *Der p* and *iDer p* mothers were similarly sensitized to *Der p* and harbored equivalent amount of *Der p* specific IgG in breastmilk, only breastfeeding by *Der p* exposed mothers resulted in Th2 immune imbalance in offspring. Altogether, these observations point to the role of early oral exposure to *Der p* allergens themselves through breastmilk for increased allergy risk in offspring.

Only a few studies have considered the possibility of being orally exposed to HDM allergens and its potential contribution to digestive allergic reactions. We found *Der p* allergen to be present in digestive fluid of healthy individuals and demonstrated that it can increase gut permeability (17). Scala *et al* reported vomiting symptoms triggered by environmental exposure to HDM (48) and a few reports described anaphylaxis after ingestion of wheat flour contaminated with mite in HDM-sensitized individuals (49-52). The clinical and experimental evidence that HDM can be a cause of eosinophilic esophagitis (53) and the often reported gastrointestinal complaints during sublingual immunotherapy with HDM (54) further suggest a possible role for HDM in digestive allergies. Others have also reported that sublingual immunotherapy with HDM could promote new sensitization to some food allergen or worsen symptoms of food allergy because of significant homology between tropomyosin from HDM and

from crustacea and mollusk (55). Here, we identify a new mechanism by which HDM can promote food allergy in early life. We show that early oral exposure to HDM *Der p* allergen through breastmilk can favor development of food allergy to the unrelated food allergen OVA because of *Der p* protease induced-dysregulated mucosal immunity. We found *Der p* in breastmilk capable of triggering all the critical immunological events for development of food allergy, by promoting the Th2 immune response and by inhibiting the induction of Tregs. For this, *Der p* in breastmilk increased gut permeability by disrupting tight junction, stimulated the production of IL-33 by gut epithelial cells and activated ILC2 in the neonatal gut lamina propria.

Strikingly, we demonstrated a causal role of *Der p* proteases in maternal environment in the induction of dysregulated immunity in breastfed offspring. Indeed, our data show increased protease activity in breast milk and intestinal fluid of mice nursed by *Der p* exposed mothers and we find that only mice nursed by mothers exposed to *Der p* with intact *Der p* protease activity have dysregulated gut immunity. However, we cannot formally conclude that *Der p* protease are the sole responsible for the cascade of immune events that are occurring in offspring nursed by *Der p* exposed mothers and other immune modulatory molecules in breastmilk of *Der p* exposed mothers may play an additional role (56, 57).

The observation that milk of mothers exposed to *Der p* displayed an increased proteolytic activity using *Der p 1* preferential substrate as compared to milk of PBS-exposed mothers or to milk of mothers exposed to *Der p* allergen with no protease activity (*iDer p*), suggests that maternal exposure to *Der p* protease results in its transfer to the milk. We further confirmed that the increased proteolytic activity found in *Der p* containing breastmilk was not related to the potential release of mast cell Tryptase by neonates exposed to *Der p* as similar levels of Tryptase were found in milk collected from the stomach of pups nursed by mothers exposed to PBS or

Der p (Data not shown). The surprising presence of intact protein with conserved proteolytic activity in milk is supported by findings both in mice experimental model and in the human setting. We previously showed that *Der p* protease activity was maintained up to the large intestine upon its oral administration to adult mice (17). Hale *et al* also showed that ingested bromelain, a protein with protease activity found in pineapple, retains proteolytic activity within the murine gastrointestinal tract *in vivo* (58). Important for the translatability of our data to the human are the studies reporting the presence of un-degraded dietary antigen in blood in healthy humans (59, 60), including protein with intact protease activity (59). Furthermore, the presence of intact dietary antigen in human milk is well-described (61-64). This stresses the need to identify which factors control the transport of intact protein to breastmilk. Previous data in mice showed that maternal antibodies are not necessary for antigen transfer to breastmilk (8) and may decrease it (9). Other factors such as microbiota and maternal diet will need to be addressed. Finally and importantly, although breastmilk is known to contain protease inhibitors (65), these appear not to be sufficient to fully neutralize *Der p* protease activity. This strengthens the need to identify protease inhibitors that efficiently neutralise *Der p* activity and how to increase their activity. Previous studies also showed that endotoxin content of *Der p* was important for its allergenicity (66). However, endotoxin content of the *Der p* extract we used in the present study, was very low and the neutralisation of protease was sufficient to abolish the Th2 cascade in neonates breastfed by mothers exposed to *Der p*. This indicates that endotoxin content of *Der p* is not required for *Der p* to alter mucosal immunity in early life.

In conclusion, our study identifies a potential novel risk factor for food allergy, i.e. the presence of respiratory allergen from *Der p* in breastmilk, which may interfere with oral tolerance induction to bystander food antigen. The next steps will be to validate our observation

in a large human study. If validated, we propose that manipulating the levels of *Der p* allergen in breastmilk and/or its protease activity will be necessary to endow breast milk with the capacity to protect from food allergy by oral tolerance induction. This should add an important string to the bow of the beneficial effects of breastfeeding in an era where allergic diseases are surpassing childhood infectious disease morbidity.

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Figure Legends

Fig.1: Neonatal exposure to allergens through breastmilk

(A). *Der p* transfer through breastmilk. Future dams were exposed intra-nasally (IN) to *Der p* extracts before mating to mimic natural sensitization. To address the influence of *Der p* and its protease in breastmilk on offspring gut immunity, dams continued to be exposed to *Der p* or protease inactivated *Derp* (*iDer p*) during the whole lactation and pups were sacrificed on day 16. For control groups, mothers were exposed to PBS by IN route. **(B)** OVA transfer through breast milk. Future dams were immunized by two intra-peritoneal (IP) injections of OVA adsorbed on Alum before mating to mimic natural sensitization. After delivery and during the whole lactation period, mothers continued to be exposed to OVA by intra-gastric (IG) gavage. Control groups were breastfed by naïve mothers given water by IG gavage. Offspring that had been injected at birth with OVA specific T cells, were analysed at 28 days for OVA specific T cell expansion and conversion into FoxP3 Tregs in MLN. Others were analysed at 6 weeks for egg food allergy induced by IP immunization and oral challenges with OVA.

In the case of transfer of both OVA and *Der p* allergens through breast milk, mice were breastfed by mothers exposed to both *Der p* and OVA as described for individual exposure.

To avoid maternal *in utero* effect, all mice were delivered by naïve unexposed mothers and were fostered at birth by *Der p* exposed, OVA exposed, OVA and *Der p* exposed or unexposed mothers

Figure 2. *Der p* allergen and its protease are present in breastmilk of mice inhaling *Der p*

Mice were breastfed by mothers exposed to PBS, to *Der p* or to *iDer p* extracts by Intra-nasal (IN) route. Two hours after last exposure, 16 days-old pups were sacrificed and milk harvested

from stomach of pups. **(A)**. Concentration of *Der p 1* in breastmilk determined by ELISA. Data represent mean $\pm$ sem with $n = 4-8$ milk sample per group. **(B)** Proteolytic activity of milk using *Der p 1* preferential substrate. Lines represent the kinetics of the catalytic activity of milk of mothers exposed to PBS (black circle), to *Der p* extract (red circle) and to *iDer p* extract, which proteases activity have been inactivated (red open circle). One representative experiment out of 3 is shown. p-value calculated with one way Anova - multiple comparisons test. ** $p < 0.01$; *** $p < 0.001$

Figure 3. Early oral exposure to *Der p* through breast milk induces a transient and protease-dependent small intestine gut barrier dysfunction

Mice were breastfed by mothers exposed to PBS, *Der p* or *iDer p* extracts by IN route and neonatal gut integrity assessed at day 16. **(A)** *In vivo* transfer of FITC-Dextran was assessed in 16 days-old pups, 2h or 24h after last maternal treatment. FITC-Dextran was administered to offspring by IG and one hour later, blood was collected and fluorescence measured. **(B)** Representative image of immuno-staining of tight junction protein, ZO-1 (red), in small intestine of neonates harvested 2 hours after maternal treatment. (C). Quantitative assessment of percentage area positive for ZO-1 and mean intensity of ZO-1 staining.

Data represent mean $\pm$ sem. (A) $n = 3$ exp with 5-6 mice per group (B,C) one representative experiments with $n = 5-6$ mice per group. p-value calculated with one way Anova - multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$

Figure 4. Early oral exposure to *Der p* through breast milk induces a transient and protease-dependent increase in IL-33 in small intestine

Mice were breastfed by mothers exposed to PBS, *Der p* or *iDer p* extracts by IN route and IL-33 in gut assessed at day 16. **(A)** IL-33 quantified by ELISA in the small intestine tissue of pups exposed to PBS, *Der p* or *iDer p* through breastmilk after 2 hours or 24 hours. **(B)** Representative image of immuno-staining of IL-33 (green) in small intestine of neonates harvested 2 hours after maternal treatment. Epithelial cell staining with E-cadherin is shown in red.

Data represent mean $\pm$ sem. (A) n=2 exp with 5-6 mice per group (B,C) one representative experiments with n=5-6 mice per group. p-value calculated with one way Anova - multiple comparisons test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

Figure 5. Early oral exposure to *Der p* through breastmilk induces a protease-dependent activation of ILC2 and Th2 expansion in small intestine

Mice were breastfed by mothers exposed to PBS, *Der p* or *iDer p* extracts by IN route. *Lamina propria* cells were isolated from 16 days old mice and ILC2 and CD4⁺ T cells analyzed. **(A)** ILC gating strategy by flow cytometry. **(B)** GATA3 expression in ILC. **(C)** IL-13 and IL-5 expression in ILC after PMA-ionomycin stimulation. **(D)** IL-5 expression in ILC according to their GATA3 expression. **(E)** Intra-cellular GATA3, FoxP3 and IL-5 expression were assessed by flow cytometry after PMA-Ionomycin stimulation. Data show representative dot plots and mean $\pm$ sem of cell numbers obtained in 5-7 mice per group; n= 2 exp. p-value calculated with one way Anova - multiple comparisons test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

Figure 6. Early oral exposure to *Der p* through breastmilk inhibits oral tolerance induction to bystander dietary antigen

Neonates were breastfed by mothers exposed to PBS, Der p, OVA, OVA and Der p or OVA and iDer p, during breastfeeding. (A) OVA-specific T cell expansion and OVA specific FoxP3 Treg induction. Neonates were injected IV at day 1 with OVA specific KJ1.26⁺ TCR transgenic CD4⁺ T cells. One week after the last OVA administration to lactating mothers, mesenteric lymph nodes (MLN) were harvested and the expansion of OVA specific CD4 T cells was analysed by flow cytometry using KJ1.26 mAb. Induction of OVA specific Tregs was assessed by analyzing FoxP3 expression among injected OVA specific KJ1.26⁺ CD4⁺ T cells. Data show representative dot plots and mean $\pm$ sem of cell number obtained in 6-8 mice per group; n= 2 exp. p-value calculated with one way Anova - multiple comparisons test. (B) Susceptibility to food allergy to OVA was assessed in 6 week-old mice after 2 IP immunization with OVA and Alum and 6 oral OVA challenges. Data show mean $\pm$ sem of diarrhea score, levels of MMCP1 and OVA-specific IgE. n=2 exp with 6-8 mice per group. p-value calculated with one way Anova - multiple comparisons test. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001

Figure 7. Heterogeneity of human breastmilk content in Der p and OVA

(A). Analysis of Der p and OVA levels in human milk and egg allergy risk in offspring. (B) Der p 1 and OVA levels in human milk samples. Data represent Der p 1 and OVA levels in maternal milk samples (n=100) as determined by ELISA. Dot lines represent the detection level and solid line median value among positive samples. (C) Correlation of OVA and Der p 1 levels in breastmilk samples. For logarithmic representation of antigen levels, an arbitrary value of 1 was given to samples with undetectable OVA or Der p 1. The lines indicate the detection levels of OVA and Der p and the quadrants are defined by the detection/ non detection of OVA and Der p 1. The numbers in each quadrant indicate the number of positive sample among the whole

798 population. **(D)** IgE-mediated egg allergy prevalence in 1 year-old infant being exposed to the 4
799 groups of breastmilk as defined in (C). The numbers above each bar indicate the n and % of egg
800 allergy cases in each group as well as the RR and CI for egg allergy with the group of
801 undetectable OVA and Der p taken as reference.

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804 **Table 1: Population characteristics of human breast milk samples**

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	Whole population (n=100)	OVA ⁻ / Der p ⁻ (n=27)	OVA ⁻ / Der p ⁺ (n=28)	OVA ⁺ / Der p ⁻ (n=22)	OVA ⁺ / Der p ⁺ (n= 23)	p _{3 DF}
Maternal age at birth (year)*	34.1 (3.9)	34.3 (3.0)	34.1 (3.6)	34.1 (4.2)	33.7 (5.1)	0.96
Gestational age at birth (weeks)**	39.5 (1.2)	39.6 (1.2)	39.2 (1.3)	39.7 (1.2)	39.5 (1.1)	0.62
Infant birth weight (g)*	3486 (445)	3567 (460)	3323 (468)	3493 (400)	3582 (408)	0.12
Caesarian section **	26 (26)	6 (22)	10 (36)	4 (18)	6 (26)	0.52
Breastfed at 12 months of age **	55 (55)	17(63)	13 (46)	13 (59)	12 (52)	0.63
Duration of breastfeeding *	10.4 (2.3)	10.4 (2.6)	10.5 (1.9)	10.7 (2.2)	10.1 (2.5)	0.83
Infant male sex **	45 (45)	10 (37)	12 (43)	10 (45)	13 (57)	0.58
introduced egg in the first 12 months data available for n=83)**	74 (89)	20 (91)	22 (96)	15 (79)	17 (89)	0.41
Season of birth (spring or summer vs. the remainder)	44 (45)	14 (54)	13 (48)	8 (36)	9 (39)	0.59
Egg allergy at 12 months**	14 (14)	4 (14.8)	6 (21.4)	1 (4.5)	3 (13)	0.40

806 *Mean (±SD) ** n (per cent) *** The 3 DF test (F-test for continuous, Chi-Square or Fisher's exact test for
807 categorical variables) is comparing all 4 groups . Two-sided tests were used.

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Fig.1: Neonatal exposure to allergens through breastmilk

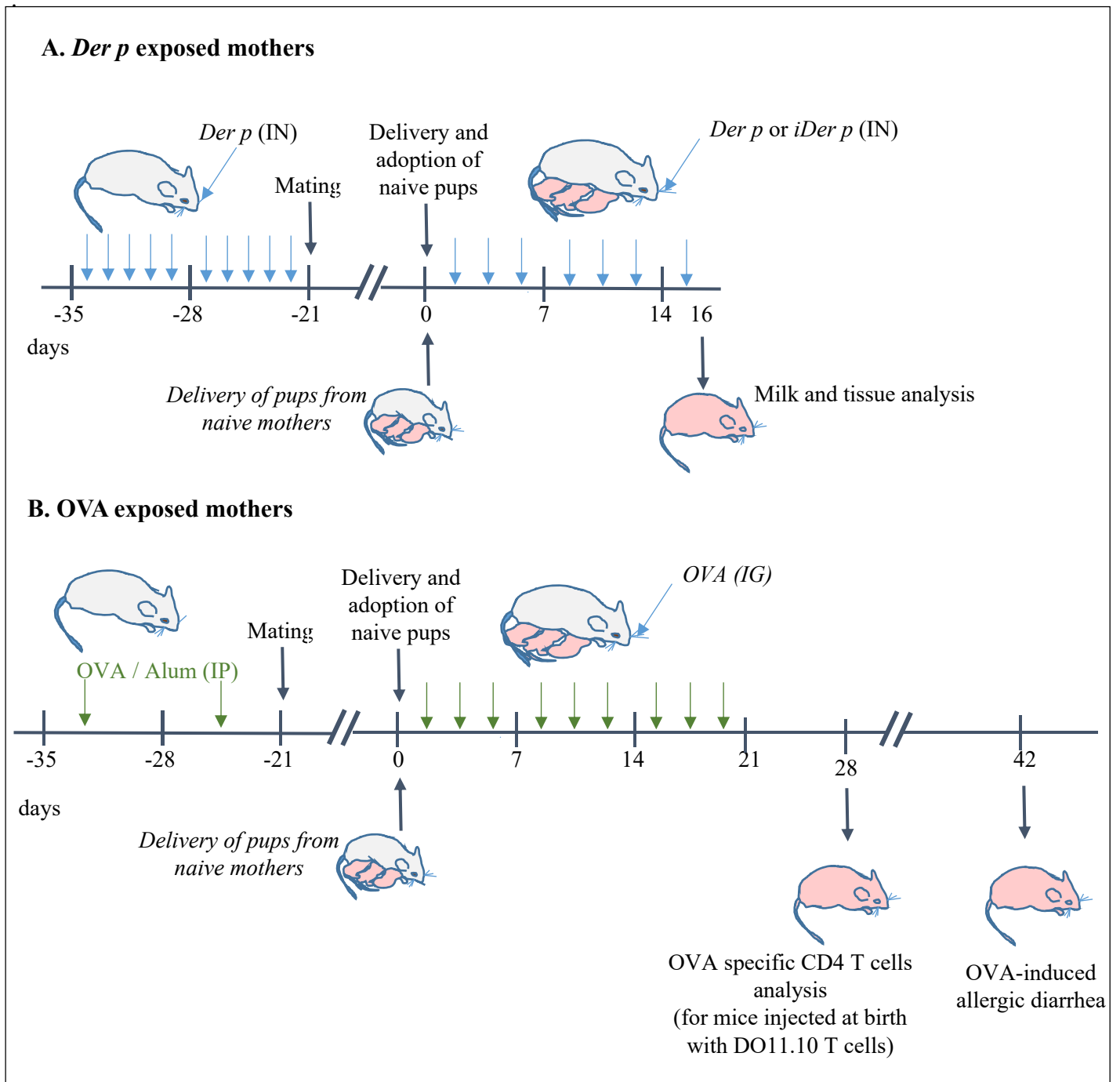


Figure 2. Der p allergen and its protease are present in breastmilk of mice inhaling Der p

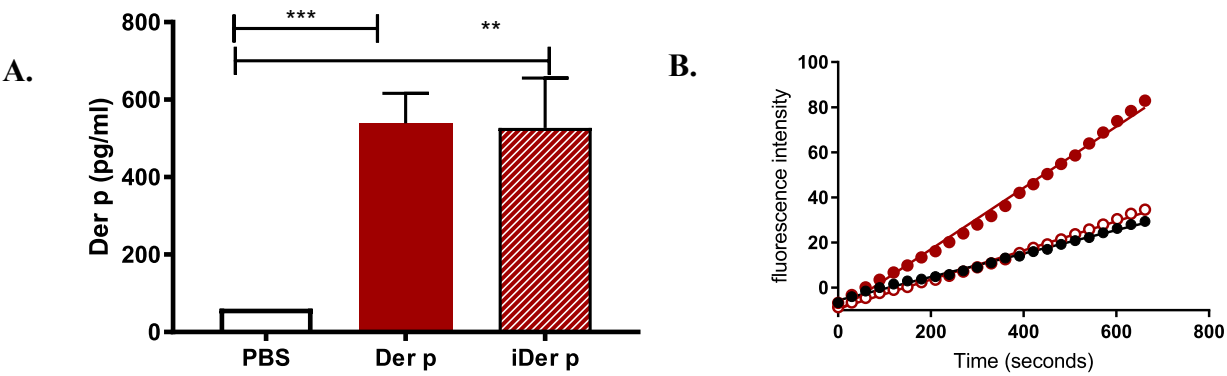


Figure 3. Early oral exposure to *Der p* through breast milk induces a transient and protease-dependent small intestine gut barrier dysfunction

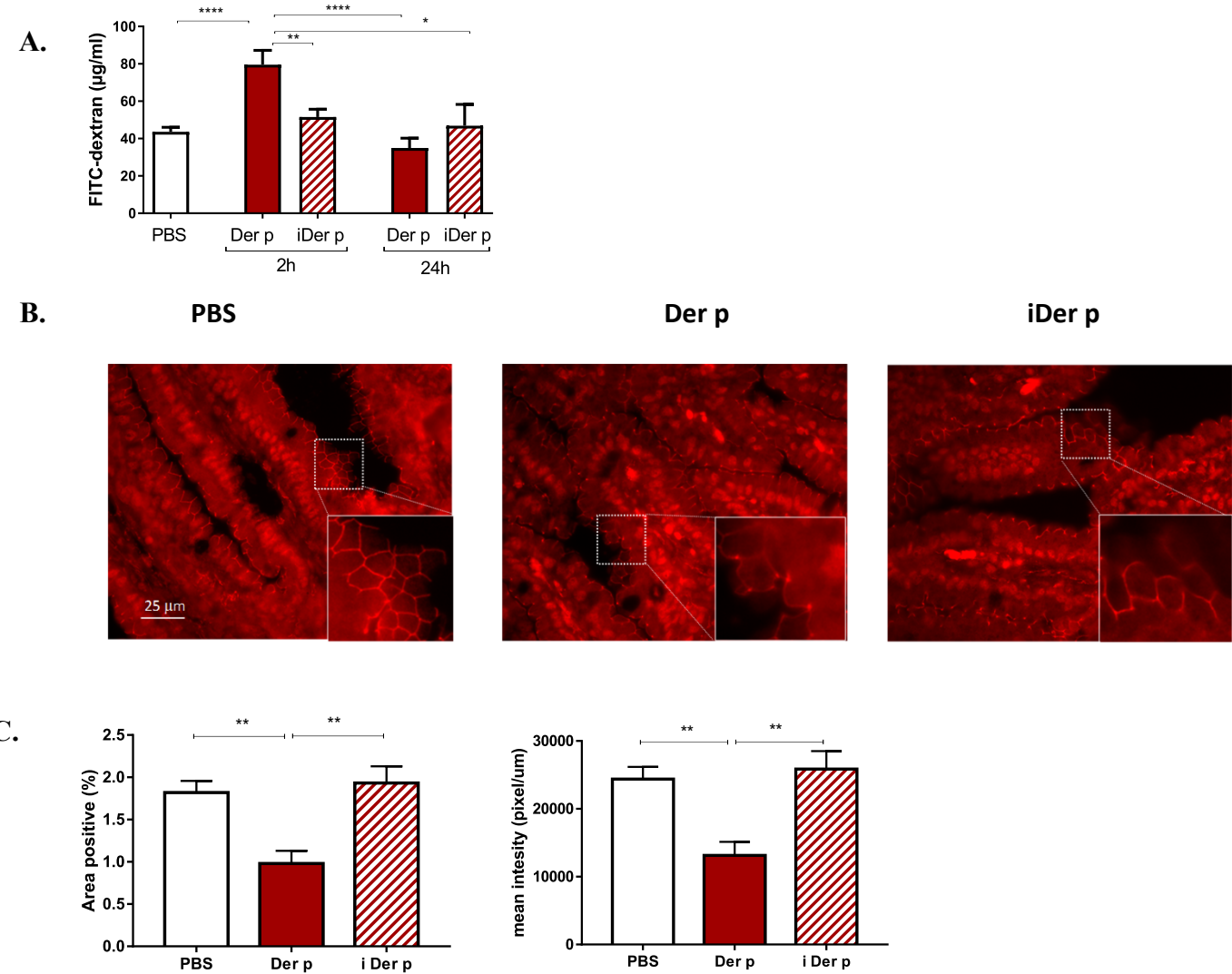


Figure 4. Early oral exposure to *Der p* through breast milk induces a transient and protease-dependent increase in IL-33 in small intestine

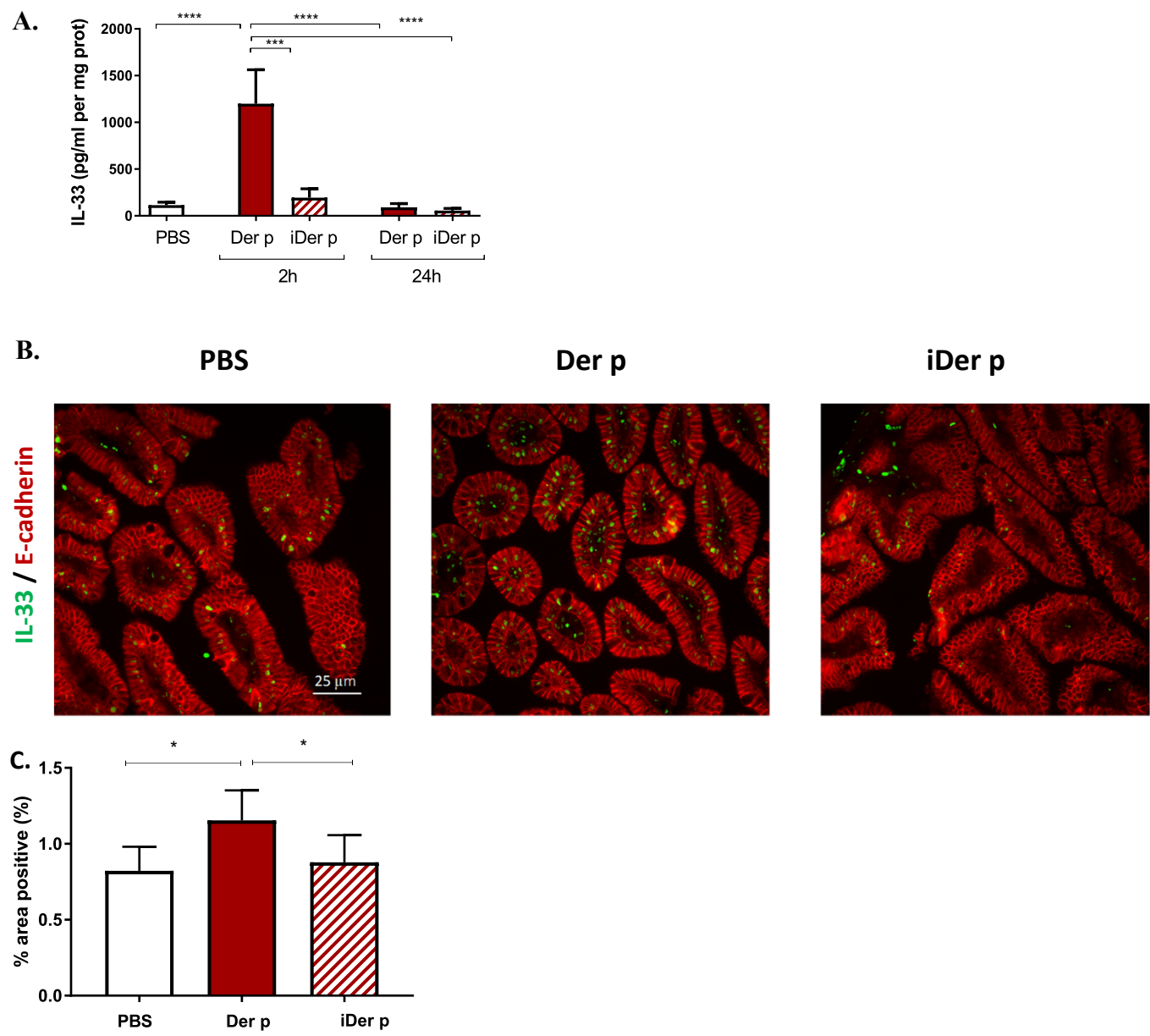


Figure 5. Early oral exposure to Der p through breastmilk induces a protease-dependent activation of ILC2 and Th2 expansion in small intestine

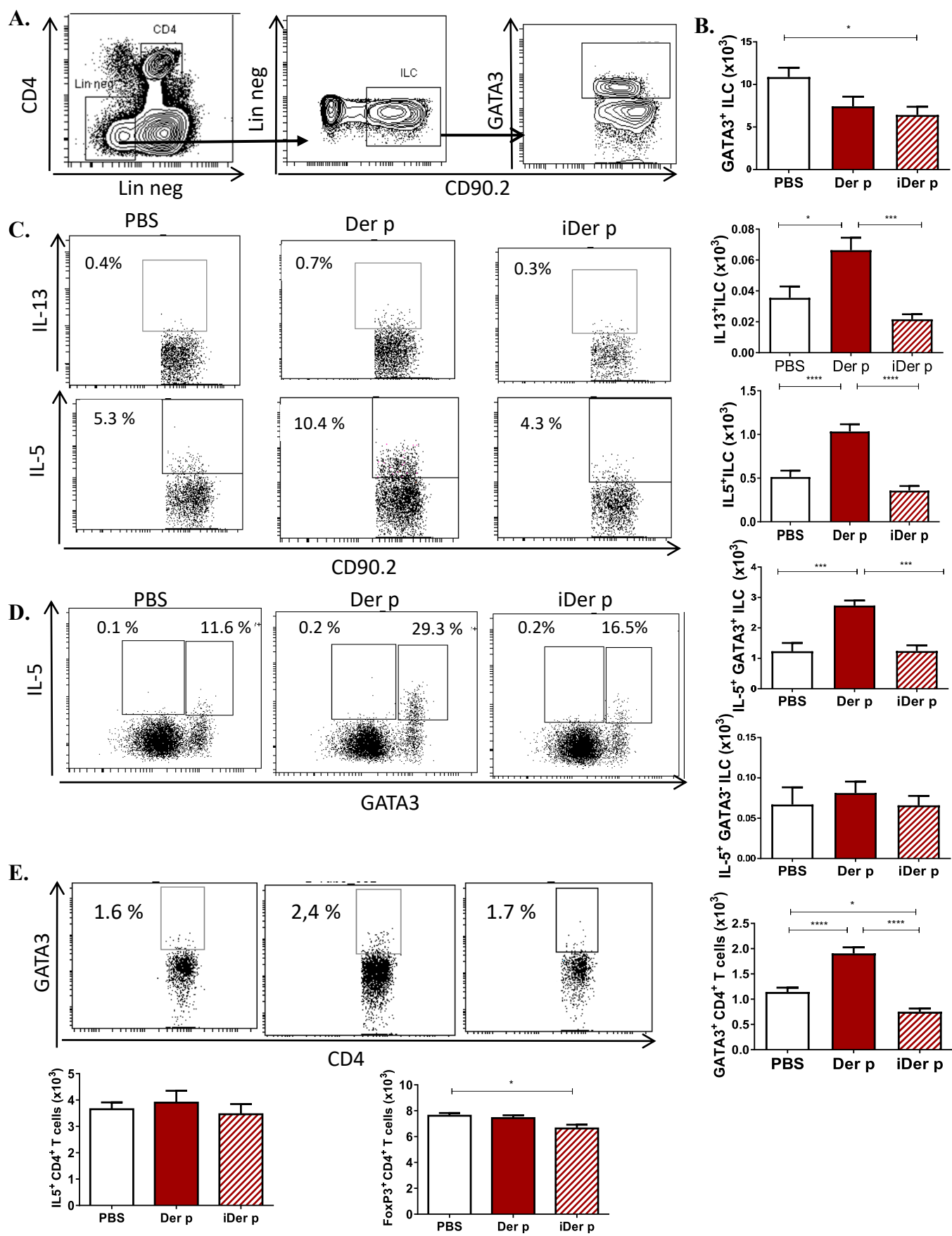


Figure 6. Early oral exposure to Der p through breastmilk inhibits oral tolerance induction to bystander dietary antigen

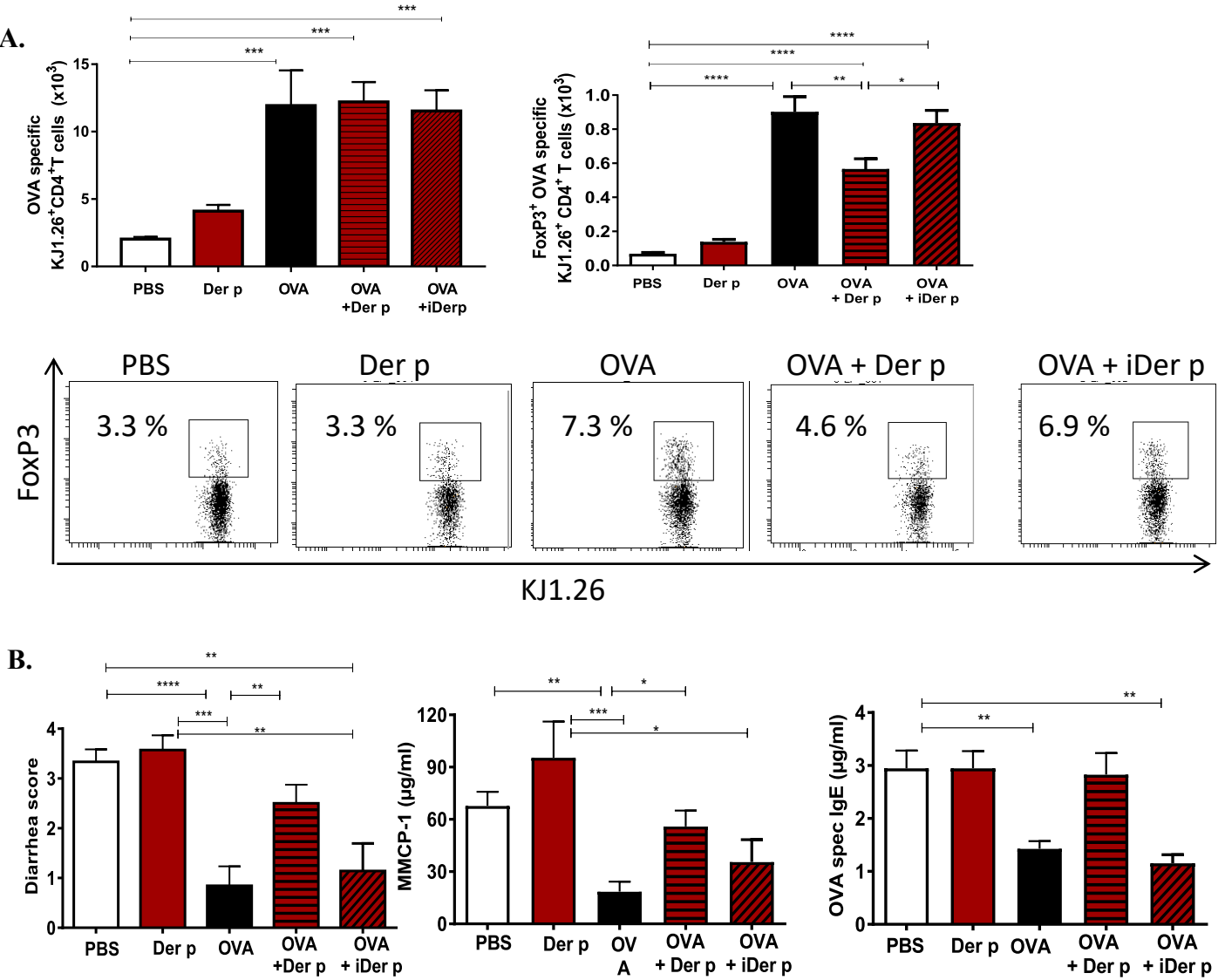
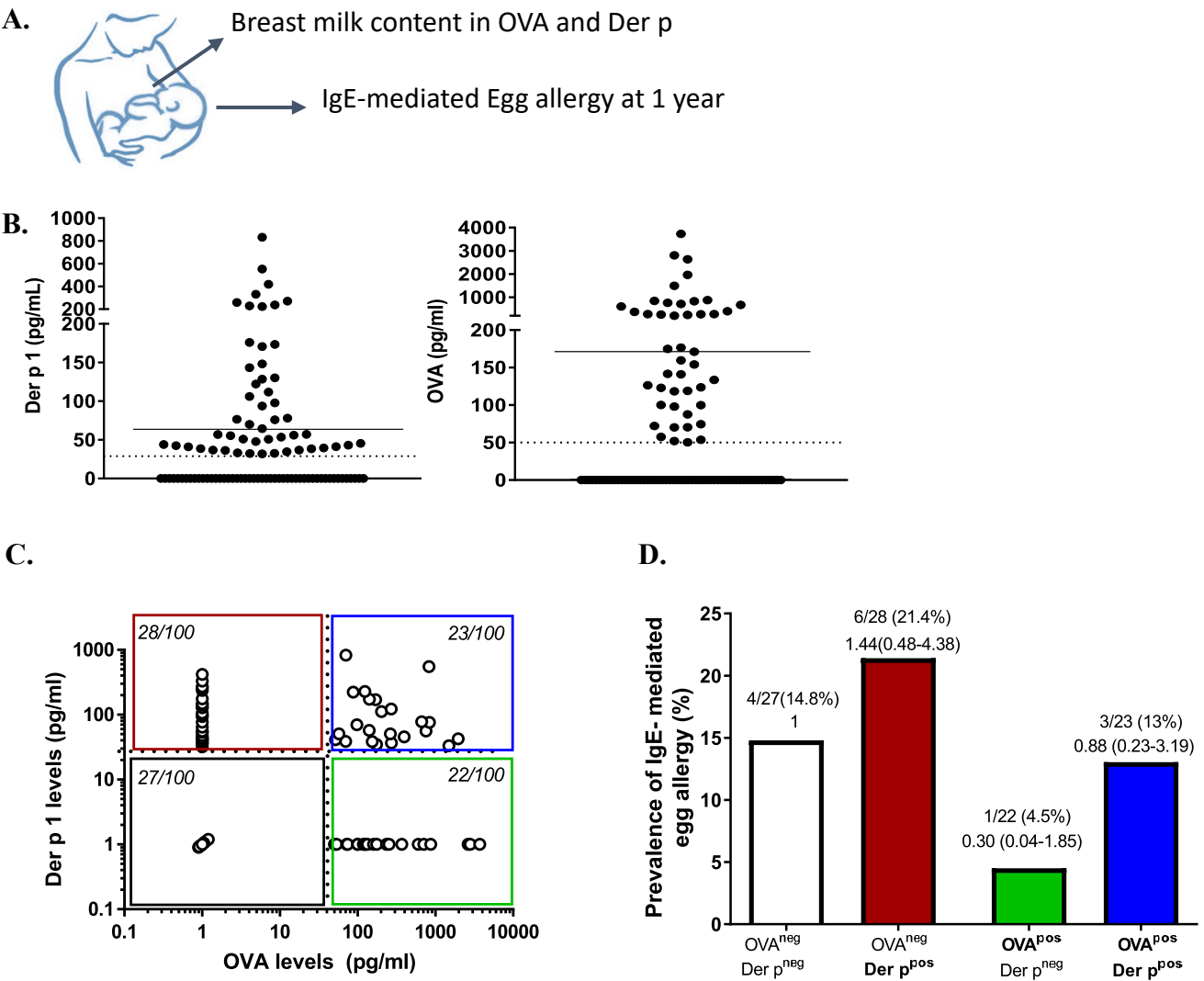


Figure 7. Heterogeneity of human breastmilk content in *Der p* and OVA



Online Repository Material

MATERIAL AND METHODS

Mouse model of Der p and OVA allergens transfer through breast milk

Der p and OVA allergen transfer to neonates through breastmilk of immunized mothers was induced as previously described (1, 2) and as illustrated in Fig.1. Briefly, for *Der p* allergen transfer, mice were exposed intranasally (IN) to *Der p* extract (Stallergenes, Anthony, France) containing 20µg of *Der p* 1 for 10 days before mating (Fig. 1). Two days after the last exposure, *Der p*-exposed mice were mated and after delivery, they continued to be exposed 3 times a week to 20 µg of *Der p* or *Der p* extract with inactivated protease (*iDer p*) during the whole lactation period (1). Control groups were exposed to PBS by IN route.

For OVA transfer (2), mice were immunized by two intra-peritoneal (IP) injections with 10 µg chicken OVA (grade V, Sigma-Aldrich, Lyon, France) adsorbed on 2 mg aluminium hydroxide (Alum) (Thermo Fisher Pierce, Illkirch, France) at intervals of one week and were mated two days after the last sensitization (Fig.1). After delivery, mothers were given 2 mg OVA by intra-gastric gavage, 3 times a week during whole lactation period. Control groups were breastfed by naïve mothers given water by intra-gastric gavage.

For transfer of both OVA and *Der p* allergens through breast milk, mice were exposed to both *Der p* and OVA before mating and during lactation as described above. A 4 hours interval was allowed between *Der p* and OVA administration to the lactating mother.

To avoid maternal *in utero* effect, all mice were delivered by naïve unexposed mothers and were fostered at birth by *Der p* exposed, OVA exposed, OVA and *Der p* exposed or unexposed mothers.

Small intestine ILC and CD4 T cells isolation and characterization

Two week-old pups were sacrificed by lethal IP administration of Pentothal and the small intestine was harvested for Lamina Propria (LP) cells analysis using a protocol adapted to neonates. Briefly, fat and Peyer's patches were removed, and the small intestine was extensively and carefully washed with RPMI 1640 (GIBCO, Thermo Scientific, Courtaboeuf, France) containing antibiotics, 3% Fetal bovine serum (FBS Saint Aubin, France) and 2mM EDTA (referred here below as complete medium). Epithelial cells were removed by a first incubation of small intestinal pieces in complete medium containing 0,145mg/mL dithiothreitol (Sigma; Aldrich, Lyon, France) for 10 minutes at 37°C. After vigorous agitation, gut pieces were harvested and submitted to a second round of incubation in complete medium for a period of 10min. Gut pieces were then digested with 10 µg/mL liberase (Roche Diagnostics, Meylan, France) in the presence of 100 µg/mL DNase I (Roche Diagnostics, Meylan, France) for 10 min minutes at 37°C under agitation. Cell suspensions were recovered after filtration through a 70µm nylon filter. Then the cell are centrifuged for 7 min at 1300 rpm and filtered through a 40µm nylon filter and counted

Flow cytometry Analysis of phenotype and cytokine production by innate lymphoid cells and T lymphocytes

ILC were identified among live cells by the expression of CD45 (30-F11), CD90.2 (53-2.1) and the non expression of lineage markers (CD4 (RM4-5), CD3 (17A2), CD11b (M1/70), B220 (RA3-6B2), CD49b (DX5)). ILC2 were further identified by the intra-nuclear expression of GATA3 (TWAJ) after cell fixation and permeabilization using transcription factor buffer set (ebioscience, Paris, France). CD4 T cells were identified among CD45 expressing cells using CD4 (RM4-5) and CD3 (17A2) mAbs. Th2 cells and Tregs were further identified after cell fixation and permeabilization using transcription factor buffer set

(ebioscience, Paris France) using mAbs directed against GATA-3 (TWAJ) and FoxP3 (FJK-16S) respectively. IL-5 (TRFK5) and IL-13 (eBio13a) production was analyzed in ILC and CD4 T cells after PMA-ionomycin stimulation and cell fixation and permeabilization. Cell labelling was analysed by flow cytometry using FACS Canto flow cytometer (BD).

Immunofluorescence staining in neonatal small intestine

Immunofluorescence staining and analysis was performed by a blinded operator of the iBV Experimental Histology Platform at Valrose Institute, Nice, France. ZO-1, IL-33 and E-cadherin immunofluorescence staining was performed on 5 µm thick paraffin sections of jejunum section from 2 weeks old mice. All sections were then deparaffinized in xylene, rehydrated through alcohol, and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. Antigen retrieval was performed in EDTA buffer pH 9.0, in a de-cloaking chamber (Dako, S2367). Section were blocked in 5% BSA for 1H and then incubated overnight with antibody against ZO-1 (Thermoscientific, 40-2300, 1/250). Donkey anti-rabbit secondary antibodies (donkey Anti Rabbit-A594, Invitrogen, A21207, 1/500) was then applied. For IL-33 staining, the section were incubated overnight with antibody against IL-33 goat (R&D, AF3626, 1/40) and with antibody against E-Cadherin rabbit (New England Biolabs, 31955, 1/1000). Donkey anti-goat secondary antibody (donkey anti-Goat-A488, Invitrogen, A11055, 1/500) and Donkey anti secondary rabbit antibodies (donkey Anti Rabbit-A594, Invitrogen, A21207, 1/500) were then applied. Images were collected on an Axioplan2 upright microscope (20X, NA 0.3; 40X NA,0.75). Quantitative analysis of immunostained whole gut sections were performed using ImageJ processing software.

In vivo induction of FOXP3 expressing OVA specific T cells

We followed the induction of FOXP3 expression in OVA specific T cells injected in 1 day old mice following a previously published protocol (3). Splenocytes and lymph nodes cells from naïve RAG-2^{-/-} DO11.10 TCR transgenic mice were prepared and CD4⁺ T cells were purified by negative selection using the CD4⁺ T cells isolation kit (Stemcell, Grenoble, France). One million CD4⁺ T cells were injected intravenously into 1-day-old BALB/c pups that were breastfed by mothers exposed to PBS, *Der p*, OVA, *Der p* and OVA or *iDer p* (protease-inactivated) and OVA. One week after last OVA administration to mothers, mesenteric lymph nodes were harvested and cell suspension analyzed by flow cytometry. Injected DO-11.10 CD4 T cells were identified using anti-CD3, anti-CD4 and KJ1.26 mAb (ebioscience, Paris, France). OVA specific CD4 T cells were analyzed for FoxP3 expression after cell fixation and permeabilization using transcription factor buffer set (ebioscience, Paris, France).

OVA-induced allergic diarrhea in the offspring

Mice were tested for their susceptibility to OVA-induced allergic diarrhea when 6-8 week old using a protocol previously published (4). Female mice received two IP injections with 50 µg chicken OVA and 1 mg Alum (Sigma-Aldrich, Lyon, France) at 2-week intervals. Two weeks after the second sensitization, the mice were fasted for 3 hours and challenged every 2 to 3 days with 50 mg OVA by intra-gastric gavage for a total of 7 challenges. Mice were placed in individual cages and monitored for diarrhea symptoms for one hour. The mice were scored according to feces appearance as follows: score 0= no change; score 1= well formed; score 2= soft, non-formed; score 3= one episode of liquid diarrhea; score 4= at least two episodes of liquid diarrhea. One hour after the last challenge, mice were sacrificed by lethal IP administration of Pentothal and blood and gut tissue harvested for further analyses. Scoring was performed independently by 2 examiners. One hour after the last OVA

challenge, mice were euthanized and serum collected. MMCP1 levels in serum were quantified by ELISA (ebiosciences, Paris, France) to evaluate mast cells degranulation (4). Allergic sensitization to OVA was evaluated by measuring the levels of OVA-specific IgE as previously described (3).

TABLE E1. Association between Der p 1 levels in breast milk and risk of IgE mediated allergic disease

	Whole population n=100 (%)	Der p - n=49 (%)	Der p + n=51 (%)	RR (95% CI)	Pre vale nce of alle rgy in
Food allergy	17 (17)	7 (14)	10 (20)	1.2 (0.8 - 1.9)	
Hen's Egg	14 (14)	5 (10)	9 (18)	1.3 (0.8 - 2.1)	
Cow's Milk	3 (3)	1 (2)	2 (4)	1.3 (0.6 - 3.0)	
Peanut	3 (3)	2 (4)	1 (2)	0.7 (0.1 - 3.3)	
Atopic Dermatitis	16 (16)	8 (17)	8 (16)	1.0 (0.6 - 1.6)	

one year-old infant with detectable (Der p +) versus non detectable (Der p -) Der p in maternal milk. Relative Risk (RR) with confidence interval (CI) are also shown.

FIGURE LEGENDS

Fig. E1: Catalytic activity of Der p and iDer p extract. Catalytic activity was measured in a continuous rate assay using the fluorogenic peptide substrate Boc- Gln-Ala-Arg-AM. Lines represent the progression curves of the catalytic activity of Der p extract (red circle), of Der p extract with addition of serine protease inhibitor, AEBSF, and cysteine protease inhibitor, E-

64, in the assay (black open circle) and of iDer p extract, which protease activity have been inactivated by pre-incubation with AEBSF and E-64 (red open square). Both Der p and iDer p have been passed on desalting column before being tested for protease activity.

Figure E2. Der p allergen and its protease are present in breastmilk of mice inhaling Der p. Mice were breastfed by mothers exposed to PBS, to Der p or to protease-inactivated Der p (iDer p) extracts by Intra-nasal (IN) route. Two hours after last exposure, 16 days-old pups were sacrificed and milk harvested from stomach of pups for measurement of proteolytic activity using Der p 1 preferential substrate. Data are expressed as the slope of the curve of fluorescence accumulation and represent the mean $\pm$ SD of 3 experiments. p-value calculated with one way Anova - multiple comparisons test. * $p < 0.05$.

Fig. E3: Der p allergen and its protease are present in intestinal fluid of neonated nursed by mothers inhaling Der p

Mice were breastfed by mothers exposed to PBS, *Der p* or to i*Der p* extracts by Intra-nasal (IN) route. Two hours after last exposure, 16 days-old pups were sacrificed and intestinal fluid collected after instillation of 0.5 ml of PBS. **(A).** Concentration of *Der p 1* in intestinal fluid determined by ELISA. Data represent mean $\pm$ sem with $n = 4-6$ sample per group. **(B)** Proteolytic activity of intestinal fluid using *Der p 1* preferential substrate. Lines represent the kinetics of the catalytic activity of fluids collected from pups exposed to PBS (black circle), to *Der p* extract (red circle) or to i*Der p* extract. One representative experiment out of 2 is shown.

Fig.E4: Der p and iDer p exposed mothers have similar levels of Der p specific IgG in breastmilk and Der p IgE and IL-5 in serum.

Data represent mean $\pm$ sem of levels of Der p Specific IgG in breastmilk (n=3), Der p specific IgE in sera (n=15) and IL-5 in sera (n=5) of mothers exposed to PBS, *Der p* or *iDer p* extract

Fig.E5. Cell frequency in lamina propria from small intestine and MLN.

Mice were breastfed by mothers exposed to PBS, *Der p* or protease-inactivated *Der p* (*iDer p*) extracts by IN route (A,B) or by mothers exposed to PBS, OVA, OVA and *Der p* or OVA and *iDer p* (C). *Lamina propria* cells (A,B) and MLN cells (C) were isolated from 16 days old mice, and analyzed by flow cytometry. Data show mean $\pm$ sem of results obtained in 5-7 mice per group; n= 2 exp

Figure E6. Early oral exposure to Der p through breastmilk induces an IL-33 - dependent activation of gut ILC2

Mice were breastfed by mothers exposed to PBS or *Der p* extracts by IN route. To neutralize IL-33, we adapted a protocol previously published by Kim et al (5). Neonates were injected with 0.15 μ g/ g (mice weight) of neutralizing IL-33 IgG (RD) or control goat IgG (RD) every 2 days during the lactation period . *Lamina propria* cells were isolated from 16 days old mice and ILC2 and CD4⁺ T cells analyzed. IL-5 expression in ILC after PMA-ionomycin stimulation is shown. Data show mean $\pm$ sem of cell numbers obtained in 6 mice per group; n= 1 exp. p-value calculated with one way Anova - multiple comparisons test

Fig. E7. OVA levels in breastmilk of mice

Mice were breastfed by mothers exposed to PBS, to OVA, OVA + Der p or to OVA + protease-inactivated Der p (iDer p) extracts . OVA was given by IG route and Der p by IN route. Two hours after last exposure, 16 days-old pups were sacrificed and milk harvested from stomach of pups. Concentration of OVA in breastmilk was determined by ELISA. Data represent mean +/- sem with n= 3 milk sample per group

Fig.E8. Prevalence of IgE-mediated allergy in children according to the presence of Der p 1 in breast milk

Prevalence of IgE-mediated food allergy to egg, cow milk (CM) or peanut and of atopic eczema in children with undetectable levels of Der p 1 in maternal milk (open column; <30 pg/ml) or detectable levels of Der p 1 (red columns; ≥ 30 pg/ml).

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Fig. E1: Catalytic activity of *Der p* and *iDer p* extract..

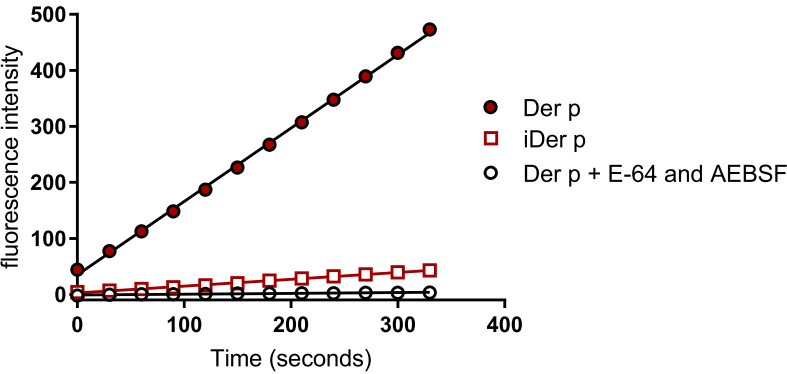


Figure E2. Der p protease are present in breastmilk of mice inhaling Der p

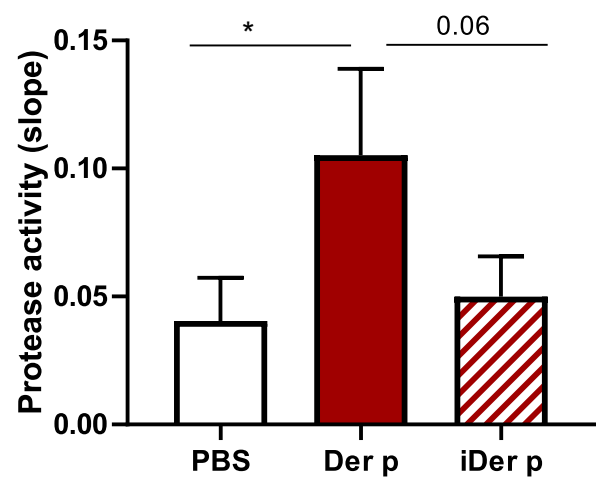


Fig. E3: Der p allergen and its protease are present in intestinal fluid of neonates nursed by mothers inhaling Der p

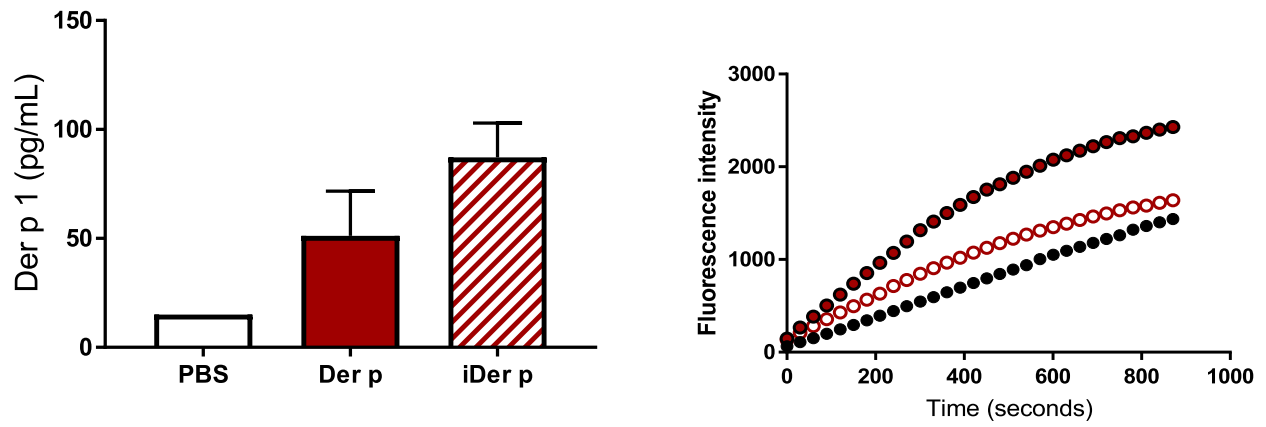


Fig.E4: Der p and iDer p exposed mothers have similar levels of Der p specific IgG in breastmilk and Der p IgE and IL-5 in serum.

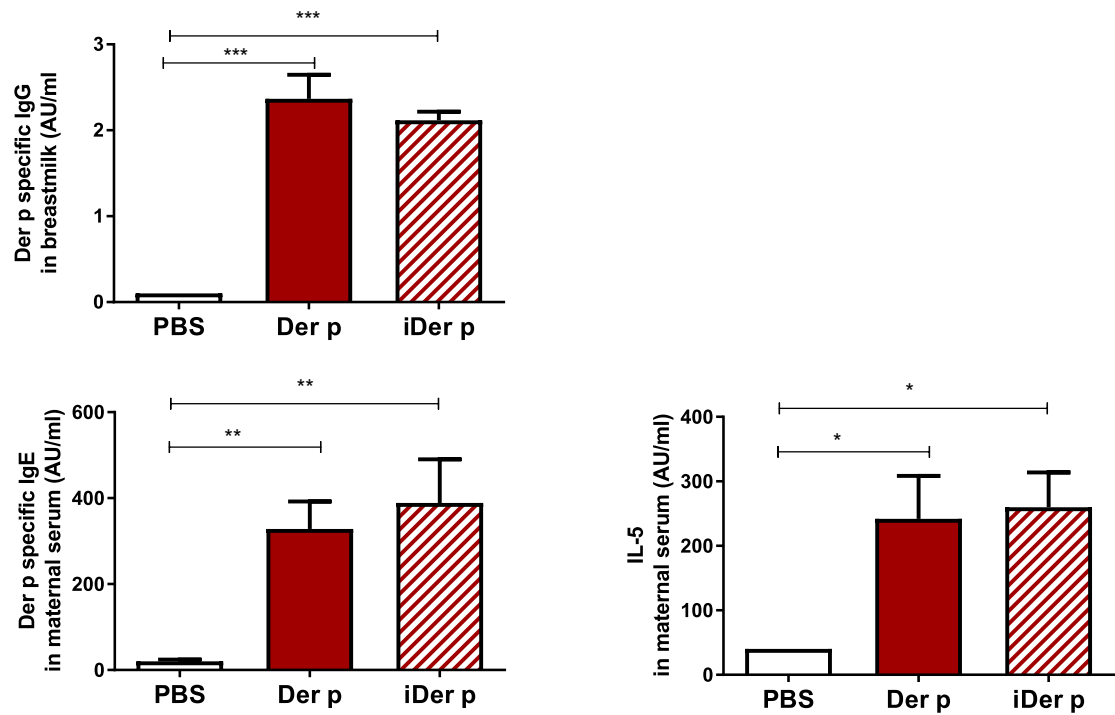
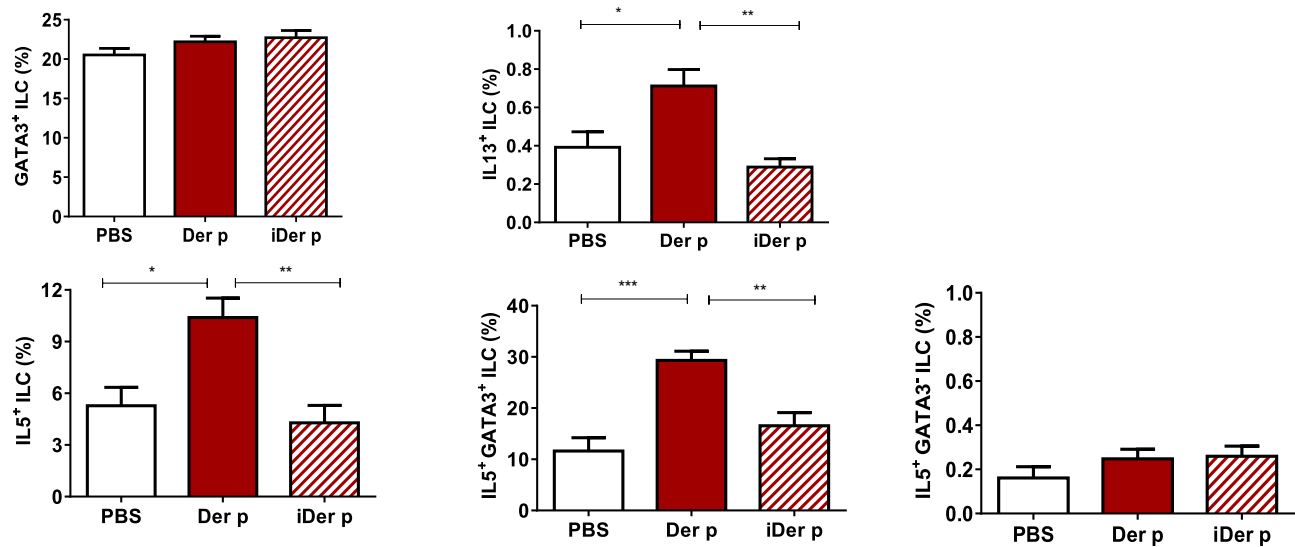
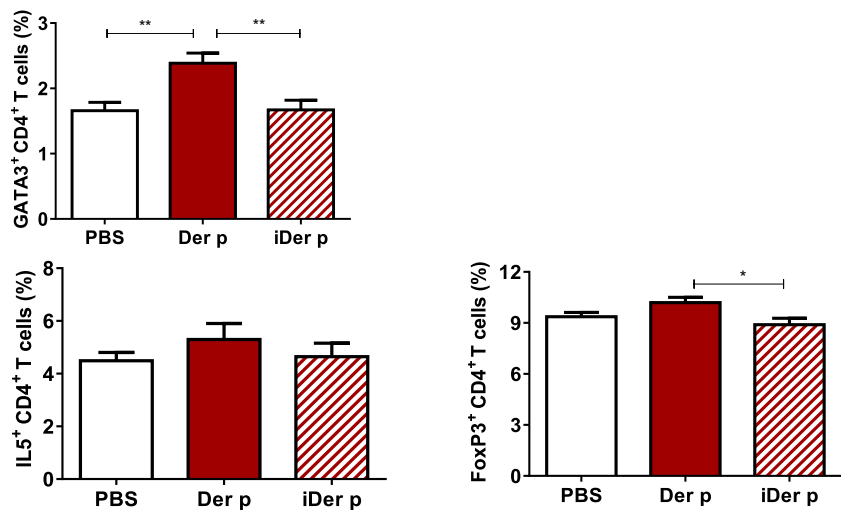


Fig.E5. Cell frequency in lamina propria from small intestine and MLN.

A.



B.



C.

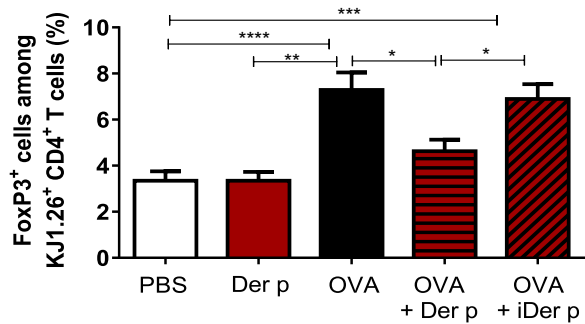


Figure E6. Early oral exposure to Der p through breastmilk induces an IL-33 -dependent activation of gut ILC2

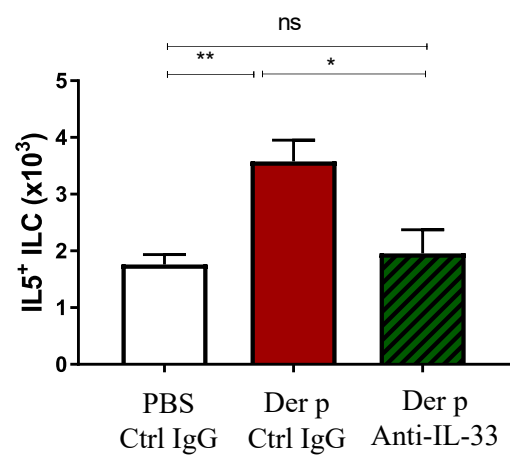


Fig. E7. OVA levels in breastmilk of mice

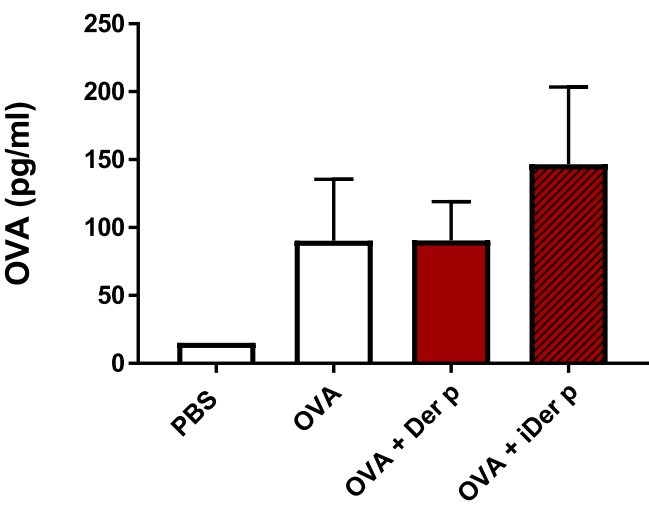


Fig.E8. Prevalence of IgE-mediated allergy in children according to presence of Der p 1 in breast milk

