A potential role for CD25^{+} regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates

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Abstract

Dietary non-digestible carbohydrates reduce the development of cows' milk allergy in mice. In the present study, the contribution of CD25^{+} regulatory T-cells (T_{reg}) was investigated using *in vivo* T_{reg} depletion and adoptive transfer studies. Mice were orally sensitised with casein and fed a diet containing 2 % short-chain galacto-, long-chain fructo- and acidic oligosaccharides (GFA) or a control diet. Donor splenocytes of mice sensitised with casein and fed the GFA or control diet were adoptively transferred to naive recipient mice, which were casein- or sham-sensitised and fed the control diet. In addition, *in vivo* or *ex vivo* CD25^{+} T_{reg} depletion was performed using anti-CD25 (PC61). The acute allergic skin response upon intradermal casein challenge and casein-specific Ig were determined. Furthermore, T-helper (T_H) 1 and T_H2 cell numbers were analysed in the mesenteric lymph nodes. The oligosaccharide diet strongly reduced the development of the acute allergic skin response, which was abrogated by the *in vivo* anti-CD25 treatment. The diet enhanced the percentage of T_H1 cells and tended to reduce the percentage of T_H2 cells in casein-sensitised mice. Recipient mice were protected against the development of an acute allergic skin response when transferred with splenocytes from casein-sensitised GFA-fed donor mice before sensitisation. *Ex vivo* depletion of CD25^{+} T_{reg} abrogated this transfer of tolerance. Splenocytes from sham-sensitised GFA-fed donor mice did not suppress the allergic response in recipient mice. In conclusion, CD25^{+} T_{reg} contribute to the suppression of the allergic effector response in casein-sensitised mice induced by dietary intervention with non-digestible carbohydrates.

Key words: Cows' milk allergy: Prebiotics: Non-digestible carbohydrates: Oligosaccharides: Regulatory T-cells: Casein

One of the first manifestations of atopic disease is the development of cows' milk allergy (CMA) in young infants. Casein, in particular αS1-casein, is the main allergenic protein in cows' milk[1,2]. Non-digestible carbohydrates such as short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) mimic structural and functional properties of neutral oligosaccharides such as present in human breast milk and are added to clinical nutrition and infant milk formulas since they may have health and immune-promoting capacities[3,4]. Previously, in a mouse model of orally induced CMA this 9:1 scGOS/lcFOS mixture (Immunofortis®; Nutricia, Zoetermeer, The Netherlands) was found to reduce the allergic effector response to whey protein[5]. Furthermore, in a clinical study with infants at high risk of developing allergic disease, the same carbohydrate mixture reduced the incidence of atopic dermatitis significantly[6]. Acidic oligosaccharides derived from pectin (pAOS) are

non-digestible carbohydrates that mimic properties of human milk acidic oligosaccharides[7]. The combined use of neutral and acidic oligosaccharides was most effective in supporting the vaccination response in mice and has been shown to increase faecal *Bifidobacteria* and *Lactobacilli*[8,9].

The present study aims to address whether this particular scGOS/lcFOS/pAOS (9:1:1) mixture protects against the development of orally induced casein allergy in mice and to gain further insight into the underlying mechanism by which these oligosaccharides exert their effect.

In patients affected with food allergy, oral tolerance for harmless food protein such as casein is lost, resulting in local and systemic symptoms upon allergen challenge. Oral tolerance is in majority generated within the gut-associated lymphoid tissue. Regulatory T-cells (T_{reg}) generated in the mesenteric lymph nodes (MLN) are involved in the transfer of tolerance to the systemic immune system[10,11]. Cells with regulatory properties

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include TGF-β-producing T-helper (T_H) 3 cells, IL-10-producing T_{reg}1 cells and invariant natural killer T-cells[12–15]. However, naturally occurring or *de novo*-generated $CD4^+CD25^+Foxp3^+$ T_{reg} were described to be important for maintenance of peripheral or acquired tolerance to, for example, food antigens[16–18]. The contribution of $CD25^+$ T_{reg} to the induction of oral tolerance has been shown in children who have outgrown CMA; however, other mechanisms might be involved as well[19–21].

In the present study, mice were fed a diet containing 2% scGOS/lcFOS/pAOS during oral sensitisation with casein aiming to reduce the allergic effector response. *In vivo* anti-CD25 depletion and adoptive transfer studies were performed to determine the contribution of $CD25^+$ T_{reg} to the preventive effect of the diet.

Experimental methods

Diets

Standard synthetic semi-purified cows' milk protein-free (cows' milk proteins are replaced by soya proteins) AIN-93G-based diets (specified by Reeves[22]) were mixed with the oligosaccharides (Research Diet Services, Wijk bij Duurstede, The Netherlands). The scGOS/lcFOS/pAOS diet contained 2% (9:1:1, w/w) scGOS (Vivinal GOS, FrieslandCampina Domo, Zwolle, The Netherlands), lcFOS (Raftiline HP, Orafti, Wijchen, The Netherlands) and pAOS (Südzucker AG, Mannheim, Germany[9]). scGOS/lcFOS spray-dried powder consisted of approximately 50% scGOS and lcFOS (9:1), 19% maltodextrin (Glucidex 2, Roquette, France), 16% lactose, 14% glucose and 1% galactose[8]. pAOS consists of approximately 75% galacturonic acid oligomers, 10% monomers and 15% moisture and ash. All oligosaccharides were exchanged for the same amount of total carbohydrates, which resulted in a comparable overall carbohydrate composition in the diets.

Oral sensitisation and challenge of mice

Specific pathogen-free female C3H/HeOuJ mice (3 weeks old) were purchased from Charles River Laboratories (Maastricht, The Netherlands) and housed in the animal facility in accordance with the guidelines of the Dutch Committee of Animal Experiments. Mice were fed the control or scGOS/lcFOS/pAOS diet during the whole study period, starting 2 weeks before the first sensitisation. Mice were sensitised intragastric with 0·5 ml homogenised casein (40 mg PBS/ml; DMV International, Veghel, The Netherlands) with cholera toxin (20 μg PBS/ml; Quadratech Diagnostics, Epsom, UK) as an adjuvant, using a blunt needle. Control mice received cholera toxin alone. Mice were boosted (intragastric) once a week for a period of 5 weeks[23]. At 1 week after the last sensitisation, mice were challenged intradermally in the ear pinnae to determine the acute allergic skin response and challenged intragastric with 0·5 ml homogenised casein (200 mg PBS/ml). After 24 h, blood samples were collected in Minicollect® Z Serum Sep vials (Greiner bio-one BV, Alphen a/d Rijn, The Netherlands) and mice were killed by cervical dislocation.

The collected blood samples were centrifuged for 15 min at 13 500 rpm and sera were stored at −70°C.

Acute allergen-specific skin response

The acute allergic skin response was measured after intradermal injection with 20 μl homogenised casein protein (0·5 mg/ml in PBS) in the ear pinnae. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands), at $t = 0$ and 1 h after challenge. Ear swelling (μm) was calculated by subtracting the basal ($t = 0$ h) from the specific casein-induced ear swelling after 1 h ear thickness and additionally (Δ ear swelling) subtracting the mean ear swelling from the group of sham-sensitised mice fed the corresponding diet.

Anti-CD25 depletion studies

In the first set of experiments, *in vivo* depletion of $CD25^+$ T_{reg} was performed. A day before the first sensitisation, sham- and casein-sensitised mice fed the control or scGOS/lcFOS/pAOS diet were intraperitoneally injected with 200 μg (100 μl of 2 mg/ml solution) rat anti-mouse CD25 mAb or rat IgG_1 isotype antibody as a control. A second CD25 depletion was carried out one day before the third (day 14) sensitisation.

Adoptive transfer experiments

To determine whether the protective effect by scGOS/lcFOS/pAOS was transferable to naive recipient mice, an adoptive transfer study was performed using splenocytes in the presence or absence of *ex vivo* depleted $CD25^+$ T_{reg}. After killing, single cell suspensions were prepared of spleens from sham- or casein-sensitised mice fed the control or scGOS/lcFOS/pAOS diet (n 18, pooled). For each group, two-third part of the splenocytes was treated with 600 μg rat anti-CD25 (PC61) mAb, a kind gift of Bioceros BV (Utrecht, The Netherlands) in 60 ml Roswell Park Memorial Institute (RPMI) culture medium with 5% fetal calf serum for half an hour and washed. The other one-third part was untreated (non-depleted). Biomag Goat anti-rat IgG magnetic beads (2 ml; Bangs Laboratories, Inc., Fishers, IN, USA) in RPMI culture medium/5% fetal calf serum were added to the PC61-treated cells and rotated for 45 min. The bead–cell mixture was placed in a magnetic separator (Bangs Laboratories, Inc.) for 5 min; this procedure was repeated for the supernatant. Depletion of CD25-positive cells was analysed using flow cytometric analysis. Cells were counted using the Coulter Z1 particle counter (Beckman Coulter, The Netherlands). Splenocytes of the donor mice *ex vivo* depleted for $CD25^+$cells or non-depleted splenocyte suspensions were intravenously injected (100 μl, 1×10^7 cells/ml) in sixteen different groups of recipient mice, n 6 (Table 1). The recipient mice were sham- or casein-sensitised as described earlier. The acute allergic skin response and casein-specific serum Ig were measured to determine allergic sensitisation.

Table 1. Overview of the experimental set-up of adoptive transfer experiments

Sensitisation of donors	Diets	Treatment of splenocytes	Sensitisation of recipients	Diets
Sham	Control	No	Sham	Control
	Control	No	Casein	Control
	Control	Yes	Sham	Control
	Control	Yes	Casein	Control
	scGOS/lcFOS/pAOS	No	Sham	Control
	scGOS/lcFOS/pAOS	No	Casein	Control
	scGOS/lcFOS/pAOS	Yes	Sham	Control
	scGOS/lcFOS/pAOS	Yes	Casein	Control
Casein	Control	No	Sham	Control
	Control	No	Casein	Control
	Control	Yes	Sham	Control
	Control	Yes	Casein	Control
	scGOS/lcFOS/pAOS	No	Sham	Control
	scGOS/lcFOS/pAOS	No	Casein	Control
	scGOS/lcFOS/pAOS	Yes	Sham	Control
	scGOS/lcFOS/pAOS	Yes	Casein	Control

scGOS, short-chain galacto-oligosaccharides; lcFOS, long-chain fructo-oligosaccharides; pAOS, pectin-derived acidic oligosaccharides.

Flow cytometry

Single cell splenocyte suspensions were prepared and blocked for 30 min in PBS/1 % bovine serum albumin/5 % fetal calf serum. In addition, for the isolation of MLN lymphocytes, MLN were treated with RPMI culture medium containing collagenase D and DNAse (Roche Diagnostics, Almere, The Netherlands) and incubated for 30 min at 37°C twice; enzymes were inactivated using RPMI culture medium/5 % fetal calf serum. Cells were counted (Coulter Z1 particle counter, Beckman Coulter) and 2×10^6 cells/ml were incubated for 30 min at 4°C with different Abs (BD Biosciences, Alphen aan den Rijn, The Netherlands), unless otherwise stated. Anti-CD4-FITC (1:100), anti-CD4-PerCP-CY5.5 (1:50), anti-CD25-PE (1:100), anti-CD69-FITC (1:50), anti-CD69-PE (1:50), anti-CXCR3-PE (1:50) and anti-T1/ST2-FITC (1:50) (MD Biosciences, St Paul, MN, USA) and isotype controls were used. Foxp3 staining was performed following the manufacturer's protocol (eBioscience, Breda, The Netherlands). Flow cytometry analysis was performed using FACSCalibur and CellQuest Pro Analysis software (BD Biosciences) and isotype controls were subtracted.

Measurement of specific serum Ig

Concentrations of casein-specific IgE, IgG_1 and IgG_{2a} were determined in serum by means of ELISA as described earlier[23]. In short, plates were coated with 100 μl casein (20 mg/l), washed and several serum dilutions were applied for 1 h. Plates were washed and incubated with biotin-labelled rat anti-mouse IgE, IgG_1 and IgG_{2a} (1 mg/l; BD Biosciences), washed and incubated with streptavidin–horseradish peroxidase using diaminobenzidine as a substrate. For detection, absorption at A490 nm was determined using a spectrophotometer (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Results are expressed as arbitrary units, calculations were based on titration curves of pooled sera from casein and alum-intraperitoneally immunised mice as a positive reference serum.

Statistics

All data were analysed using the one-way ANOVA and *post hoc* Bonferroni's comparison test or Mann–Whitney test. For the Ig levels, ANOVA was performed on log-transformed data. Statistical analyses were conducted using GraphPad Prism software (version 4.03; GraphPad Prism, La Jolla, CA, USA). $P < 0.05$, $P < 0.01$ and $P < 0.001$ were indicated when analyses reached statistical significance. Data are presented as means with their standard errors.

Results

Effect of dietary intervention and in vivo CD25+ Treg depletion on the acute allergic skin response

Starting 2 weeks before the first sensitisation, mice were fed the control or 2 % (w/w) scGOS/lcFOS/pAOS diet. To study the contribution of CD25+ T_{reg} to the possible preventive effects of the scGOS/lcFOS/pAOS diet on the acute allergic skin response, sham- and casein-sensitised mice were injected (intraperitoneally) with the anti-CD25 antibody or isotype control. The Δ ear swelling in casein-sensitised mice fed the control diet was significantly increased compared with sham-sensitised mice (data not shown) and the scGOS/lcFOS/pAOS diet largely prevented this ($P < 0.001$, isotype control group; Fig. 1(a)). The protective effect of the scGOS/lcFOS/pAOS diet was completely abrogated upon partial *in vivo* depletion of CD25+ T_{reg} using the anti-CD25 mAb treatment. To confirm the depletion of CD25+ cells, two groups of casein-sensitised mice were killed at day 15, 2 d after the second anti-CD25 or isotype control treatment (Fig. 1(b)). The CD4+CD25+Foxp3+ T_{reg} percentages in the spleen declined by more than 65 % using the anti-CD25 mAb treatment compared with the isotype control (4·7 (SEM 0·5) *v.* 2·2 (SEM 0·3) % of CD4+ cells, $P < 0.01$, scGOS/lcFOS/pAOS group). The scGOS/lcFOS/pAOS diet did not affect the CD25+ T_{reg} cell percentages (data not shown). The anti-CD25 treatment did not reduce CD4+CD25+Foxp3− cells, which includes the effector T-cell population (5·1 (SEM 0·4) *v.* 5·5 (SEM 0·5) % of CD4+ cells, scGOS/lcFOS/pAOS

group). The percentage of $CD4^+$ cells was reduced in the anti-CD25-treated group compared with the isotype controls (12·9 (SEM 1·0) $v.$ 10·1 (SEM 0·3) % of total lymphocytes, $P<0·05$).

Casein-specific Ig levels in mice upon in vivo regulatory T-cell depletion

Serum samples were collected and analysed for casein-specific Ig levels. As described before[23] casein-specific IgE levels were not enhanced in casein-sensitised mice in comparison with sham-sensitised mice, and casein-specific IgG_1 was not enhanced due to two non-responders. However, after T_{reg} depletion, casein-specific IgE and IgG_1 levels were enhanced in casein-sensitised mice ($P<0·05$ and $P<0·01$; Fig. 2) in comparison with sham-sensitised mice. This was observed in mice fed the control diet as well as the scGOS/lcFOS/pAOS diet. Casein-specific IgG_{2a} levels were not increased upon sensitisation; however, after T_{reg} depletion, in both diet groups, casein-specific IgG_{2a} levels were enhanced, although this only reached significance in the scGOS/lcFOS/pAOS diet
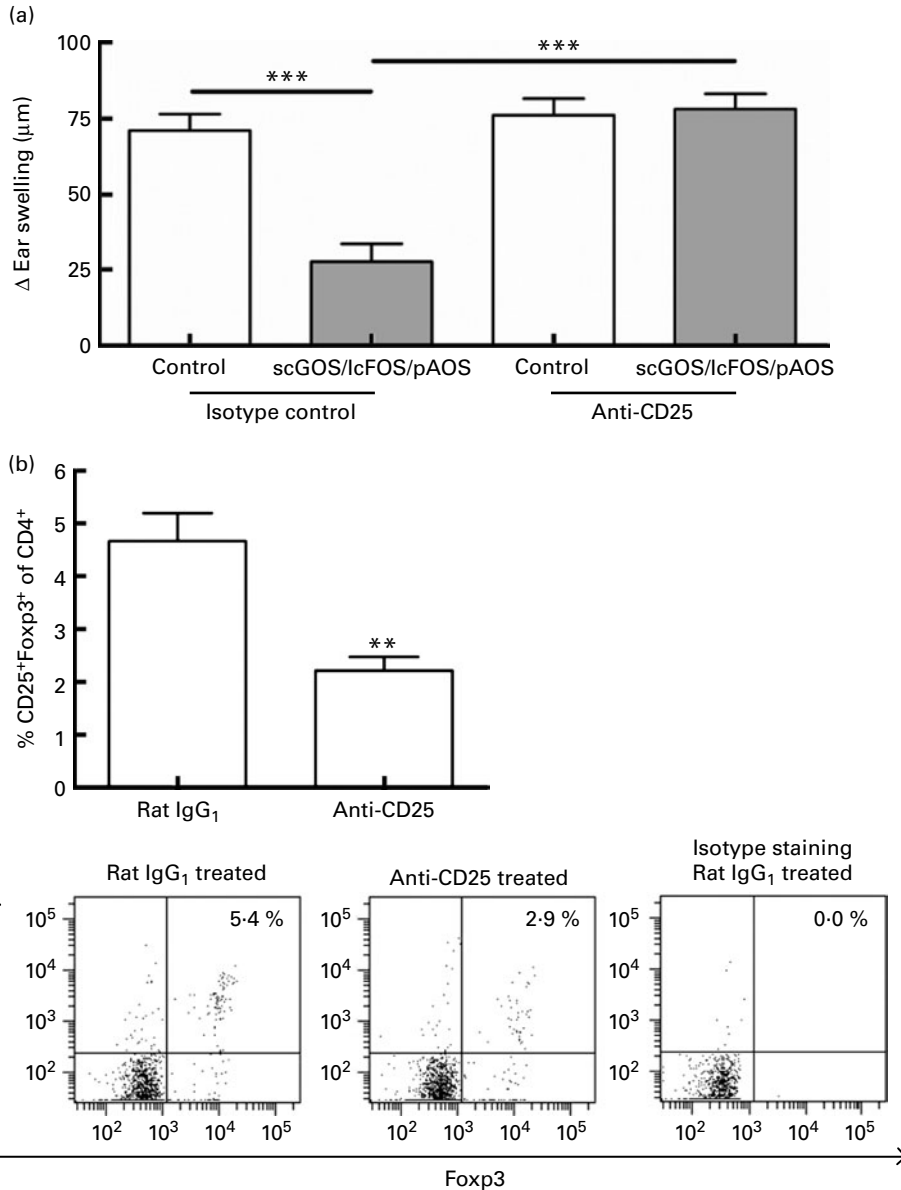

Fig. 1. Short-chain galacto-oligosaccharide (scGOS)/long-chain fructo-oligosaccharide (lcFOS)/pectin-derived acidic oligosaccharide (pAOS) diet effectively reduced the acute allergic skin response, which was abrogated by the anti-CD25 mAb treatment. (a) The casein-induced acute skin response in casein-sensitised mice fed the control or scGOS/lcFOS/pAOS diet was measured 1 h after intradermally challenge 1 week after the last sensitisation. Mice were fed a control or oligosaccharide diet and casein- or sham-sensitised for five consecutive times using cholera toxin as an adjuvant. Before the first and third sensitisation, mice were intraperitoneally injected with the anti-CD25 or isotype control antibody. Values are means of two identical experiments, with their standard errors represented by vertical bars (n 6). *** Mean values were significantly different ($P<0·001$). (b) Confirmation of in vivo depletion of $CD4^+CD25^+Foxp3^+$ regulatory T-cells in the spleen after the second anti-CD25 treatment. Representative dot plots of $CD25^+Foxp3^+$ cells in the $CD4^+$ population of mice in vivo treated with the anti-CD25 or isotype control antibody. The percentage of $CD4^+CD25^+Foxp3^-$ cells was unaffected. Values are means, with their standard errors represented by vertical bars (n 5–6). ** Mean values were significantly different ($P<0·01$).

group compared with sham-sensitised controls fed the scGOS/lcFOS/pAOS diet ($P<0.01$; Fig. 2(c)). In addition, upon anti-CD25 treatment, casein-specific IgG$_{2a}$ levels of casein-sensitised mice fed the scGOS/lcFOS/pAOS diet were enhanced compared with T$_{reg}$-depleted casein-sensitised mice fed the control diet ($P<0.05$; Fig. 2(c)). However, also sham-sensitised mice fed the scGOS/lcFOS/pAOS diet tended to have enhanced IgG$_{2a}$ (Fig. 2(c)).

Adoptive transfer experiments; donor mice

To further study CD25$^+$ T$_{reg}$ cell involvement in the preventive effects of the scGOS/lcFOS/pAOS diet, an adoptive transfer study was performed (Table 1). In the donor mice, the scGOS/lcFOS/pAOS diet effectively reduced the acute allergic skin response of casein-sensitised donor mice when compared with the control diet (108·9 (SEM 4·3) v. 60·8 (SEM 5·0) μm, $P<0.001$). The scGOS/lcFOS/pAOS diet did not change the
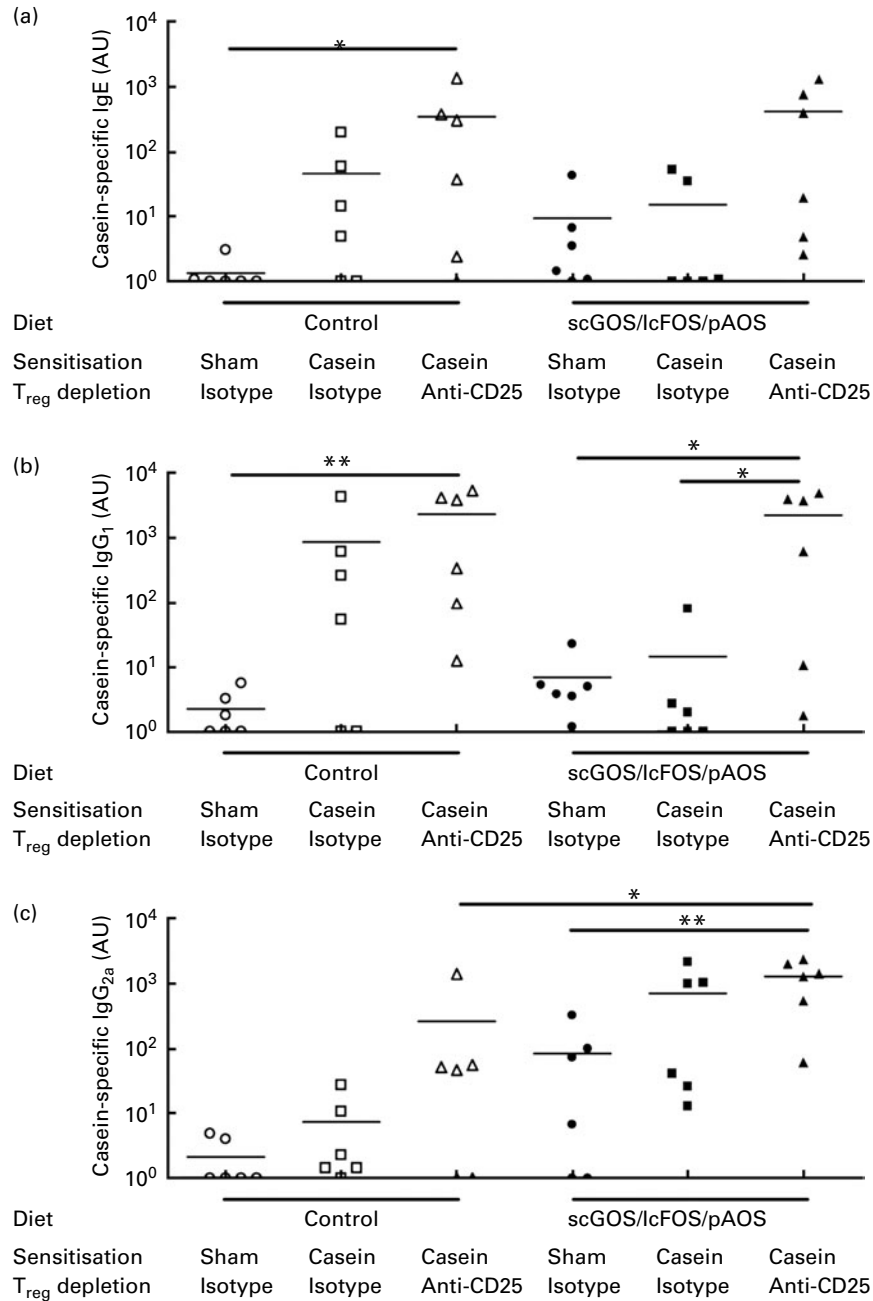
Fig. 2. Casein-specific Ig levels in sham- or casein-sensitised mice. (a) Casein-specific IgE, (b) casein-specific IgG$_1$ and (c) casein-specific IgG$_{2a}$. Mice were fed a control or oligosaccharide diet and casein- or sham-sensitised for five consecutive times using cholera toxin as an adjuvant. Before the first and third sensitisation, mice were intraperitoneally injected with the anti-CD25 or isotype control antibody. At 1 week after the last sensitisation, mice were orally challenged with casein and serum was collected. Values are means, with their standard errors represented by vertical bars (*n* 6). Mean values were significantly different: *$P<0.05$ and **$P<0.01$. AU, arbitrary units; scGOS, short-chain galacto-oligosaccharides; lcFOS, long-chain fructo-oligosaccharides; pAOS, pectin-derived acidic oligosaccharides; T$_{reg}$, regulatory T-cell.

ear-swelling response in sham-sensitised donor mice compared with control diet mice (36·6 (SEM 2·9) v. 39·8 (SEM 2·5) μm) nor the body weight of casein- or sham-sensitised mice (data not shown). scGOS/lcFOS/pAOS did enhance the percentage of activated T_H1 cells of casein-sensitised mice when compared with control diet-fed casein-sensitised mice, although this was not observed in all mice ($P < 0.05$; Fig. 3(a)). The percentage of activated T_H2 cells tended to be reduced in scGOS/lcFOS/pAOS-fed casein-sensitised mice (Fig. 3(b)).

Acute allergic skin response of recipient mice in adoptive transfer experiments

Splenocytes of sham- or casein-sensitised mice fed the control or scGOS/lcFOS/pAOS diet were adoptively transferred to control diet-fed recipient mice, which were consequently casein-sensitised (Table 1). The recipient mice developed an acute allergic skin response, which was almost completely prevented by adoptive transfer using splenocytes of casein-sensitised mice fed the scGOS/lcFOS/pAOS diet (Fig. 4). In contrast, adoptive transfer with splenocytes of control

diet-fed casein-sensitised donor mice did not dampen the acute allergic skin response. The protective effect was dependent on the sensitisation with casein since splenocytes of sham-sensitised mice fed the scGOS/lcFOS/pAOS diet did not transfer this tolerance. Ex vivo $CD25^+$ T_{reg} cell depletion completely abrogated the protective effect ($P < 0.001$).

Casein-specific Ig of recipient mice in adoptive transfer experiments

Casein-specific IgE and IgG_1 levels of sham-sensitised recipient mice receiving splenocytes from sham-sensitised donor mice were low and did not enhance upon sensitisation. Transfer with splenocytes from casein-sensitised donors (control as well as scGOS/lcFOS/pAOS fed) tended to enhance casein-specific IgE and IgG_1, and this was not affected by ex vivo depletion of $CD25^+$ T_{reg} cells (Fig. 5(a) and (b)). Casein-specific IgG_{2a} levels of casein-sensitised recipient mice transferred with splenocytes from casein-sensitised donor mice were enhanced compared with recipients transferred with splenocytes of sham-sensitised donor mice either
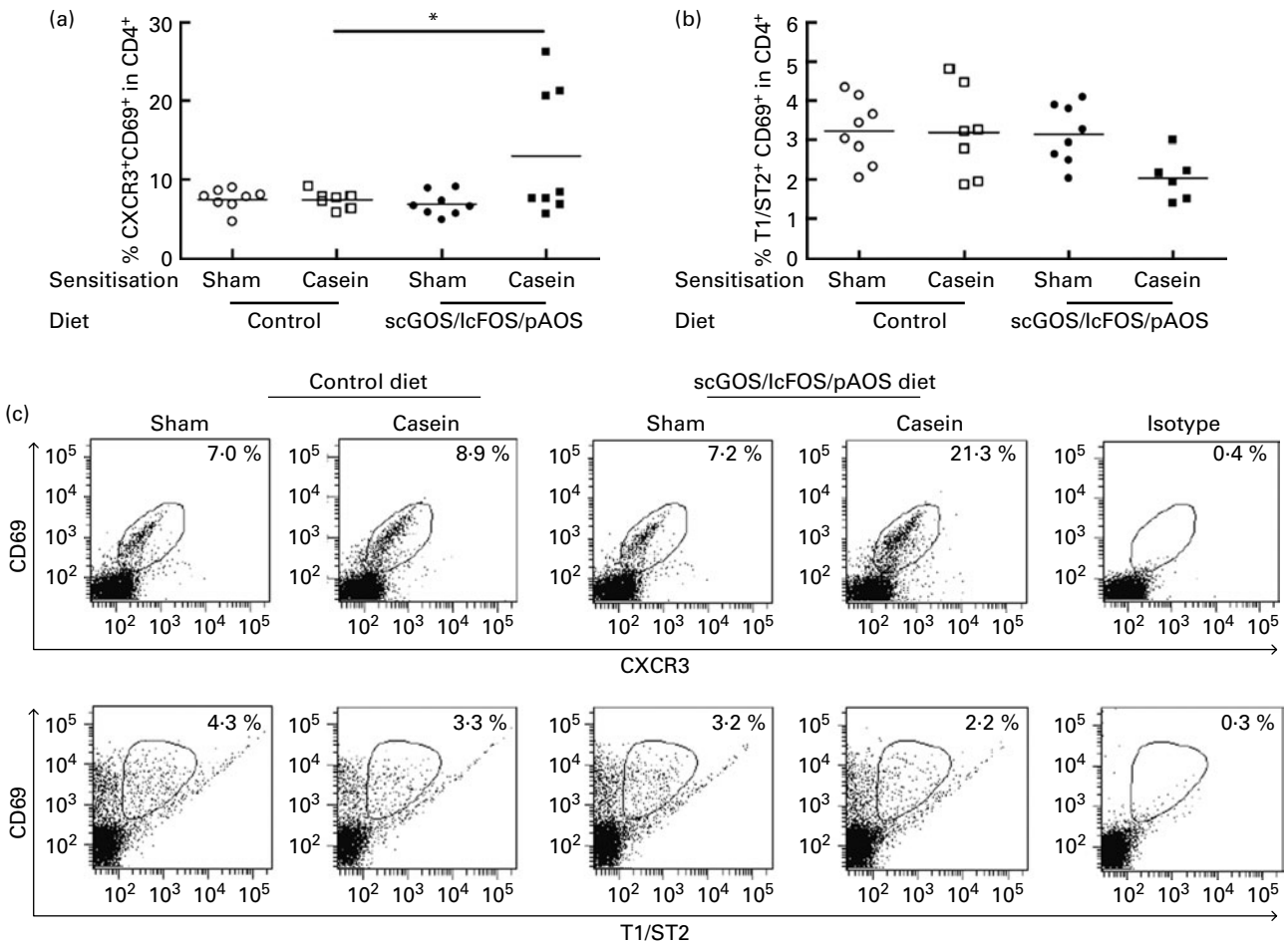

Fig. 3. Flow cytometric analysis of activated T-helper (T_H) 1 and T_H2 cells in mesenteric lymph node (MLN) cells of sham- and casein-sensitised mice fed the control or short-chain galacto-oligosaccharide (scGOS)/long-chain fructo-oligosaccharide (lcFOS)/pectin-derived acidic oligosaccharide (pAOS) diet. (a) $CD4^+CD69^+$ $CXCR3^+$ (T_H1) cells and (b) $CD4^+CD69^+T1/ST2^+$ (T_H2) cells. (c) Representative dot plots of $CXCR3^+CD69^+$ and $T1/ST2^+CD69^+$ in the $CD4^+$ population. Mice were fed a control or oligosaccharide diet and casein- or sham-sensitised for five consecutive times using cholera toxin as an adjuvant. At 1 week after the last sensitisation, mice were orally challenged with casein and killed, and MLN were collected. Values are means, with their standard errors represented by vertical bars (n 6–8). Mean values were significantly different: *$P < 0.05$.
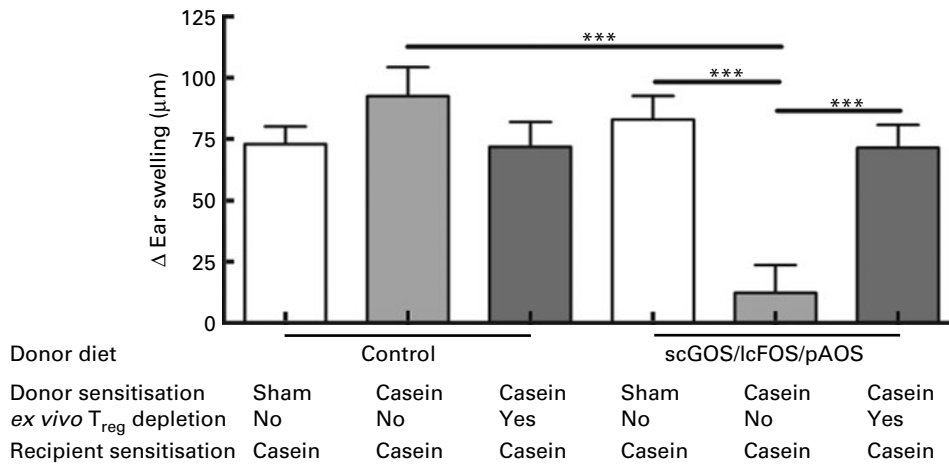
Fig. 4. Adoptive transfer of splenocytes in the absence or presence of *ex vivo* depleted CD25$^+$ regulatory T-cells (T$_{reg}$). Casein-sensitised recipient mice adoptively transferred with splenocytes from casein-sensitised short-chain galacto-oligosaccharide (scGOS)/long-chain fructo-oligosaccharide (lcFOS)/pectin-derived acidic oligosaccharide (pAOS)-fed donor mice were protected against the development of an acute allergic skin response, which was abolished by *ex vivo* depletion of CD25$^+$ T$_{reg}$ cells. Splenocytes of sham-sensitised donors fed the oligosaccharide diet or donor cells of casein-sensitised mice fed the control diet did not protect against the development of the acute allergic skin response in recipient mice. Recipient mice were fed the control diet and transferred with donor splenocytes in the absence or presence of CD25$^+$ T$_{reg}$ before sham or casein sensitisation. Δ Ear swelling was calculated by subtracting the mean ear swelling of corresponding sham-sensitised recipient mice. Values are means, with their standard errors represented by vertical bars (n 4–6). Mean values were significantly different: ***$P < 0.001$.

fed the control or scGOS/lcFOS/pAOS diet ($P < 0.001$ and $P < 0.05$; Fig. 5(c)). *Ex vivo* CD25$^+$ T$_{reg}$ cell depletion did not alter this response.

In general, no differences in casein-specific Ig levels were found between recipient mice transferred with splenocytes from control or scGOS/lcFOS/pAOS-fed mice and *ex vivo* CD25$^+$ T$_{reg}$ cell depletion did not affect the Ig levels.

Discussion

In the present study, the involvement of CD25$^+$ T$_{reg}$ is demonstrated in the protective effects of scGOS/lcFOS/pAOS for the prevention of CMA in mice orally sensitised with casein.

Mice fed a scGOS/lcFOS/pAOS diet before and during oral sensitisation with casein showed a strong reduction in the allergic effector response when compared with mice fed a control diet. To study whether the non-digestible carbohydrates may protect against the development of casein allergy via CD25$^+$ T$_{reg}$, *in vivo* depletion studies were performed. In accordance with previous studies, monoclonal anti-CD25 antibody PC61 treatment selectively decreased the percentage of regulatory CD4$^+$CD25$^+$Foxp3$^+$ cells in the spleen for more than 65 %[24–27]. *In vivo* depletion of CD25$^+$ T$_{reg}$ was found to abrogate the protective effects of the scGOS/lcFOS/pAOS diet. scGOS/lcFOS/pAOS-fed mice treated with anti-CD25 developed a severe acute allergic skin response, indicating a role for CD25$^+$ T$_{reg}$ in the preventive effects induced by the diet. In agreement with the present study, Sun *et al.*[28] showed that the oral administration of antigens induces oral tolerance via the induction of CD4$^+$CD25$^+$Foxp3$^+$ T$_{reg}$.

We hypothesised that the diet affects the mucosal immune system and actively interferes in oral tolerance induction. Since allergies develop as a consequence of a disparity in the T$_H$1 *v.* T$_H$2 balance towards T$_H$2[29], the percentage of T$_H$1 and T$_H$2 cells was determined in the MLN. Indeed, in casein-sensitised

mice, the scGOS/lcFOS/pAOS mixture was found to enhance the percentage of activated T$_H$1 cells, while the percentage of activated T$_H$2 cells tended to reduce. This may indicate active regulation of the mucosal immune response occurring upon sensitisation with casein as a consequence of dietary intervention with scGOS/lcFOS/pAOS. Although further studies are needed to substantiate these findings, other studies have supported the involvement of T$_H$1 and CD25$^+$ T$_{reg}$ in the effects generated with scGOS/lcFOS/pAOS. Vos *et al.*[30] described scGOS/lcFOS/pAOS to alleviate ovalbumin-induced allergic asthma while stimulating the influenza vaccination response in mice. In addition, recently it has been established that CD25$^+$ T$_{reg}$ depletion abrogated the stimulatory effects of the oligosaccharide mixture in the murine vaccination model[31]. This implies the help of T$_{reg}$ in the generation of an effective T$_H$1-driven vaccination response and the support of scGOS/lcFOS/pAOS on both T$_H$1 and T$_{reg}$ cells while skewing away from a T$_H$2-type allergic response.

Although casein-specific IgE levels tended to increase upon sensitisation, this did not reach significance as described previously[23]. However, the depletion of CD25$^+$ T$_{reg}$ resulted in enhanced casein-specific IgG$_1$ and a similar tendency was shown for IgE, indicating an exacerbated T$_H$2 response upon *in vivo* anti-CD25 treatment. Consistent with the present study, Sumi *et al.*[32] and van Wijk *et al.*[12] described that selective *in vivo* depletion of CD25$^+$ T$_{reg}$ cells results in exacerbation of allergic sensitisation in a murine model for allergic conjunctivitis and peanut allergy, respectively, as reflected by increased levels of specific IgE. Hence, *in vivo* anti-CD25 mAb treatment may have enhanced T$_H$2 activity and T$_H$2 polarisation. Since *in vivo* anti-CD25 mAb treatment increased the antibody levels, which could have underlain abrogation of the protective effect of the scGOS/lcFOS/pAOS diet, adoptive transfer experiments were conducted.

Food antigen-responsive T-cells that are generated in the MLN subsequently travel through the bloodstream and home
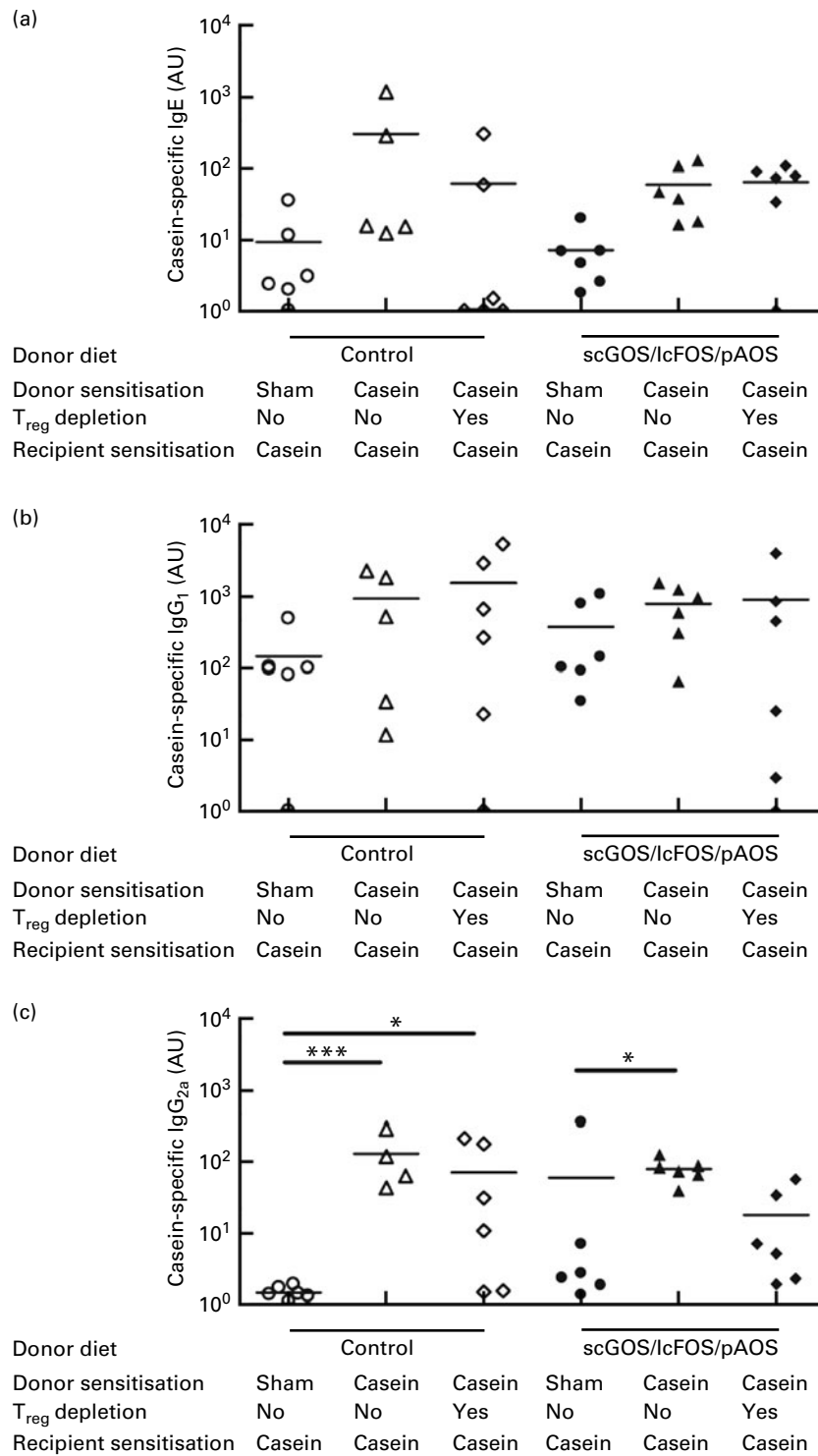
Fig. 5. Casein-specific Ig levels in the serum of recipient mice after adoptive transfer with whole spleen suspension or *ex vivo* CD25^{+} regulatory T-cell (T$_{reg}$)-depleted splenocytes. No differences in Ig levels were observed in mice transferred with splenocytes of control and short-chain galacto-oligosaccharide (scGOS)/long-chain fructo-oligosaccharide (lcFOS)/pectin-derived acidic oligosaccharide (pAOS)-fed mice and *ex vivo* T$_{reg}$ depletion did not affect Ig levels. (a) Casein-specific IgE, (b) casein-specific IgG$_1$ and (c) casein-specific IgG$_{2a}$. Recipient mice were fed the control diet and transferred with donor splenocytes in the absence or presence of CD25^{+} T$_{reg}$ before sham or casein sensitisation. Values are means, with their standard errors represented by vertical bars (*n* 6). Mean values were significantly different: *$P < 0.05$ and ***$P < 0.001$. AU, arbitrary units.

back into the mucosa or remain in the periphery (e.g. spleen)[10,11]. In this respect, local induced tolerance for food-derived antigens can be transferred to systemic non-responsiveness. In particular, CD25$^+$ T$_{reg}$ cells have been implicated to contribute to this phenomenon[11]. To confirm that dietary intervention with scGOS/lcFOS/pAOS resulted in a systemic tolerogenic effect on the adaptive immune response, the adoptive transfer experiments were carried out with splenocytes. Casein-sensitised recipients transferred with splenocytes from scGOS/lcFOS/pAOS-fed casein-sensitised donor mice were protected from the development of an acute allergic casein-specific skin response. This was abrogated after ex vivo anti-CD25 mAb treatment of the splenocytes before transfer. The anti-CD25 antibody PC61 is known for its high affinity for CD25$^+$ T$_{reg}$[12,32–34]. Indeed, in the present study, we show a partial but selective in vivo depletion of T$_{reg}$ while effector T-cell populations were unaffected. Recently, published studies[35,36], however, have shown that a high dose of anti-CD25 completely depletes the CD25$^+$ T$_{reg}$ cell population, which coincides with a partial depletion of effector T-cells. Hence, the ex vivo T$_{reg}$ depletion may have resulted in the loss of some effector T-cells. However, CD25$^+$ T$_{reg}$ are highly sensitive for the depleting antibody and most probably involved in the protective effect of the scGOS/lcFOS/pAOS diet since the reduction in the acute allergic skin response is lost upon ex vivo CD25$^+$ T$_{reg}$ depletion in the adoptive transfer studies, and these effects mimic the selective but partial in vivo CD25$^+$ T$_{reg}$ depletion. Furthermore, transfer of ex vivo CD25$^+$ T$_{reg}$-depleted splenocytes did not affect casein-specific Ig levels in the recipient mice, hence the adoptive transfer studies confirmed CD25$^+$ T$_{reg}$ to be involved in the protective effects induced by the scGOS/lcFOS/pAOS diet. The same mixture of oligosaccharides also protected mice against the development of whey allergic symptoms, which could be adoptively transferred depending on the presence of CD25$^+$ T$_{reg}$[37]. Recently, Ig-free light chains were found to contribute to the acute allergic skin response of casein-sensitised mice while for whey, this most probably is IgE[38]. Therefore, it is likely that CD25$^+$ T$_{reg}$ induced by the oligosaccharide diet protect against the development of allergic symptoms, which may be applicable to both IgE-mediated and non-IgE-mediated acute-type hypersensitivity responses.

Gri et al.[39] showed that T$_{reg}$ are able to inhibit acute allergic responses, which may relate to the reduced allergic skin response observed in the present study in mice fed the scGOS/lcFOS/pAOS diet. Previous studies have shown that the acute allergic skin response is a robust reproducible parameter which relates to systemic anaphylaxis scores upon intradermally challenge and mucosal mast cell degranulation upon oral challenge[5,23]. It remains elusive how the CD25$^+$ T$_{reg}$ may exert their effect on the allergic effector response in the murine model of casein allergy since Ig titres remain highly unaltered. However, one of the possible explanations could be that CD25$^+$ T$_{reg}$ have altered the mast cell phenotype. Mast cells are most probably involved in the acute skin response and T$_{reg}$ are believed to alter Fc-receptor expression and mast cell degranulation by IL-10 production or cell–cell contact[39–41].

Both the in vivo and ex vivo CD25$^+$ T$_{reg}$ cell depletion studies have suggested a central participation of CD25$^+$ T$_{reg}$ in the preventive effects of the scGOS/lcFOS/pAOS diet on casein-induced acute allergic responses. Only splenocytes from casein-sensitised and not splenocytes from sham-sensitised donor mice fed the scGOS/lcFOS/pAOS diet could prevent the allergic response in recipient mice. This may imply that antigen-specific T$_{reg}$ were induced and protected against CMA, antigen-specific T$_{reg}$ have been shown to be generated under several conditions and are associated with resolution of milk allergy in human subjects[20,24,42–44].

In conclusion, dietary intervention with scGOS/lcFOS/pAOS reduces the development of an acute allergic skin response upon antigen challenge, which was abrogated by in vivo CD25$^+$ T$_{reg}$ depletion or ex vivo depletion before adoptive transfer. Only splenocytes of scGOS/lcFOS/pAOS-fed casein-sensitised donor mice transferred these protective effects, suggesting the involvement of dietary scGOS/lcFOS/pAOS-induced antigen-specific CD25$^+$ T$_{reg}$ in the suppression of the allergic effector response.

Acknowledgements

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