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A dietary intervention with non-digestible oligosaccharides and partial hydrolysed whey protein prevents the onset of food allergic symptoms in mice

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ABSTRACT

Strategies to prevent food allergy to common food such as cow's milk are important because there is no causal treatment available yet. Oral tolerance induction is of great importance for allergy prevention which is strongly dependent on allergen exposure and proper immune environment. Improving the efficacy of oral tolerance with adjuvants is a promising new strategy. In the current study, we investigated non-digestible oligosaccharides (NDO) on their capacity to enhance oral tolerance induced by partial hydrolyzed whey protein (pWH). Mice were treated orally with PBS, pWH, NDO or pWH + NDO for 6 days and subsequently fed a control diet while sensitized to whey protein. Acute allergic skin responses and mast cell activation were measured after whey challenge. pWH + NDO prevented acute allergic skin responses, mast cell activation and induced lower whey-specific IgE compared to NDO fed mice. In mice supplemented with either pWH or NDO, respectively Foxp3+ regulatory T-cells or galectin-9 were enhanced. Increased CD103+ dendritic cells percentages were measured in the mesenteric lymph nodes of mice treated with pWH + NDO. These data show the capacity of NDO, if combined with a pWH, to prevent the onset of allergic symptoms. NDO could act as adjuvant in preventing allergic responses to harmless food proteins.

1. Introduction

Food allergy develops when the body fails to develop oral tolerance, which allows allergic sensitization and subsequently the production allergen-specific IgE. Allergies to cow's milk and hen's egg are the most common food allergies in children and prevention strategies for food allergy have been mainly focused on avoidance of the offending food. This changed during the last decade to strategies aiming at supporting oral tolerance by early introduction of solid foods. In children suffering from severe atopic dermatitis or allergic to hen's egg, it was shown that early introduction of peanut protected against the development of peanut allergy [1–3]. For food proteins like hen's egg or cow's milk proteins, the effects of early introduction on oral tolerance induction and the prevention of allergic symptoms remains uncertain and early introduction might lead to unwanted and dangerous allergic reactions [4–6]. The possibility of severe side effects is one of the major drawbacks of using whole protein for oral tolerance induction. Many processing techniques are used for reducing the allergenicity of food proteins. For cow's milk allergy hydrolyzation processes are used to reduce the allergenicity of cow's milk proteins. It has been demonstrated that pWH retained the capacity to induce allergen specific oral tolerance to the native whey protein. Protective effects coincided with increased Foxp3+ regulatory T-cells that could confer the protective effects to naïve recipient mice [7,8]. Formulas containing hydrolyzed proteins are commonly used for infants with a high risk of developing allergic diseases.

CD103+ dendritic cells (DC) are present in the small intestine and they migrate to the mesenteric lymph nodes (MLN), where they initiate and support oral tolerance [9]. CD103+ DC are described as important regulators of oral tolerance by driving gut homing of

Abbreviations: NDO, non digestible oligosaccharides; pWH, partial hydrolysed whey protein; MLN, mesenteric lymph nodes; DC, dendritic cells.

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Foxp3+ regulatory T-cells [10–13]. Evidence suggests that environmental factors like dietary components, mucosal epithelium, luminal bacteria and other immune cells can condition CD103+ DC [9]. The contribution of CD103+ DC in oral tolerance induction to food proteins like hen's egg has been shown recently [14].

Breastfeeding is considered to be the gold standard for the prevention of allergic disease. It provides a unique combination of lipids, proteins, carbohydrates, vitamins and minerals. Furthermore, there are numerous bioactive compounds present in human milk with immunological properties. One of the potential protective mechanisms of breastfeeding is the activity of oligosaccharides which are abundantly present in human milk [15,16]. To mimic some of the health and immune promoting properties of human milk oligosaccharides, a mixture of neutral and acidic non-digestible oligosaccharides was developed resembling the structure of human milk oligosaccharides. This mixture of non-digestible oligosaccharides (NDO) exerts immune modulatory effects in several experimental disease models [17–19]. Based on these data, it can be hypothesized that NDO fulfill a supportive role in preventive strategies for food allergy. NDO could act as adjuvant by supporting allergen-specific oral tolerance to harmless food proteins.

In the current study, it was hypothesized that the introduction of pWH + NDO before sensitization is more effective in preventing the onset of allergic symptoms than pWH alone. In a mouse model for cow's milk allergy, we analyzed the effects of NDO in oral preventive protocols on the development of allergic symptoms and effects on immune regulatory mechanisms, such as mucosal epithelium derived galectin-9, Foxp-3+ regulatory T-cells and CD103+ DC to support oral tolerance induction.

2. Methods

2.1. Cow's milk proteins and pWH

Whey was obtained from DMV International, Veghel, the Netherlands and hydrolyzed with an established mixture of endopeptidases and exopeptidases (confidential enzyme composition used by Nutricia Research) resulting in partial hydrolyzed whey protein (pWH). The enzymatic process was stopped by fast cooling. The pWH was characterized by analysis of the peptide size (85% < 1 kD, 8% < 2 kD, 4% < 5 kD, 1% < 10 kD, 0.6% < 0 kD and 1.4% > 20 kD) by means of high pressure liquid chromatography. This experimental whey hydrolysate was used in the animal studies as mentioned below.

2.2. Reagents and antibodies

Cholera toxin was purchased from Quadratech Diagnostics, Epsom, UK. Biotin labeled rat anti-mouse IgE, IgG_1, FITC-conjugated anti-CD4 (L3T4), PE-conjugated anti-CD25, APC-conjugated anti-CD103, PerCP-Cy5.5-conjugated anti-CD11c and isotype controls were obtained from Pharmingen, Alphen a/d Rijn, the Netherlands. APC-conjugated Foxp3 was obtained from eBioscience, San Diego, CA, USA. PBS was obtained from Cambrex Bio Science (Verviers, Belgium), streptavidin-horse radish peroxidase was obtained from Sanquin, Amsterdam, the Netherlands. Collagenase IV and DNAse 1 were obtained from Roche Diagnostics, Almere, the Netherlands. All other chemicals were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands.

2.3. Diets

Semi-purified cow's milk protein free AIN-93G-based diets were composed and mixed with NDO by Research Diet Services (Wijk bij Duurstede, the Netherlands). NDO of less complex structures than human milk oligosaccharides have been used as components in several dietary products to resemble the beneficial effects of human milk oligosaccharides. A specific mixture of short-chain-galacto- and long-chain-fructo-oligosaccharides enriched with acid-oligosaccharides was used in the current study. Structural aspects show similarity to human milk oligosaccharides in sugar chain length and molecular weight ranges. Moreover, human oligosaccharides start with a lactose moiety just as a prominent fraction of the galacto-oligosaccharides. The NDO mixture contained a 2 w/w% (9:1:2) mixture of short-chain galacto-oligosaccharides (scGOS, obtained by enzymatic elongation of lactose with galactose by β-galactosidase), long-chain fructo-oligosaccharides (lcFOS, derived from chicory inulin) and acidic oligosaccharides (pAOS, produced from pectin) as described previously [19] and is indicated as NDO throughout the manuscript. All oligosaccharides were exchanged for the same amount of total carbohydrates resulting in a comparable carbohydrate composition in the diets. The diets were stored at −20° C prior to use.

2.4. Animals

Three- to 4-week-old pathogen free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maastricht, the Netherlands), maintained on cow's milk protein free standard mouse chow (AIN-93G soja, Research Diets Services, Wijk bij Duurstede, the Netherlands). Animal care and use of animals were performed in accordance with and approved by the guidelines of the Dutch Committee of Animal Experiments (2010.III.02.023).

2.5. Oral tolerance induction, oral sensitization and challenge of mice

Prior to whey sensitization mice were fed a control diet and orally treated (daily; day −7 until day −2) with PBS or pWH (50 mg, once a day) using a blunt needle. Another group of mice were fed the NDO for 6 days with or without additional oral administration of the pWH. Subsequently, mice were fed a control diet and orally sensitized by gavage dosing, on day 0, 7, 14, 21 and 28 with whey (20 mg) per animal homogenized in PBS (0.5 ml) mixed with cholera toxin (10 μg), as previously described [20,21]. Non-sensitized mice received cholera toxin in PBS only. At day 33, five days after the last sensitization, the acute allergic skin response (ear swelling at 1 h) after intradermal whey challenge was measured. Mice were subsequently orally challenged with 0.5 ml of whey (100 mg/ml PBS) and 18 h later blood samples were collected, centrifuged for 15 min at 20,000*g* and stored at −70 °C until further analyses. A schematic representation of the timeline of tolerance induction, oral sensitization and challenge protocol is provided in Fig. 1.

2.6. Acute allergic skin response

An acute allergen-specific skin response was determined in all whey-sensitized mice, 1 h after intradermal challenge with whey (10 μg) in the ear pinnae. As a negative control, non-sensitized mice were challenged in the ear with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice. The ear swelling is expressed as delta μm.

2.7. Measurement of serum specific antibodies, mouse mast cell protease-1 (mMCP-1) and Galectin-9

Whey-specific IgE and IgG_1 levels were measured in serum by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, the

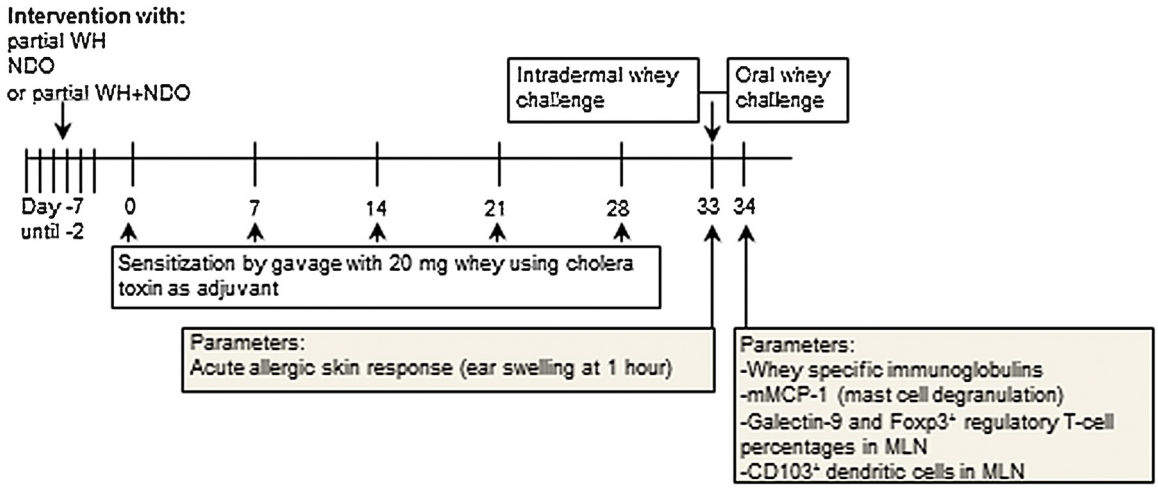

Fig. 1. Experimental set-up.
Mice fed a control diet were sensitized orally by gavage dosing, on day 0, 7, 14, 21 and 28 using cholera toxin as an adjuvant. Prior to whey sensitization mice were treated orally (daily; day −7 until day −2) with PBS or pWH while being fed the control diet, the NDO diet or pWH + NDO for 6 days (50 mg, once a day). Five days after the last sensitization (day 33), the acute allergic skin response was determined after intradermal whey challenge. At day 33 also an oral challenge was given with 50 mg whey and at day 34 the mice were sacrificed and whey-specific antibodies, serum mMCP-1 concentrations (as a reflection of mucosal mast cell activation), regulatory T-cell percentages in MLN, epithelium derived galectin-9 and CD103+ DC in MLN were determined.

Netherlands) were coated with 20 μg of whey in coating buffer for 18 h at 4 °C. Plates were washed and blocked for 1 h with buffer containing 50 mM Tris, 2 mM EDTA en 137 mM NaCl/0.05% Tween and 0.5% BSA. Serum samples were incubated for 2 h at room temperature. Plates were washed and incubated with 1 μg biotin labeled rat anti-mouse IgE or IgG_1 for one hour at room temperature. After washing the plates were incubated with streptavidin-horse radish peroxidase for one hour, washed and developed with o-phenylendiamine. The reaction was stopped after 10 min with 4 M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Biorad, Veenendaal, the Netherlands). Results were expressed as absorbance units (AU). Serum concentrations of mMCP-1 as a reflection of local intestinal mast cell degranulation [20] were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK). Galectin-9 was measured in serum by means of ELISA as described previously [22].

2.8. Flow cytometric analysis of regulatory T-cells and DC

MLN were collected and cut into small pieces and incubated with 0.2% collagenase IV and 2kU/ml DNAse 1 for 30 min at 37 °C, resuspended and incubated for another 30 min. The enzymatic reaction was stopped by adding 50 μl FCS. Single-cell suspensions (5×10^5) of MLN were blocked with PBS containing 5% FCS, 1% BSA and subsequently incubated for 20 min with either mAb against CD4 and CD25 for regulatory T-cells, mAb against CD103 and CD11c for the detection of DC or isotype controls. For detection of Foxp3 (FJK-16), CD4 and CD25-stained cells were permeabilized and incubated with anti-Foxp3 according to the manufacturer's

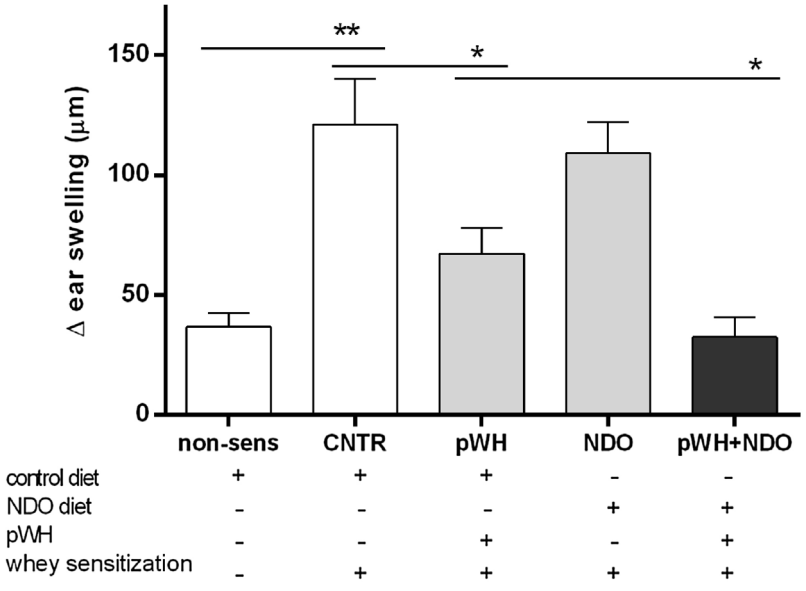

Fig. 2. NDO enhanced the protective effect of pWH on the acute allergic skin response.
At day 33, an acute allergic skin response was measured 1 h after intradermal whey challenge in the ears of non-sensitized and whey sensitized mice on a control diet (CNTR) and in ears of mice treated with pWH, NDO or pWH + NDO prior to sensitization. Data are expressed as mean values ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$.

protocol. Stained cells were analyzed using a BD FACSCantoII flow cytometer. Data are expressed as percentage of total cells

2.9. Statistics

The data were analyzed using one way ANOVA and post-hoc Dunnett's test for the acute allergic skin response, regulatory T-cells and DC percentages. Data were log-transformed and analyzed using one way ANOVA and post-hoc Dunnett's test for whey-specific antibody levels, and mMCP-1 concentrations. A probability value of $P < 0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism software.

3. Results

3.1. Acute allergic skin response after intradermal ear challenge

In order to study whether the NDO increased the tolerizing effects of the pWH, the acute allergic skin response was measured in mice after intradermal whey challenge (Fig. 2). In sensitized mice fed the control diet (CNTR), the ear challenge with whey induced a significant ear swelling at 1 h (121.1 ± 19.0 μm; $p < 0.01$) compared to non-sensitized mice (36.7 ± 5.7 μm). Administration of the pWH induced a lower acute ear swelling response compared to CNTR mice (67.2 ± 10.7 μm; $p < 0.05$). Interestingly, in pWH + NDO treated mice, the acute skin response remained similar to the level of non-sensitized mice and was significantly different from pWH-treated mice on a control diet (32.6 ± 8.1 μm; $p < 0.05$). Results indicate that pWH + NDO successfully prevented the onset of acute allergic skin responses and that supplementary feeding of NDO increased the tolerizing capacities of pWH.

3.2. Mast cell activation (mMCP-1 serum concentrations) after oral challenge

To assess whether the NDO mixture affected local intestinal mast cell activation, mMCP-1 serum concentrations were determined as a reflection of mucosal mast cell activation (Fig. 3). Administration of the pWH showed a tendency towards reduced mMCP-1 levels (42.1 ± 16.2 ng/ml) compared to sensitized mice on a control diet (CNTR; 69.2 ± 14.6 ng/ml). When the pWH was combined with NDO, mast cell activation was prevented. Serum mMCP-1 concentrations were significantly different from CNTR mice (15.3 ± 2.0 ng/ml; $p < 0.01$) remaining at the level of non-sensitized mice (14.0 ± 3.8 ng/ml).

3.3. Whey-specific antibodies

In sensitized mice fed the control diet (CNTR), whey-specific IgE (Fig. 4A) and IgG_1 (Fig. 4B) were increased in serum when compared to non-sensitized mice. No effect on IgE or IgG_1 levels was observed if mice were treated with the pWH or NDO prior to sensitization. However, combining pWH + NDO, showed significantly lower levels of whey-specific IgE levels (Fig. 4A) compared to sensitized mice on NDO diet. A tendency towards a lower production of IgE was observed in pWH + NDO treated mice compared to control mice (CNTR). No effect was observed on whey-specific IgG1 levels (Fig. 4B).

3.4. Galectin-9 in serum

In previous studies, it was shown that allergic symptoms (acute skin response, anaphylaxis) were reduced in mice fed with a mixture of neutral NDO in combination with *Bifidobacterium breve* [23]. Epithelium-derived galectin-9 was hypothesized as one of the regulatory mechanisms underlying this effect [22,24]. In the current study, increased concentrations of epithelium-derived galectin-9 were measured in mice fed the NDO. No effect on galectin-9 levels was observed in mice fed with the combination of pWH + NDO (Fig. 5B).

3.5. Percentage of $Foxp3^+$ regulatory T-cells and $CD11c^+CD103^+$ DC in MLN

To investigate whether the NDO diet in combination with pWH influenced immune regulatory cells, $Foxp3^+$ regulatory T-cells (Fig. 5A) and CD11c + CD103+ DC (Fig. 6) were determined in MLN. FoxP3 regulatory T-cells were only increased in mice treated with

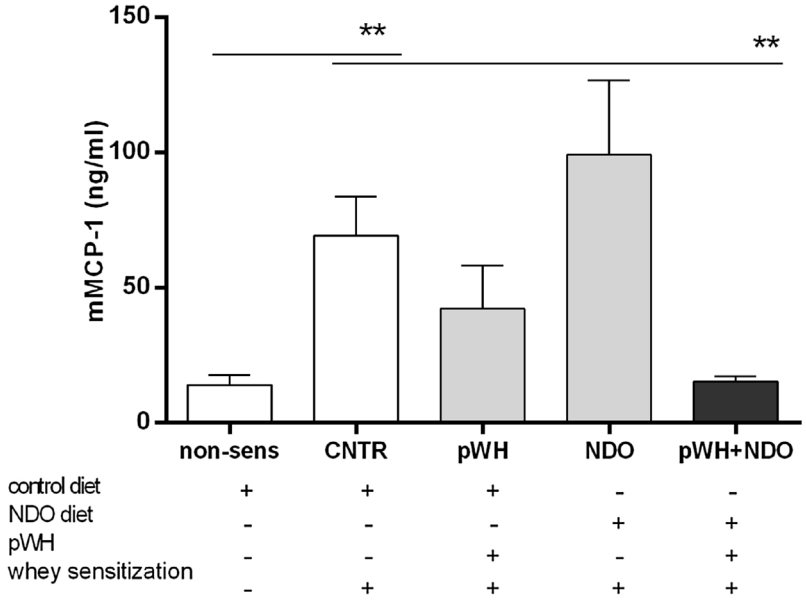

Fig. 3. NDO + pWH treatment prior to sensitization prevented mast cell activation.
mMCP-1 serum concentrations were measured 18 h after oral whey challenge in non-sensitized and whey sensitized mice on a control diet (CNTR) and in mice treated with pWH, NDO or pWH + NDO prior to sensitization as a reflection of mast cell degranulation. Data are expressed as mean values ± SEM; $^{**}p < 0.01$.

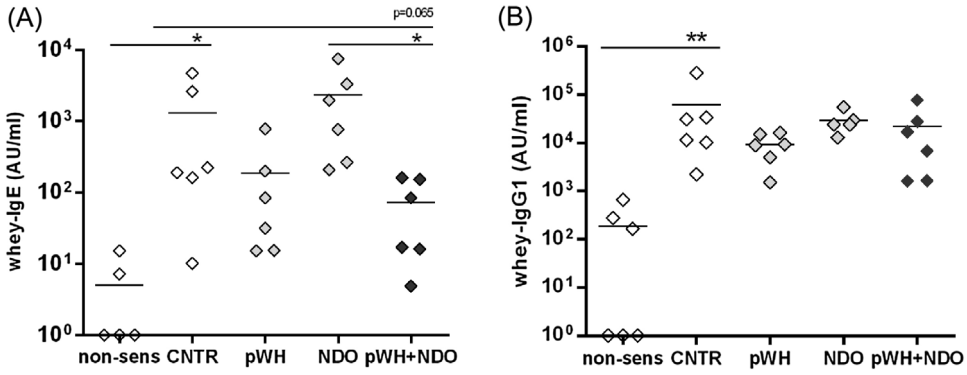

Fig. 4. Allergen-specific IgE and −IgG1.
At day 34, whey-specific IgE (A) and IgG_1 (B) concentrations were measured in serum of non-sensitized and whey sensitized mice on a control diet (CNTR) and in mice treated with pWH, NDO or pWH + NDO prior to sensitization. Data are expressed as individual values; $^{*}p < 0.05$, $^{**}p < 0.01$.

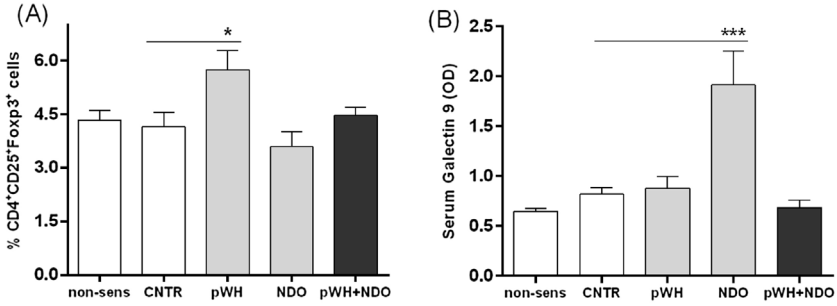

Fig. 5. Regulatory T-cells and galectin-9.
Regulatory T-cell percentages (A) and galectin-9 (B) were determined in the MLN (A) or serum (B) of non-sensitized and whey sensitized mice on a control diet (CNTR) and in mice treated with pWH, NDO or the combination of both. Data are expressed as mean values ± SEM; $^{*}p < 0.05$ $^{***}p < 0.001$.

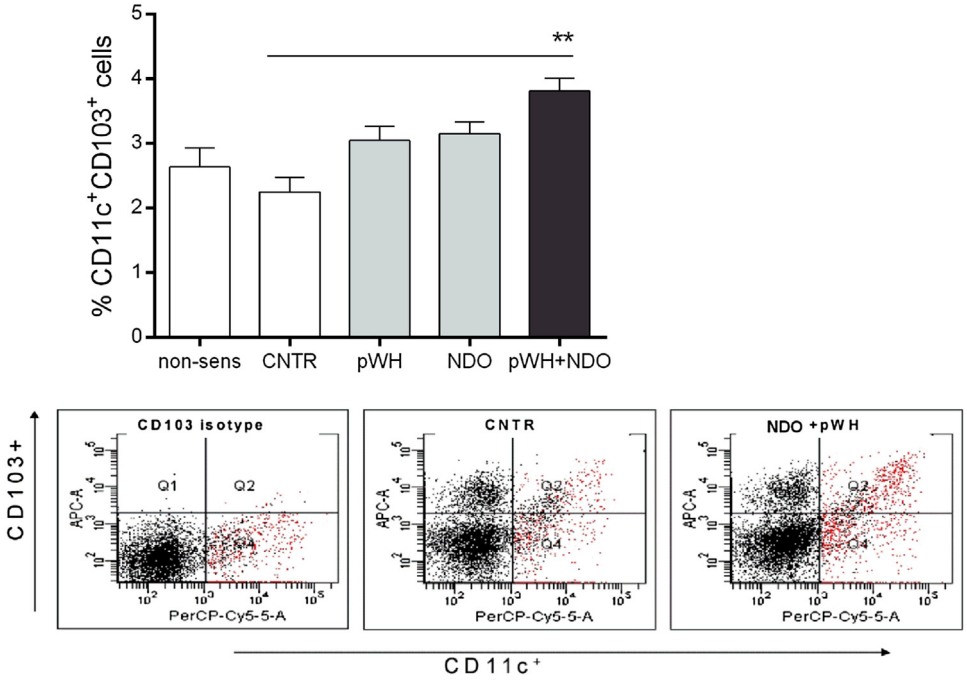

Fig. 6. NDO + pWH treatment prior to sensitization enhanced CD103+ DC in MLN.
$CD11c^{+}CD103^{+}$ DC percentages were determined in the MLN of non-sensitized and whey sensitized mice on a control diet (CNTR) and in mice treated with pWH, NOD or pWH + NDO prior to sensitization. Data are expressed as mean values ± SEM; $^{**}p < 0.01$.

the pWH (Fig. 5A). Interestingly, by combining pWH with NDO a significant increase in the percentage of CD11c + CD103+ DC was observed ($3.8 \pm 0.2\%$; $p < 0.01$) compared to CNTR mice ($2.3 \pm 0.2\%$ of total cells).

4. Discussion

In the current study, we found evidence that NDO, which resemble structural and functional properties of human NDO in breast milk, support specific oral tolerance induced by hydrolyzed whey proteins. The increased induction of tolerance is reflected by decreased IgE production and a prevention of clinical responses (e.g. acute skin response and mast cell activation) after antigen challenge. The tolerance supporting activity of NDO may be related to its effects on intestinal CD103+ dendritic cells in the intestines. This study suggests that NDO have a potential as an adjuvant to support induction of allergen-specific immune tolerance to food proteins or protein fragments.

Allergen crosslinking to the high-affinity receptor for IgE (FcεRI) on mast cells leads to mast cell degranulation resulting in allergic symptoms. Allergen-specific IgE concentrations were decreased in mice fed pWH in combination with NDO compared to NDO only. No significant decrease was found between IgE levels in pWH and pWH + NDO, indicating that additional mechanisms may be involved in reducing mast cell activation (Fig. 3) and ear swelling responses (Fig. 2). Increasing evidence suggests that NDO exert a direct effect on intestinal epithelial cells and/or immune cells [25,26]. Eiwegger et al. showed epithelial transport of human milk oligosaccharides in an in vitro model through a CaCo-2 cells monolayer, indicating that milk oligosaccharides can pass the epithelial lining of the intestine [26], thereby enabling direct contact of NDO with cells from the immune system. Mast cells are resident cells of the intestine and important regulators in acute allergic responses. In a recent study, it was shown that human milk oligosaccharides reduced food allergic symptoms in an experimental model for food allergy [27] with a direct effect of human milk oligosaccharides on mast cell activation in vitro. Since we did not find inhibition of mast cell activation and antigen-induced skin responses in NDO-fed mice, other mechanisms than direct effects of NDO on mast cells might be responsible for the observed effects in mice fed the combination of pWH + NDO. Receptors on epithelial cells which might be involved in the recognition of NDO are lectins. One family of soluble type lectins expressed by mucosal epithelium, are galectins [24]. Galectin-9 is described to have IgE antagonizing effects [28], thereby inhibiting mast cell degranulation [29] and it was previously shown to be correlated with reduced allergic responses in cow's milk allergic mice in a preventive setting [22]. In the current study, NDO stimulated galectin-9 release from intestinal epithelial cells in a transwell co-culture system [24] and mice fed with NDO have indeed increased galectin-9 in serum, but this did not result in decreased allergic symptoms after allergen exposure. On the other hand, combination of partial hydrolyzed whey with NDO is not accompanied with increased serum galactin-9. Although we cannot exclude local production of galactin-9, these findings suggest that increased tolerance may not be caused by the inhibitory effects of galectin-9.

Previous animal studies showed that prolonged feeding (50 days) with NDO induce immune modulation in allergic disease. For example, Vos et al. show that a diet supplemented with NDO is effective in decreasing allergic symptoms in murine models of allergic asthma [17]. Moreover, in a mouse model for cow's milk allergy, a change in Th1 and/or regulatory T-cell numbers was underlying the beneficial effect of the NDO [18,19,30]. Current findings show a prevention of the onset of allergic symptoms comparable to the level of non-sensitized mice by feeding the mice the NDO in combination with pWH. These observations suggest that NDO in the presence of partially hydrolyzed whey proteins promote the intestinal milieu towards the development of tolerance (i.e. increased CD103+ DC). Both in mice and humans $CD103^+$ DC drive preferentially the expansion of Foxp3+ regulatory T-cells by mechanisms involving TGF-β and retinoic acid and Foxp3+ regulatory T-cells are important for oral tolerance induction to food proteins [11–13,31]. DC trafficking from the intestinal mucosa to the MLN have been shown to be inevitable for the induction of oral tolerance indicating the important role of the MLN in this process [32–35]. Moreover, CD103+ DC induce gut homing of regulatory T-cells [9,36]. Although Foxp3+ regulatory T-cell numbers in the MLN were not increased in pWH + NDO treated animals, measurement of presence of Foxp3+ regulatory T-cells in lamina propria would be important to monitor in future studies. Increased CD103+ DC percentages were observed in mice fed NDO in the presence of pHW. If the latter is true in humans, results demonstrate a possible role for CD103+ DC in creating a tolerogenic milieu induced by NDO.

In conclusion, the current study demonstrates that a combination of NDO and pWH prevents the onset of allergic symptoms and that dietary NDO may optimize immune modulation and support oral tolerance induction by (hydrolyzed) food proteins.

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