

Oligosaccharide-Induced Whey-Specific CD25⁺ Regulatory T-Cells Are Involved in the Suppression of Cow Milk Allergy in Mice^{1,2}

Bastiaan Schouten,³ Betty C. A. M. van Esch,^{3,4} Gerard A. Hofman,³ Louis Boon,⁵ Léon M. J. Knippels,⁴ Linette E. M. Willemsen,³ and Johan Garssen^{3,4*}

³Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht 3584 CA, The Netherlands; ⁴Danone Research, Centre for Specialised Nutrition, Wageningen 6704 PH, The Netherlands; and ⁵Bioceros BV, Utrecht 3584 CM, The Netherlands

Abstract

Dietary intervention with a unique prebiotic nondigestible carbohydrate mixture has been shown to reduce the development of allergic disease in infants at risk. In this study, the involvement of CD25⁺ regulatory T-cells (Treg) in the carbohydrate-induced effects was investigated in mice orally sensitized with whey using adoptive transfer experiments. Donor mice were sensitized with whey and fed a diet containing short-chain galacto-, long-chain fructo- and acidic-oligosaccharides, or a control diet starting 2 wk before sensitization. The acute allergic skin reaction upon intradermal whey challenge was determined and whey-specific Ig were measured. Splenocytes of the donor mice were transferred to naïve recipient mice after partial ex vivo depletion of CD25⁺ Treg. The prebiotic diet clearly diminished the acute allergic skin reaction ($P < 0.001$). Whey-sensitized recipient mice transferred with splenocytes from whey-sensitized, prebiotic-fed donor mice displayed almost complete prevention of the acute allergic skin reaction compared with mice receiving cells from sham-sensitized, prebiotic-fed donor mice ($P < 0.001$). Partial depletion of CD25⁺ T-cells inhibited these effects ($P < 0.001$), although IgE sensitization was not prevented. This study indicates the involvement of whey-specific CD25⁺ Treg in the suppression of the allergic effector response induced by dietary intervention with prebiotics. J. Nutr. 140: 835–841, 2010.

Introduction

The gastrointestinal tract is exposed to a wide variety of dietary proteins. In general, these proteins are tolerized by the gastrointestinal immune system, resulting in local and systemic nonresponsiveness. This orally induced tolerance may be a result of anergy or active suppression, which is determined by both antigen dosage (1) and age (2). In food allergic patients, the oral tolerance to specific dietary proteins is broken or not established (3). Currently, there is no specific therapy for the treatment of food allergy. The only option to avoid symptoms is to circumvent exposure to the responsible food. The most common food allergy in childhood is cow milk allergy, which affects ~2–3% of all infants (4). It is caused by an allergic inflammatory response to milk proteins (5). Cow milk proteins (0.35 g/L in milk) consist of casein and whey proteins. Whey proteins comprise β -lactoglobulin (the main allergen), α -lactalbumin, albumin, lactoferrin, Ig, and proteoseptone (6). In Europe, most of the infant formulas are whey based and

supplemented with prebiotics, whereas in North America, most are not and are casein based.

In food allergy, the delicate balance between oral tolerance and hypersensitivity is regulated by cells of the immune system. Cells with regulatory properties include transforming growth factor- β -producing Th3 cells, interleukin-10-producing Tr1 cells, and natural killer T-cells (7–9). In addition, naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T-cells (Treg)⁶ were described to be important for maintaining peripheral tolerance and for suppression of proliferation of CD4⁺ and CD8⁺ T cells (10,11). This subset of Treg can also be generated de novo in the periphery (e.g. the intestinal mucosa) (12). Treg represent 5–10% of the CD4⁺ T lymphocyte population and express the transcription factor forkhead box p3 (Foxp3), which is confirmed as a key gene for the generation and maintenance of Treg (12). Currently, the involvement of Treg in tolerance induction by dietary components is unknown. Human milk consists of numerous components, including but not limited to oligosaccharides, which may have many immunological properties and

¹ Author disclosures: B. Schouten, B. C. A. M. van Esch, G. A. Hofman, L. Boon, L. M. J. Knippels, L. E. M. Willemsen, and J. Garssen, no conflicts of interest.

² Supplemental Figures 1–3 are available with the online posting of this paper at jn.nutrition.org.

* To whom correspondence should be addressed. E-mail: j.garssen@uu.nl.

⁶ Abbreviations used: CT, cholera toxin; dp, degree of polymerization; Foxp3, forkhead box 3; lcFOS, long-chain fructooligosaccharide; pAOS, pectin-derived acidic oligosaccharide; scGOS, short-chain galactooligosaccharide; T_H, T helper cell; Treg, regulatory T-cell.

might facilitate tolerance (13,14). Prebiotic fibers manufactured to resemble some of these oligosaccharides are defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” (15). Short-chain galactooligosaccharides (scGOS) and long-chain fructooligosaccharides (lcFOS) are dominating the prebiotic literature. Beneficial immunological properties of these scGOS and lcFOS are confirmed by an improved vaccination response in a murine model (16) and lowered incidence of atopic dermatitis in children (17). In addition, a similar prebiotic mixture reduced the acute allergic skin response in mice orally sensitized with whey (18). Besides scGOS and lcFOS, the pectin-derived acidic oligosaccharides (pAOS) were identified as a potential prebiotic fiber (19).

In this study, we used a mouse model for orally induced sensitization to whey proteins to study the immunological mechanisms that might underlie the induction of tolerance against whey using prebiotics (20). The role of CD25⁺ Treg in the preventive capacities of dietary intervention with a prebiotic mixture [2 g/100 g (9:1:1) scGOS, lcFOS, and pAOS] was assessed using adoptive transfer experiments.

Materials and Methods

Chemicals. Whey proteins were obtained from DMV International. Cholera toxin (CT) was purchased from Quadrant Diagnostics and PBS was from Cambrex Bio Science. Biotin-labeled rat anti-mouse IgE, IgG₁, and IgG_{2a} were from BD Biosciences. PC61 (anti-CD25), a monoclonal antibody from a rat PC61 hybridoma cell line, was a kind gift of Bioceros BV. All other chemicals were obtained from Sigma-Aldrich-Chemie.

Diets. Semipurified protein-free AIN-93G-based diets containing cow milk were composed and mixed with prebiotics by Research Diet Services (21). The prebiotic diet contained 2 g/100 g (9:1:1) short-chain GOS (scGOS; enzymatic elongation of lactose with galactose by β -galactosidase), long-chain FOS (lcFOS; from chicory inulin) and pAOS (produced from pectin). scGOS (Vivinal GOS, FrieslandCampina Domo) contained oligosaccharides with a degree of polymerization (dp) of 3–8. lcFOS (Raftiline HP, Orafit) contained oligosaccharides with an average dp >23. The scGOS/lcFOS spray-dried powder consisted of ~50% scGOS and lcFOS, 19% maltodextrin (glucidex 2, Roquette), 16% lactose, 14% glucose, and 1% galactose (16). The pAOS powder (Südzucker) had a dp of 1–20 and consisted of ~75% galacturonic acid oligomers, 10% monomers, and 15% of moisture and ash (19). All prebiotics were exchanged for the same amount of total carbohydrates; this resulted in a comparable overall carbohydrate composition in the diets.

Oral sensitization and challenge of mice. Three-week-old specific pathogen-free female C3H/HeOuJ mice were purchased from Charles River Laboratories and housed in the animal facility. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Dams were fed the cow milk-containing protein-free diets for at least 2 generations. Two weeks before the first sensitization, mice received the different diets and consumed them throughout the study. They were orally sensitized with whey (20 mg) 5 times at weekly intervals, using CT (10 μ g) as an adjuvant. The total gavage volume was 0.5 mL (18,20). Sham-sensitized mice received CT alone. One week after the last sensitization, the mice were challenged in the ear to determine the allergic skin response and after 24 h, an oral challenge was performed using 100 mg whey in 1.0 mL PBS. After 24 h, blood samples were collected in Minicollect Z Serum Sep vials (Greiner bio-one) and mice were then killed by cervical dislocation. Collected blood was centrifuged for 15 min at 16,000 $\times$ g and sera were stored at –70°C. Details of the protocol are in Supplemental Fig 1.

Acute allergen-specific skin reaction. The acute allergen-specific skin response was measured after an intradermal injection with 20 μ L homogenized whey protein (0.5 g/L in PBS) in the ear pinnae. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo) at $t = 0$ and 1 h after challenge. Ear swelling (μ m) was calculated by subtracting the basal ear thickness ($t = 0$ h) from the specific whey-induced ear thickness after 1 h and additionally (Δ ear swelling) subtracting the mean ear swelling from the group of sham-sensitized mice fed the corresponding diet.

Adoptive transfer experiments. To test whether splenocytes are able to transfer prebiotic-induced tolerance, adoptive transfer experiments were carried out. After the prebiotic-supplemented (donor) mice were killed, the spleens were obtained and pooled per group ($n = 12$). Single cell suspensions of the 4 groups (sham, sham prebiotic, whey, and whey prebiotic) were i.v. injected (100 μ L, 1×10^{10} cells/L) in naïve mice. In total, 8 recipient groups of $n = 6$ /group were formed (Expt. A; Table 1). The recipient mice were fed the control diet and sensitized (sham or whey) as described above. The acute allergic skin reaction was measured and whey-specific serum Ig were measured to determine allergic sensitization.

In another set of experiments (Expt. B), single spleen cell suspensions of whey-sensitized donor mice fed the control or prebiotic diet were pooled ($n = 16$) and one-half of the cells were treated using 50 μ g rat anti-CD25 (PC61) per spleen. Biomag goat anti-rat IgG (2 mL) (Bangs Laboratories) was added to the treated cells and placed on a rotator for 45 min. The bead-cell suspension was placed in a magnetic separator for 5 min and this was repeated with the unbound fraction. Depletion of CD25 positive cells was analyzed using flow cytometric analysis. The splenocytes of the 4 groups were i.v. injected (100 μ L, 1×10^{10} cells/L) into recipient mice ($n = 6$ /group, 8 groups in total) (Table 1, Expt. B). The recipient mice were sham or whey sensitized as described above. Variables measured to determine allergic sensitization were the acute allergic skin reaction and whey-specific serum Ig.

Flow cytometry. Single cell suspensions were prepared from mouse spleens. Cells were counted using a Coulter Z1 particle counter (Beckman Coulter) and diluted to a concentration of 2×10^9 cells/L. For identification of CD4⁺CD25⁺Foxp3⁺ cells, anti-CD4-FITC (1:100) and anti-CD25-PE (1:100) were used (BD). Foxp3 staining was performed following the manufacturer's protocol (eBioscience). Flow cytometry analysis was performed using FACSCalibur (BD) and CellQuest Pro Analysis software (BD) and isotype controls were subtracted.

TABLE 1 Overview of the experimental designs

Experiment	Sensitization of donors	Diet	Treatment of splenocytes	Sensitization of recipients	Diet
A	Sham	Control	—	Sham	Control
		Control	—	Whey	Control
		Prebiotic	—	Sham	Control
		Prebiotic	—	Whey	Control
	Whey	Control	—	Sham	Control
		Control	—	Whey	Control
		Prebiotic	—	Sham	Control
		Prebiotic	—	Whey	Control
B	Whey	Control	—	Sham	Control
		Control	Anti-CD25	Sham	Control
		Control	—	Whey	Control
		Control	Anti-CD25	Whey	Control
		Prebiotic	—	Sham	Control
		Prebiotic	Anti-CD25	Sham	Control
		Prebiotic	—	Whey	Control
		Prebiotic	Anti-CD25	Whey	Control

Measurement of specific serum Ig. Concentrations of whey-specific IgE, IgG₁, and IgG_{2a} were measured in serum using ELISA as described before (20). In short, plates were coated with 100 μ L whey (20 mg/L). Plates were incubated with biotin-labeled rat anti-mouse IgE, IgG₁, and IgG_{2a} (1 mg/L) and with streptavidin-horseradish peroxidase. Results were expressed as arbitrary units, calculations were based on titration curves of pooled sera from whey and alum-i.p. immunized mice as a positive reference serum.

Statistics. The data were analyzed using 2-way ANOVA or the Mann-Whitney test using GraphPad Prism software (version 4.03) and SAS statistical software. The former tested the main effects of diet and sensitization or Treg depletion. In addition, interactions between the donor diet and sensitization or Treg-depletion were analyzed. Bonferroni's post hoc test was used to identify the means that differed. To obtain normal distribution, the Ig levels were log-transformed prior to statistical analyses. Differences were considered significant at $P < 0.05$ (2-tailed). Data in the text are means $\pm$ SEM.

Results

Acute allergic skin reaction of donor mice. To investigate the preventive effect of the prebiotic diet on sensitization, we measured Δ ear swelling in the whey-sensitized mice (157 ± 11 μ m). The prebiotic diet largely prevented the acute allergic skin reaction of the whey-sensitized mice (67 ± 5.8 μ m; $P < 0.001$). The diet did not affect ear swelling of sham-sensitized mice (data not shown).

Whey-specific Ig levels of donors. Whey-specific serum IgE, IgG₁, and IgG_{2a} levels were greater in whey-sensitized mice compared with sham-sensitized mice (data not shown). Whey-specific IgE was reduced in whey-sensitized mice fed the prebiotic diet compared with mice fed the control diet ($P < 0.05$; Fig. 1). Whey-specific IgG₁ also was reduced by the prebiotic diet ($P < 0.05$). Dietary intervention did not affect whey-specific IgG_{2a} levels in whey-sensitized mice fed the control diet compared with the prebiotic diet.

Acute allergic skin reactions in recipient mice adoptively transferred with splenocytes. In sham- as well as whey-sensitized recipient mice transferred with splenocytes (i.v.) of whey-sensitized donor mice fed the control diet, severe allergic skin reactions were observed 1 h after i.d. whey injection in the ear (Expt. B) (Fig. 2). In contrast, the transfer of donor splenocytes of whey-sensitized mice fed the prebiotic diet protected against development of an acute allergic skin reaction in sham-sensitized as well as whey-sensitized recipient mice ($P < 0.001$) compared with recipients transferred with splenocytes from control diet-fed donor mice. This protection was whey-specific, because whey-sensitized recipient mice injected with splenocytes of sham-sensitized donors receiving the prebiotic diet still developed a severe ear swelling response.

Whey-specific Ig levels of recipients. Serum samples were collected from the recipient mice and analyzed for whey-specific Ig (Expt. A). Whey-specific IgE levels of recipient mice transferred with splenocytes of sham-sensitized donor mice fed the control diet were enhanced upon whey sensitization compared with sham sensitization ($P < 0.01$; Fig. 3A). Similar results were obtained when recipients were transferred with splenocytes from sham-sensitized donor mice fed the prebiotic diet ($P < 0.01$). Transfer of splenocytes of whey-sensitized mice tended to lower the capacity to develop whey-specific IgE in recipients upon sensitization with whey in both the control ($P = 0.08$) and

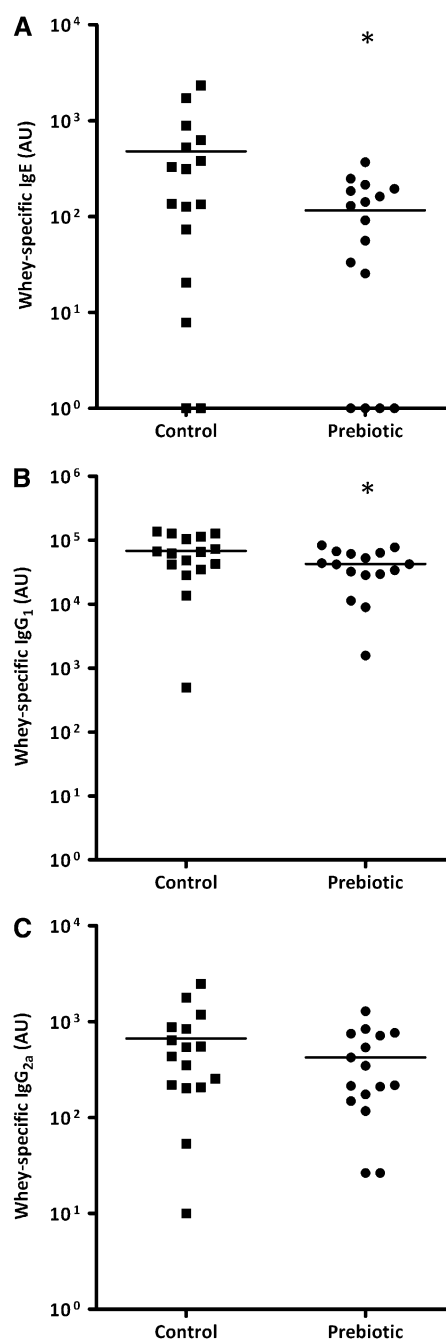


FIGURE 1 Serum whey-specific IgE (A), IgG₁ (B), and IgG_{2a} (C) concentrations of whey-sensitized mice fed the prebiotic or control diet (Expt. A). Mice were orally sensitized with whey 5 times at weekly intervals, using CT as an adjuvant. One week after the last sensitization, serum was collected 1 h after oral challenge with whey. Means and individual values are presented, $n = 16$. *Different from control, $P < 0.05$.

the prebiotic group ($P = 0.11$). However, IgE levels did not differ between recipients transferred with donor cells of mice fed the control or prebiotic diet.

Similar results were found for IgG₁ (Fig. 3B) and IgG_{2a} (Fig. 3C). Whey-sensitized recipient mice transferred with cells of sham-sensitized donor mice, regardless of the donor's diet, had enhanced IgG₁ and IgG_{2a} levels compared with sham-sensitized recipients transferred with splenocytes of sham-sensitized donors ($P < 0.01$). Whey sensitization did not further enhance recipient IgG₁ and IgG_{2a} levels except for whey IgG_{2a} of

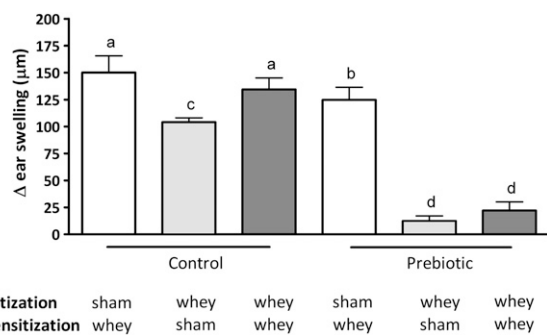


FIGURE 2 Acute allergic skin response in mice adoptively transferred with splenocytes of whey- or sham-sensitized mice fed the control or prebiotic diet (Expt. A). An allergic skin response was evoked by intradermal whey challenge in the ears of sham- or whey-sensitized recipient mice that were transferred with splenocytes of whey-sensitized donor mice fed the control diet. Values are means $\pm$ SEM, $n = 6$. P -values from the 2-way ANOVA were: diet, $P < 0.0001$; sensitization, $P < 0.05$; interaction, $P < 0.0001$. Means without a common letter differ, $P < 0.05$.

recipients transferred with splenocytes of control diet-fed donor mice ($P < 0.05$).

Overall, sensitization of the recipient mice transferred with cells of donor mice fed control or prebiotic diet, as reflected by antibody levels, did not differ.

Partial depletion of $CD4^+CD25^+Foxp3^+$ Treg in anti-CD25-treated splenocytes. Splenocytes from whey-sensitized mice fed the control or prebiotic diet were transferred with or without prior ex vivo $CD25^+$ cell depletion using the PC61 antibody (Expt. B). There was no difference in the number of $CD4^+CD25^+Foxp3^+$ Treg in the spleen of whey-sensitized mice fed the prebiotic diet ($6.1 \pm 0.3\%$) compared with the control diet ($6.4 \pm 0.3\%$). Ex vivo Treg-depletion resulted in a 67% reduction of $CD4^+CD25^+Foxp3^+$ cells in the donor splenocyte suspension of the prebiotic diet group and a 61% reduction in the control diet group compared with the nondepleted controls. Hence, in both groups, a similar partial ex vivo Treg depletion was obtained, ($P < 0.01$; Supplemental Fig. 2). Ex vivo anti-CD25 treatment also resulted in some loss of $CD4^+CD25^+Foxp3^+$ effector or regulatory type T-cells ($7.8 \pm 1.2\%$ vs $4.2 \pm 0.3\%$; $P < 0.001$). There were no differences among the mice fed the different diets.

Acute allergic skin reaction of recipients using donor cells partially depleted for Treg. Upon whey sensitization, the ear swelling response of recipient mice transferred complete spleen cell suspensions or partially Treg-depleted spleen cell suspensions from whey-sensitized donor mice fed the control diet was significantly enhanced compared with the sham-sensitized recipient mice (Expt. B) ($P < 0.001$; Fig. 4). Donor cells from whey-sensitized mice fed the prebiotic diet effectively prevented the occurrence of an acute skin response in recipients upon whey sensitization compared with sham sensitization and the ear swelling response was less than in recipients transferred with cells of mice fed the control diet ($P < 0.05$). Partial Treg depletion prior to transfer completely abolished the preventive effect of adoptively transferred donor cells of mice fed the prebiotic diet, resulting in severe ear swelling in recipient mice upon whey sensitization ($P < 0.001$).

Whey-specific Ig of recipients transferred donor cells partially depleted in Treg. Sham- or whey-sensitized recipient

mice transferred with splenocytes of control or prebiotic diet fed whey-sensitized donor mice had similar whey-specific IgE levels (Expt. B). Recipient mice receiving partially Treg-depleted spleen cells of whey-sensitized donor mice fed the control diet had higher whey-specific IgE levels in comparison with recipient mice receiving non-Treg-depleted spleen suspensions (12.5 ± 3.6 vs. 169 ± 71 arbitrary units; $P < 0.05$; Supplemental Fig. 3A).

Whey-specific IgG₁ (Supplemental Fig. 3B; $P < 0.01$) and IgG_{2a} (Supplemental Fig 3C; $P < 0.01$) levels were greater when recipient mice were administered partially Treg-depleted donor spleen suspensions compared with mice transferred with non-depleted donor splenocytes. There were no differences between recipient mice transferred with donor cells of mice fed the control or prebiotic diet either in the presence or partial absence of Treg.

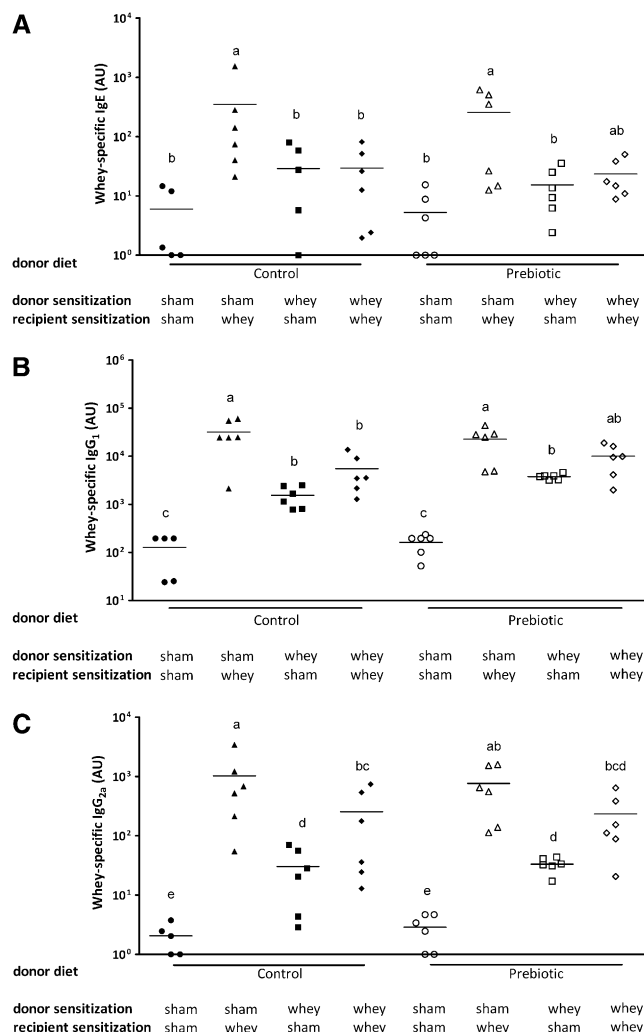


FIGURE 3 Serum whey-specific IgE (A), IgG₁ (B), and IgG_{2a} (C) in recipient mice transferred with splenocytes of sham- or whey-sensitized donor mice fed the control or prebiotic diet (Expt. A). Recipient mice were fed the control diet and after transfer with splenocytes, they were sham (CT)- or whey (whey + CT)-sensitized. One week after the last sensitization, serum was collected 1 h after oral challenge with whey. Means and individual values are presented, $n = 5-6$. Statistical analyses were performed using 2-way ANOVA on log-transformed data. P -values from the 2-way ANOVA for all Ig were: diet, $P > 0.05$; sensitization, $P < 0.0001$; interaction, $P > 0.05$. Means without a common letter differ, $P < 0.05$.

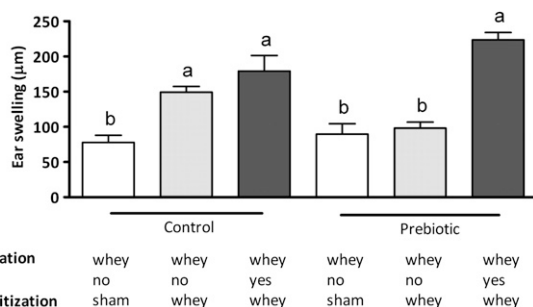


FIGURE 4 Acute allergic skin response in mice adoptively transferred with splenocytes of whey-sensitized donor mice fed the control or prebiotic diet with or without partial ex vivo CD25⁺ Treg-depletion (Expt. B). Values are means $\pm$ SEM, $n = 6$. P -values from the 2-way ANOVA were: diet, $P < 0.05$; sensitization, $P < 0.01$; interaction, $P < 0.01$. Means without a common letter differ, $P < 0.05$.

Discussion

The prebiotic mixture of scGOS/lcFOS/pAOS used in this murine model for cow milk allergy ameliorated the severity of allergic symptoms, because the acute allergic skin response in mice fed the prebiotic diet during whey sensitization was strongly reduced. A slight reduction in T helper cell (T_H) 2 Ig levels (whey-specific IgE and IgG₁) was observed, whereas T_H1 type IgG_{2a} levels were unaffected and remained high. Previously, these prebiotics were shown to improve the vaccination response in mice, which is mainly T_H1 driven (19). The results suggest an improved T_H1/T_H2 balance upon dietary intervention with prebiotics which could partly, but not conclusively, explain their protective effect (19). Naturally occurring or de novo-induced CD25⁺ Treg are important for the acquirement of tolerance (10–12) and the contribution of these cells to the protective effect of the prebiotic diet was studied.

In mice orally sensitized with whey, the acute allergic skin response determines the level of systemic response as a result of a break in oral tolerance (20). This allergen-specific ear swelling response has similarities with the human skin prick test. Because the prebiotic diet reduced the severity of the acute allergic, skin response adoptive transfer studies were performed to determine whether this tolerance could be transferred using splenocytes. Adoptive transfer of splenocytes from whey-sensitized donor mice fed the control diet transferred the allergy to recipient mice that were sham-sensitized, because they developed a whey-specific acute skin response. Guttormsen et al. (22) also found adoptively transferred whole spleen single cells to transfer immunologic memory for a vaccine. In contrast to the whey-sensitized recipient mice transferred with splenocytes of whey-sensitized mice fed the control diet, the splenocytes of prebiotic-fed, whey-sensitized mice protected against the development of allergic symptoms. The whey-specific acute allergic skin reaction was prevented; hence, tolerance for whey induced in the donor mice by the prebiotic diet could be transferred to the recipient mice.

Two phases of oral tolerance can be distinguished: clonal deletion and active suppression of the immune response by Treg cells (23). The prebiotic diet did not enhance the number of Treg cells in Peyer's patches, mesenteric lymph nodes, or spleen in mice fed the prebiotic diet (data not shown), although the prebiotic diet may have enhanced the number of whey-specific Treg within the Treg population. Indeed, in vivo anti-CD25 depletion revealed that Treg cells play an important role in the oral sensitization with whey in mice (B. van Esch, B. Schouten,

B. Blokhuis, G. Hofman, L. Boon, J. Garssen, L. Knippels, L. Willemsen, and F. Redegeld, unpublished data) (7). Furthermore, Gri et al. (24) showed that Treg cells are able to inhibit mast cell degranulation or alter mast cell phenotype via the production of interleukin-10 or cell-cell contact, which may relate to the reduced acute allergic skin reaction observed in the present study in mice fed the prebiotic diet.

The involvement of Treg in the induction of tolerance by prebiotics was studied using adoptive transfer of splenocytes of whey-sensitized mice fed the prebiotic diet that were ex vivo depleted for CD25⁺ Treg cells using an anti-CD25 antibody (PC61). Indeed, partial Treg depletion prior to transfer of these donor cells abrogated the transfer of tolerance, which protected the recipient mice from developing a severe allergic skin reaction after whey sensitization. It has been shown that the anti-CD25 mAb depletes the CD25^{high} cells in vivo, which are CD4⁺ (25). The in vivo Treg depletion is partial and selective for CD4⁺CD25⁺Foxp3⁺ cells (7,26,27). As in these studies, in our study, the ex vivo depletion resulted in a partial reduction in CD4⁺CD25⁺Foxp3⁺ cells of >60%. In the other studies, it was shown that complete depletion is not necessary to be effective and can be used to investigate Treg function (28–31). CD4⁺CD25⁺Foxp3⁺ Treg are generally immune suppressive and important for tolerance induction and hence are most likely involved in the transfer of tolerance generated by the prebiotic diet (10–12). However, the ex vivo CD25⁺ depletion also partially reduced the percentage of CD4⁺CD25⁺Foxp3⁺ cells, which may include effector T-cells and Tr1 and Th3 Treg. Furthermore, we cannot rule out a possible contribution for CD8⁺CD25⁺Foxp3⁺ suppressor T-cells, which have been shown to have regulatory functions as well (32,33). The transfer of tolerance observed in the recipient mice was whey specific, because transfer of cells of nonsensitized donor mice fed the prebiotic diet did not protect recipient mice. This suggests involvement of an antigen-specific CD25⁺ Treg cell population and there is growing evidence of a subset of Treg cells that have antigen-specific suppression characteristics (34,35).

To determine allergic sensitization, plasma levels of allergen-specific IgE are typically measured. However, we and others found that this variable is not conclusive for the generation of allergic symptoms (36,37). In contrast to the acute allergic skin reaction in whey-sensitized recipient mice, Ig levels did not differ after adoptive transfer with cells of control or prebiotic diet-fed mice. In a mouse ovalbumin model, transfer of Treg cells reduced the allergic response and numbers of eosinophils in the lung and airway remodeling was prevented. However, also in this study, IgE levels were unaffected and remained high (38). Given the long half-life of the measured antibodies, the actual experimental period might have been too short to identify differences. Alternatively, the antigen-specific CD25⁺ Treg cells, generated under support of the prebiotic diet, may have directly affected the mast cells. Treg cells have been shown to reduce Ig receptor expression levels on mast cells (10,39). Furthermore, Treg may have interfered with Ig production or the generation of plasma cells. Although whey-specific Ig are still present, the functionality of the Ig may be altered. For example, IgG-blocking antibodies or antibodies with high affinity to bind the inhibitory FcγRIIB receptor may have been generated, resulting in the suppression of mast cell degranulation (40,41). Hence, both in the donor as well as the recipient mice, antigen-specific Treg may have altered the functionality of mast cells or the Ig produced by plasma cells. Although the acute allergic skin response reflects clinical symptoms that occur upon degranula-

tion of allergic effector cells, we conclude that the reduced skin response cannot be explained by differences in antibody titers between recipients transferred with cells of prebiotic diet- or control diet-fed mice. Similar findings were obtained in a dietary intervention study using pre-, pro-, and synbiotics in mice orally sensitized with whey (18).

In this study, we showed that whey sensitization is transferable using adoptive transfer of splenocytes. In addition, the protective effect of a prebiotic mixture on allergic symptoms could be transferred as well. The prebiotic diet caused only a minor reduction in whey-specific Ig and the reduced acute allergic skin response of the recipient mice transferred with donor cells of mice fed the prebiotic diet could not be explained by a reduction in Ig levels. Partial ex vivo CD25⁺ Treg cell depletion abrogated the protective effects of the adoptive transfer with donor cells of whey-sensitized mice fed the prebiotic diet. The results suggest, at least in part, the involvement of whey-specific Treg cells in the transfer of tolerance.

Acknowledgments

We thank Simone Eussen for assistance with the statistical analyses. B.S., B.v.E., L.K., L.W., and J.G. designed research; B.S., B.v.E., and G.H. conducted research; L.B. provided essential reagents; B.S. and L.W. analyzed data and wrote the paper; and B.S. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited

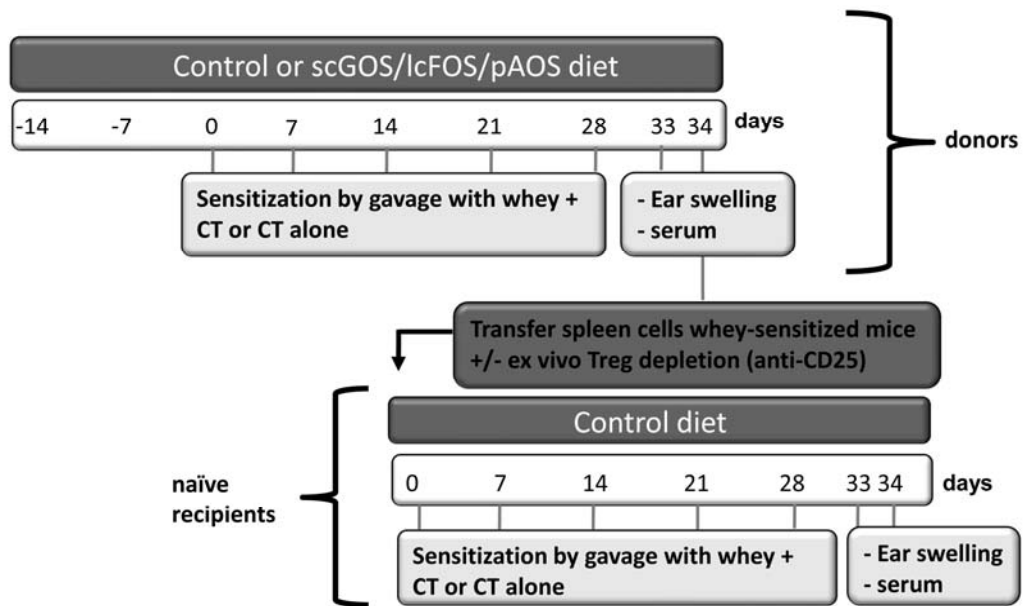
1. Friedman A, Weiner HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci USA*. 1994;91:6688–92.
2. Lundin BS, Dahlgren UI, Hanson LA, Telemo E. Oral tolerization leads to active suppression and bystander tolerance in adult rats while anergy dominates in young rats. *Scand J Immunol*. 1996;43:56–63.
3. Burks AW, Laubach S, Jones SM. Oral tolerance, food allergy, and immunotherapy: implications for future treatment. *J Allergy Clin Immunol*. 2008;121:1344–50.
4. Hol J, van Leer EH, Elink Schuurman BE, de Ruiter LE, Samsom JN, Hop W, Neijens HJ, de Jongste JC, Nieuwenhuis EE. The acquisition of tolerance toward cow's milk through probiotic supplementation: a randomized, controlled trial. *J Allergy Clin Immunol*. 2008;121:1448–54.
5. Crittenden RG, Bennett LE. Cow's milk allergy: a complex disorder. *J Am Coll Nutr*. 2005; 24 Suppl 6:S582–91.
6. Monaci L, Tregoeat V, van Hengel AJ, Anklam E. Milk allergens, their characteristics and their detection in food: a review. *Eur Food Res Tech*. 2006;223:149–79.
7. van Wijk F, Wehrens EJ, Nierkens S, Boon L, Kasran A, Pieters R, Knippels LM. CD4+CD25+ T cells regulate the intensity of hypersensitivity responses to peanut, but are not decisive in the induction of oral sensitization. *Clin Exp Allergy*. 2007;37:572–81.
8. Akbari O, Umetsu DT. Role of regulatory dendritic cells in allergy and asthma. *Curr Opin Allergy Clin Immunol*. 2004;4:533–8.
9. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med*. 2004;10:801–5.
10. Kashyap M, Thornton AM, Norton SK, Barnstein B, Macey M, Brenzovich J, Shevach E, Leonard WJ, Ryan JJ. Cutting edge: CD4 T cell-mast cell interactions alter IgE receptor expression and signaling. *J Immunol*. 2008;180:2039–43.
11. Izcue A, Powrie F. Special regulatory T-cell review: regulatory T cells and the intestinal tract: patrolling the frontier. *Immunology*. 2008;123: 6–10.
12. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*. 2009;9:83–9.
13. Field CJ. The immunological components of human milk and their effect on immune development in infants. *J Nutr*. 2005;135:1–4.

14. Vos AP, M'Rabet L, Stahl B, Boehm G, Garssen J. Immune-modulatory effects and potential working mechanisms of orally applied non-digestible carbohydrates. *Crit Rev Immunol*. 2007;27:97–140.
15. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr*. 1995;125: 1401–12.
16. Vos AP, Haarman M, Boco A, Govers M, Knol J, Garssen J, Stahl B, Boehm G, M'Rabet L. A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. *Int Immunopharmacol*. 2006;6:1277–86.
17. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch Dis Child*. 2006;91:814–9.
18. Schouten B, van Esch BC, Hofman GA, van Doorn SA, Knol J, Nauta AJ, Garssen J, Willemsen LE, Knippels LM. Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. *J Nutr*. 2009;139:1398–403.
19. Vos AP, Haarman M, van Ginkel JW, Knol J, Garssen J, Stahl B, Boehm G, M'Rabet L. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. *Pediatr Allergy Immunol*. 2007;18:304–12.
20. Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE, Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. *Int Arch Allergy Immunol*. 2008;147:125–34.
21. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr*. 1997; 127(5, Suppl):838S–41S.
22. Guttormsen HK, Wetzler LM, Finberg RW, Kasper DL. Immunologic memory induced by a glycoconjugate vaccine in a murine adoptive lymphocyte transfer model. *Infect Immun*. 1998;66:2026–32.
23. Tsuji NM, Kosaka A. Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends Immunol*. 2008;29:532–40.
24. Gri G, Piconese S, Frossi B, Manfro V, Merluzzi S, Tripodo C, Viola A, Odom S, Rivera J, et al. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40–OX40L interaction. *Immunity*. 2008;29:771–81.
25. Amu S, Gjertsson I, Tarkowski A, Brisslert M. B-cell CD25 expression in murine primary and secondary lymphoid tissue. *Scand J Immunol*. 2006;64:482–92.
26. McNeill A, Spittle E, Backstrom BT. Partial depletion of CD69low-expressing natural regulatory T cells with the anti-CD25 monoclonal antibody PC61. *Scand J Immunol*. 2007;65:63–9.
27. Couper KN, Blount DG, de Souza JB, Suffia I, Belkaid Y, Riley EM. Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice. *J Immunol*. 2007;178:4136–46.
28. Shreffler WG, Wanich N, Moloney M, Nowak-Wegrzyn A, Sampson HA. Association of allergen-specific regulatory T cells with the onset of clinical tolerance to milk protein. *J Allergy Clin Immunol*. 2009;123: 43–52 e7.
29. Kohm AP, McMahon JS, Podojil JR, Begolka WS, DeGutes M, Kasproicz DJ, Ziegler SE, Miller SD. Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol*. 2006;176: 3301–5.
30. Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, Sproles AA, Shah JS, Kohl J, et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med*. 2005;202:1549–61.
31. Matsushita N, Pilon-Thomas SA, Martin LM, Riker AI. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J Immunol Methods*. 2008;333:167–79.
32. Chaput N, Louafi S, Bardier A, Charlotte F, Vaillant JC, Menegaux F, Rosenzweig M, Lemoine F, Klatzmann D, et al. Identification of CD8 +CD25+Foxp3+ suppressive T cells in colorectal cancer tissue. *Gut*. 2009;58:520–9.
33. Mahic M, Henjum K, Yaqub S, Bjornbeth BA, Torgersen KM, Tasken K, Aandahl EM. Generation of highly suppressive adaptive CD8(+) CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation. *Eur J Immunol*. 2008;38:640–6.
34. Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med*. 2000;6:782–9.

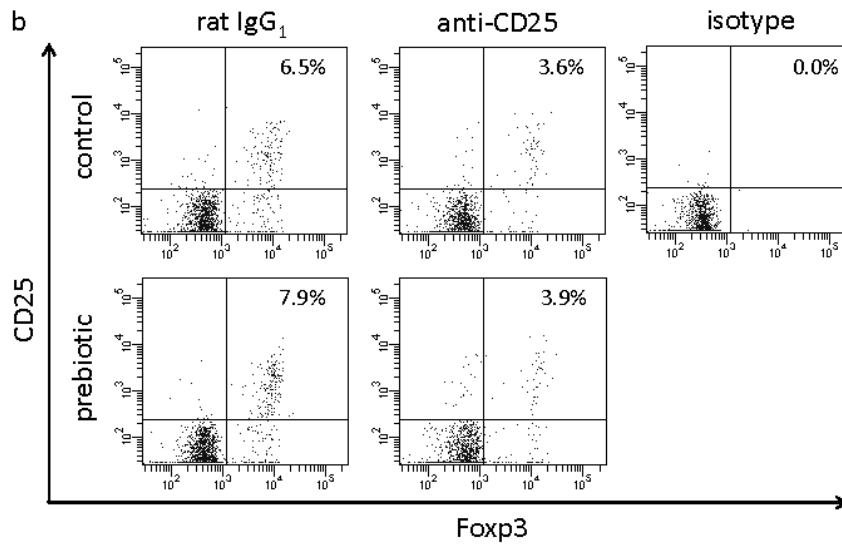
35. Koenen HJ, Joosten I. Antigen-specific regulatory T-cell subsets in transplantation tolerance regulatory T-cell subset quality reduces the need for quantity. *Hum Immunol.* 2006;67:665–75.
36. van der Gugten AC, den Otter M, Meijer Y, Pasmans SG, Knulst AC, Hoekstra MO. Usefulness of specific IgE levels in predicting cow's milk allergy. *J Allergy Clin Immunol.* 2008;121:531–3.
37. Miceli Sopo S, Radzik D, Calvani M. The predictive value of specific immunoglobulin E levels for the first diagnosis of cow's milk allergy. A critical analysis of pediatric literature. *Pediatr Allergy Immunol.* 2007;18:575–82.
38. Kearley J, Robinson DS, Lloyd CM. *CD4+CD25+* regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J Allergy Clin Immunol.* 2008;122:617–24e6.
39. Kennedy Norton S, Barnstein B, Brenzovich J, Bailey DP, Kashyap M, Speiran K, Ford J, Conrad D, Watowich S, et al. IL-10 suppresses mast cell IgE receptor expression and signaling in vitro and in vivo. *J Immunol.* 2008;180:2848–54.
40. Malbec O, Daeron M. The mast cell IgG receptors and their roles in tissue inflammation. *Immunol Rev.* 2007;217:206–21.
41. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc gamma RIIB cross-linking. *J Clin Invest.* 2006;116:833–41.

Online supporting Material

Supplemental Figure 1

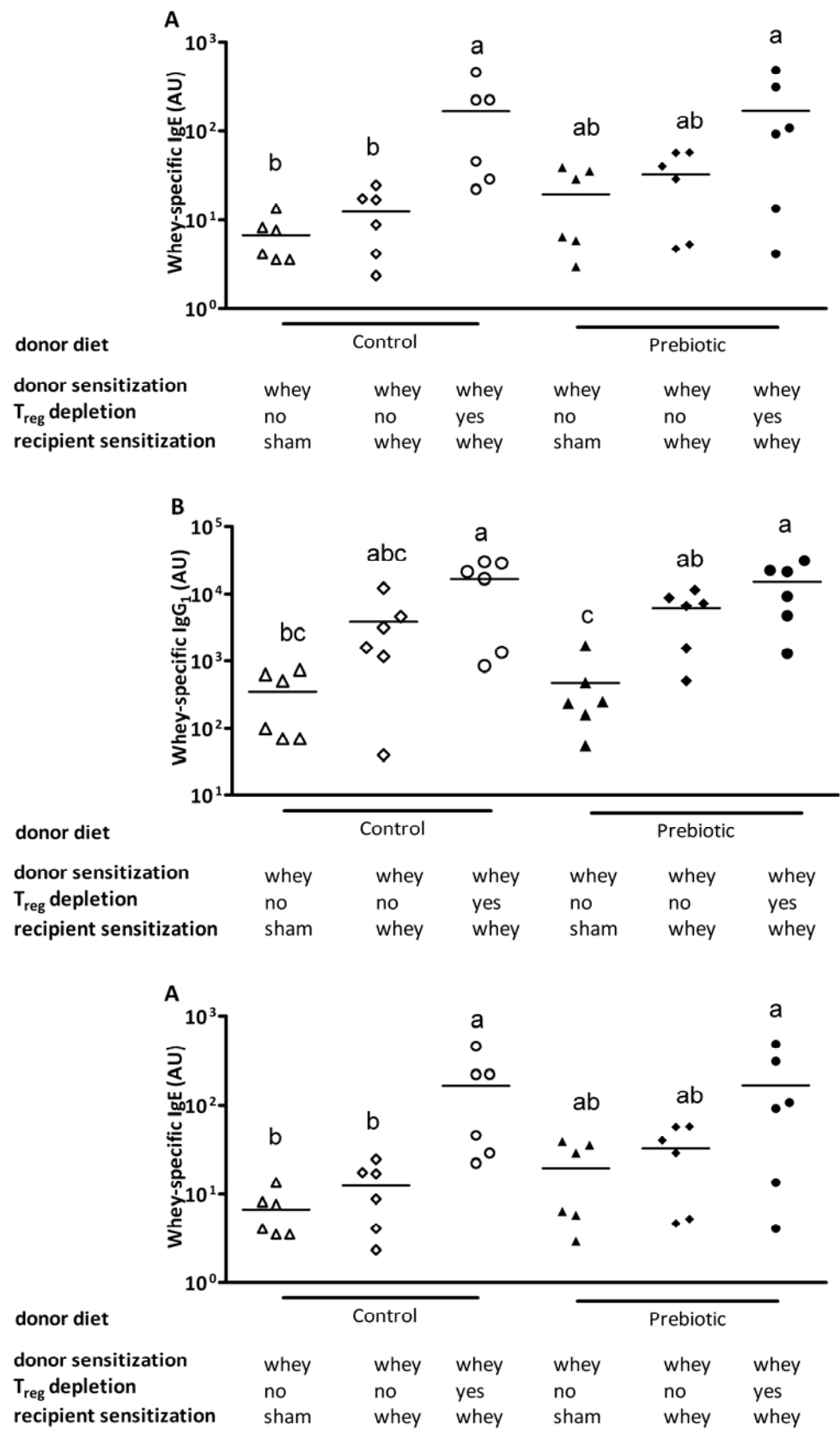


Supplemental Figure 1. Schematic diagram of the experimental set-up of the adoptive transfer experiment.

Supplemental Figure 2

Supplemental Figure 2. Representative dot plots of CD25⁺ Foxp3⁺ cells in the CD4⁺ population after *ex vivo* depletion of CD25⁺ Tregs using PC61 antibody in spleen cell suspensions.

Supplemental Figure 3.



Supplemental Figure 3. Serum whey-specific IgE (A), IgG₁ (B) and IgG_{2a} (C) concentrations in recipient mice transferred with splenocytes of whey sensitized donor mice fed the control or prebiotic diet with or without *ex vivo* CD25⁺ Treg-depletion (Expt. B). Recipient mice were fed the control diet and after transfer, they were sham- or whey-sensitized. One week after the last sensitization, serum was collected and 1h after oral challenge with whey. Means and individual values are presented, n=6. Statistical analyses were performed using 2-way ANOVA on log-transformed data. *P* values from the 2-way ANOVA for all immunoglobulins were: diet, *P*>0.05; sensitization, *P*<0.0001; interaction, *P*>0.05. Means without a common letter differ, *P*<0.05.