

Original article

Hypersensitivity and oral tolerance in the absence of a secretory immune system

Background: Mucosal immunity protects the epithelial barrier by immune exclusion of foreign antigens and by anti-inflammatory tolerance mechanisms, but there is a continuing debate about the role of secretory immunoglobulins (SIg), particularly SIgA, in the protection against allergy and other inflammatory diseases. Lack of secretory antibodies may cause immune dysfunction and affect mucosally induced (oral) tolerance against food antigens.

Methods: We used polymeric Ig receptor (pIgR) knockout (KO) mice, which cannot export SIgA or SIgM, to study oral tolerance induction by ovalbumin (OVA) feeding and for parenteral antigen sensitization in the same animal.

Results: Remarkable systemic hyperreactivity was observed in pIgR KO mice, as 50% died after intradermal OVA challenge, which was not seen in similarly sensitized and challenged wild-type (WT) mice. Oral tolerance induced by OVA completely protected the sensitized pIgR KO mice against anaphylaxis and suppressed antibody levels (particularly IgG1) as well as delayed-type hypersensitivity (DTH) to OVA. Delayed-type hypersensitivity to a bystander antigen, human serum albumin, was also suppressed and T-cell proliferation against OVA in vitro was reduced in tolerized compared with non-tolerized pIgR KO mice. This effect was largely mediated by CD25⁺ T cells. Adoptive transfer of splenic putative regulatory T cells (CD4⁺ CD25⁺) obtained from OVA-fed pIgR KO mice to naïve WT mice mediated suppression of DTH against OVA after sensitization of the recipients.

Conclusion: Compensatory regulatory T-cell function becomes critical in pIgR-deficient mice to avoid the potentially catastrophic effects of systemic immune hyperreactivity, presumably resulting from defective secretory antibody-mediated immune exclusion of microbial components.

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Key words: anaphylaxis; mucosal immunity; oral tolerance; regulatory T cells; secretory IgA.

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Accepted for publication 15 September 2009

The mucosal immune system has a complex cellular composition and tissue architecture (1). A monolayered epithelium forms a barrier against luminal antigens and bacterial penetration. When this barrier is intact, the immune system can discriminate pathogens from harmless food antigens or components of the commensal microbiota, by eliciting proinflammatory responses or mucosally induced (oral) tolerance, respectively (2). The epithelial barrier is reinforced by innate and adaptive immune mechanisms which inhibit microbial adherence and limit antigen penetration (3).

Efficient immune exclusion of foreign components requires dimeric IgA or pentameric IgM antibodies (collectively called polymeric (p)Igs) produced by mucosal plasma cells (PCs) (4). The pIgs are covalently stabilized by the joining (J) chain, which contributes to a unique binding site for membrane secretory component (SC) (4). This glycoprotein is expressed as the pIg receptor (pIgR) basolaterally on secretory epithelial cells. After interacting with pIgR, the pIgs are transcytosed to the apical surface where secretory (S)IgA and SIgM are released to the lumen with bound SC – the extracellular portion of pIgR (4). In pIgR knockout (KO) mice pIg transport is lost, resulting in leaky mucous membranes with penetration of commensal bacteria (5, 6). The present study examined whether innate immune activation because of a reduced mucosal barrier might cause systemic hyperreactivity and/or affect oral tolerance.

Although oral tolerance remains mechanistically unclear, T-cell deletion or anergy and induction of

Abbreviations: DTH, delayed-type hypersensitivity; J chain, joining chain; SC, secretory component; KO, knockout; pIg, polymeric Ig; MLN, mesenteric lymph node; pIgR, polymeric Ig receptor; SIg, secretory Ig; OVA, ovalbumin; HSA, human serum albumin; IEL, intraepithelial lymphocyte; Treg, regulatory T cell; PC, plasma cell; WT, wildtype.

regulatory T cells (T_{regs}) have been identified in experimental protocols (7). Various T_{reg} subsets have been implicated, including TGF- β -secreting Th3 cells, IL-10-secreting Tr1 cells, and $CD4^+ CD25^+ /FOXP3^+$ T cells (7–9). The $CD4^+ CD25^+$ subset can utilize TGF- β , IL-10 and/or cell contact-mediated suppression to control pro-inflammatory effector T cells – even directed against non-cognate antigens in so-called bystander suppression (10–12).

We recently showed that $CD4^+ CD25^+ T_{\text{regs}}$ prevent non-IgE-mediated (delayed type) cow's milk allergy in children (13). T_{regs} also regulate hypersensitivity in a murine model for IgE-mediated peanut allergy (14). Here, we demonstrate that systemically sensitized pIgR KO mice are hyperreactive to cognate antigen challenge, yet they can mount an efficient mucosally induced tolerogenic response – downregulating consistently the IgG1 antibody response and protecting against systemic anaphylaxis. Oral tolerance also suppressed delayed-type hypersensitivity (DTH) significantly better in this mouse strain than in wild-type (WT) mice. Our results suggested that in the absence of SIg-mediated reinforcement of the mucosal barrier, compensatory oral tolerance is induced.

Materials and methods

Mouse strains

Adult (6–8 weeks old) pIgR KO mice on a C57BL/6J background (5), were bred under standard pathogen-free conditions. Age- and sex-matched WT C57BL/6J mice, and OVA-specific TCR transgenic Balb/c (DO11.10) mice, were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and kept in the same room as the pIgR KO mice. The experiments were approved by the Regional Ethics Committee for Experimental Animals (Reference No. 17/03, Forsøksdyrutvalget, Oslo, Norway).

Induction of tolerance via the gut

To induce oral tolerance against OVA, pIgR KO and WT mice were fed an OVA-containing (9% w/w) diet (AnalyCen, Linköping, Sweden) for 1 week. Control pIgR KO, WT, and DO11.10 mice were only fed ordinary pellets (Table 1). The experiments were performed in parallel in all strains of mice.

Systemic sensitization by parenteral immunization

One week after discontinuing the OVA diet, pIgR KO and WT mice were sensitized by s.c. immunization in the hind leg with 100 μ g OVA (Grade V, Sigma, St Louis, MO, USA) mixed with 100 μ g human serum albumin (HSA; Grade V, Sigma) in 100 μ l CFA (Difco Laboratories, Detroit, MI, USA). As controls for bystander suppression, other groups of mice were sensitized with OVA (100 μ g in 50 μ l CFA/PBS solution) and HSA (100 μ g in 50 μ l CFA/PBS solution) in separate hind legs (Table 1). A booster immunization was performed in the same manner in all groups 4 weeks after the primary sensitization with the same antigen doses in incomplete Freund's adjuvant (Difco Laboratories). One week later the mice were euthanized, and blood and spleens were collected.

Table 1. Oral tolerance induction, sensitization, and hypersensitivity after challenge*

	Feeding regimen	Systemic (s.c.) sensitization	Intradermal challenge	Anaphylactic death
Weeks	0–1	2	5	5
pIgR KO	OVA diet	OVA + HSA†	OVA – HSA‡	0/10
	Ctrl. feed	None	OVA – HSA	0/5
	Ctrl. feed	OVA + HSA	OVA – HSA	5/10
	OVA diet	OVA/HSA§	OVA – HSA	2/5
WT	OVA diet	OVA + HSA	OVA – HSA	0/5
	Ctrl. feed	OVA + HSA	OVA – HSA	0/5
DO11.10	Ctrl. feed	OVA + HSA	OVA – HSA	0/5

*Oral tolerance induction and proportion of immediate anaphylactic deaths in pIgR KO, WT, and OVA-specific TCR transgenic (DO11.10) mice. Mice were fed OVA-containing food (OVA diet) or control pellets (Ctrl. feed) prior to systemic sensitization with OVA and HSA by s.c. immunization, followed by intradermal ear challenge with OVA and HSA for determination of delayed-type hypersensitivity.

†OVA + HSA, immunization with the two antigens in same hind leg.

‡OVA – HSA, challenge with the two antigens in different ears.

§OVA/HSA, immunization with the two antigens in different hind legs.

Determination of delayed type hypersensitivity

Three weeks after the primary s.c. OVA immunization, the mice were challenged with intradermal injection of 20 μ g OVA in 10 μ l PBS in one ear, and 20 μ g HSA in 10 μ l PBS in the other (Table 1). The ear thickness was measured before and 24 h after challenge with micrometer calipers (Oditest, Kroplin, Hessen, Germany). To control trauma effects, we confirmed that PBS injection caused no measurable increase of ear thickness after 24 h.

Blood samples and serum antibody determinations

Blood was collected by heart puncture. Specific IgG, IgA, and IgM serum antibody levels were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with OVA (5 μ g/ml, Grade V, Sigma) or HSA (5 μ g/ml, Grade V, Sigma) in PBS and then incubated overnight at room temperature. No blocking procedure was necessary. Serum samples diluted in PBS-Tween (0.05%) in threefold steps were added for incubation overnight at room temperature. Next, the plates were incubated for 3 h with biotinylated Abs including monoclonal rat anti-mouse IgG1 (1/10 000; Zymed, San Francisco, CA, USA), polyclonal rat anti-mouse IgG2a (1/10 000; Southern Biotechnology, Birmingham, AL, USA), monoclonal rat anti-mouse IgG2b (1/10 000; Southern), monoclonal rat anti-mouse IgG3 (1/10 000; Southern), monoclonal rat anti-mouse IgM (1/10 000; Southern), or polyclonal rabbit anti-mouse IgA (1/6000; Zymed). Streptavidin-conjugated alkaline phosphatase was added and the enzyme activity was visualized with p-nitrophenyl phosphate substrate (Sigma). Absorbance was measured with a spectrophotometer at 405 nm. Hyperimmune mouse sera were used to construct standard curves to calculate arbitrary ELISA units in individual serum samples.

Total IgE levels in serum

Plates were coated with 5 μ g/ml monoclonal rat anti-mouse IgE (Clone 6HD5). Serum samples were diluted in BSA/PBS and bound IgE was detected with 2.5 μ g/ml secondary biotinylated anti-mouse IgE (Clone RIE4). Tertiary peroxidase-conjugated streptavidin (1/1000; Pharmingen, San Diego, CA, USA) was added, and the

reaction developed with ABTS. Mouse myeloma IgE (Clone TIB-141) was used to construct a standard curve.

Proliferation assay

Splenocytes were dispersed into single-cell suspensions and cultured in 96-well round-bottom tissue culture plates (2×10^5 in 200 μ l/well). They were stimulated with 10 μ l of either OVA (20 mg/ml), HSA (20 mg/ml), or Con A (100 μ g/ml). Tritiated thymidine (1 μ Ci/well; Amersham, Uppsala, Sweden) was added after 6 days, and the cells were harvested 12–16 h later.

Depletion of CD25⁺ cells

Splenocyte suspensions were incubated with PE rat IgM mAb anti-mouse CD25 (Clone 7D4, Pharmingen) for 20 min on ice, followed by two washes. Anti-PE microbeads (Miltenyi Biotec, GmbH, Glabach, Germany) were then added at optimal dilution and incubated for 20 min on ice, followed by two washes. Magnetic columns were prepared as recommended by the manufacturer and used for depletion. The effluent cells contained <1% CD25⁺ cells as determined by flow cytometry.

Purification of CD4⁺ CD25⁺ and CD4⁺ CD25⁻ cells

T lymphocytes were enriched by incubation of splenocytes in wool columns for 30–40 min at 37°C. The columns were washed repeatedly with warm medium and the effluent cells were next incubated with rat anti-mouse CD8 (Clone YTS105.18; Serotec, Oxford, UK), followed by two washes. The cells were further incubated with sheep anti-rat IgG beads (DynaL Biotech, Oslo, Norway) and added to a magnetic column. The effluents were incubated with FITC-conjugated rat anti-mouse CD4 (Clone YTS 177.9; Serotec). Finally, the cells were incubated with biotinylated rat anti-mouse CD25 (Clone 7D4; Pharmingen) followed by PE-conjugated streptavidin. All incubations were performed on ice for 20 min and followed by washes. CD4⁺ CD25⁺ putative T_{regs} and CD4⁺ CD25⁻ helper/effector cells were finally sorted with FACS Vantage SE (Becton Dickinson, Erembodegem, Belgium). The purity of the cell populations was at least 98%.

Adoptive transfer of CD4⁺ CD25⁺ and CD4⁺ CD25⁻ cells

Tolerance against OVA was achieved by feeding donor pIgR KO mice with OVA-containing diet for 1 week. Control pIgR KO mice received ordinary pellets throughout the experiment. One day after discontinuing the OVA diet, all mice were immunized in the hind leg with 100 μ g OVA (Sigma) and 100 μ g HSA (Sigma) mixed in 100 μ l CFA. Two weeks later, the mice were killed and the spleens removed. Some OVA- or control-fed pIgR KO mice were tested for DTH reaction against OVA (see above) to verify that tolerance had been induced, while the remaining mice were used as donors for adoptive transfer. CD4⁺ CD25⁺ and CD4⁺ CD25⁻ splenic T cells were purified (see above) and separately injected intraperitoneally (3×10^5 cells/mouse) into naïve WT mice. The recipients were immunized 1 day after cell transfer (see above) and their DTH was tested 3 weeks later.

Statistical analysis

The nonparametric two-tailed Mann–Whitney *U*-test was used for statistical comparisons between groups of mice. Death rates were compared with logrank (Mantel–Cox) tests.

Results

Striking liability to systemic anaphylaxis in sensitized pIgR KO mice

We examined how immunological sensitization and oral tolerance induction are concurrently influenced by absence of secretory antibodies. As a model we used pIgR KO mice that neither export SIgA nor SIgM (5). Because priming for allergy via the gastrointestinal tract requires cholera toxin as a mucosal adjuvant, which would break oral tolerance (15–17), sensitization was performed by parenteral immunization.

Tolerization by the mucosal route was critical to avoid anaphylactic death in pIgR-deficient mice, which occurred as an unexpected event. When this strain after control feeding was systemically sensitized with a mix of OVA and HSA and 3 weeks later challenged intradermally in separate ears with the two antigens, half the animals died within 30 min after a brief period of shivering (Table 1). The rest of the control-fed pIgR KO mice showed no clinical signs of anaphylaxis, but we were not prepared to measure rectal temperature or other parameters. Notably, anaphylactic death did not occur at all in OVA-fed pIgR KO mice ($P = 0.01$) or WT control (C57BL/6) and DO11.10 mice, whether the latter were tolerized or not. The DO11.10 strain acted as a high-responder control because transgenic OVA-specific T cells proliferate excessively in mesenteric lymph nodes (MLNs) after adoptive transfer to pIgR KO mice fed relatively low doses of OVA (6).

Together, these findings demonstrated that anaphylaxis in pIgR KO mice was antigen-driven, and that antigen-specific oral tolerization protected them against hypersensitivity. Suggestive evidence of bystander suppression against HSA achieved by OVA feeding (with both antigens present in lymph nodes draining the same s.c. antigen depot, OVA + HSA) was obtained as two of five challenged pIgR KO mice died of anaphylaxis despite OVA feeding when separate hind legs had been used for sensitization (OVA/HSA, $P < 0.04$) against the two challenging antigens (Table 1). Although the three remaining mice of this small group exhibited no clinical signs of anaphylaxis, a tolerizing effect against HSA was not revealed in two other read-out systems (see later).

Suppression of humoral immunity in pIgR KO mice fed cognate antigen

It is difficult to elicit a substantial IgE response in C57BL/6J mice (16). Also, it has recently been documented that when parenteral sensitization with subsequent challenge is performed as in our study, anaphylaxis is IgG rather than IgE-dependent, although the symptoms and time course are similar (15). This is because the quantity of IgG antibody is large while the quantity of antigen used for challenge is small (in our case 20 μ g). The serum level of IgG in pIgR KO mice is around 10 mg/ml (5), whereas

IgE levels after sensitization and boosting often only showed an increased trend ($n = 11$; median 0.2 $\mu\text{g/ml}$, range 0.1–0.6 $\mu\text{g/ml}$) compared with sensitized WT mice ($n = 8$; median 0.09 $\mu\text{g/ml}$, range undetectable to 0.3 $\mu\text{g/ml}$). A substantial but still relatively low-IgE elevation was seen only in four of the sensitized pIgR KO mice (1.1, 1.1, 4.8, and 8.5 $\mu\text{g/ml}$). Similar IgE levels, increasing slightly with age, have been reported for another pIgR KO strain without sensitization (18).

In OVA-tolerized and jointly sensitized (OVA + HSA) pIgR KO mice there was significantly decreased OVA-specific antibody levels for IgG1, IgG2b, and IgA – most consistently for IgG1 (Fig. 1A). Conversely, when we analyzed antibody to OVA in WT mice, no consistent reduction of the anti-OVA titer was observed, in accordance with the fact that humoral responses are not particularly sensitive to suppression by antigen feeding in ordinary mice (19); thus, both OVA-fed and control-fed WT animals responded in the range of the control-fed pIgR KO mice, even for IgG1 (Fig. 1A). Tolerization was, therefore, exceptionally pronounced for this antibody isotype in pIgR KO mice ($P < 0.002$). The response to HSA in the OVA-fed and jointly sensitized

pIgR KO mice only tended to be reduced for some isotypes, especially IgG1, IgG2a, and IgA (Fig. 1B). This was not the case in the three surviving tolerized mice given separate sensitization (Table 1); while their anti-OVA titers were similar to the jointly sensitized and tolerized pIgR KO mice (ELISA units: 13 000, 15 000, and 19 000), their anti-HSA titers were in the upper range of control-fed pIgR mice (ELISA units: 190 000, 210 000, and 230 000).

Enhanced downregulation of cell-mediated immunity in orally tolerized pIgR KO mice

To determine whether mucosally induced tolerance was mediated by active suppression (i.e., T_{regs}) in pIgR KO mice, we measured the cellular response to OVA and HSA. We also performed T-cell depletion *in vitro* and assessed tolerance in naïve WT mice after adoptive cell transfer from OVA- or control-fed pIgR KO mice as a functional read-out for T_{regs} (see later).

Groups of pIgR KO and WT mice fed OVA-containing diet or ordinary control pellets for 1 week, were s.c. sensitized by mixed OVA and HSA and then intrader-

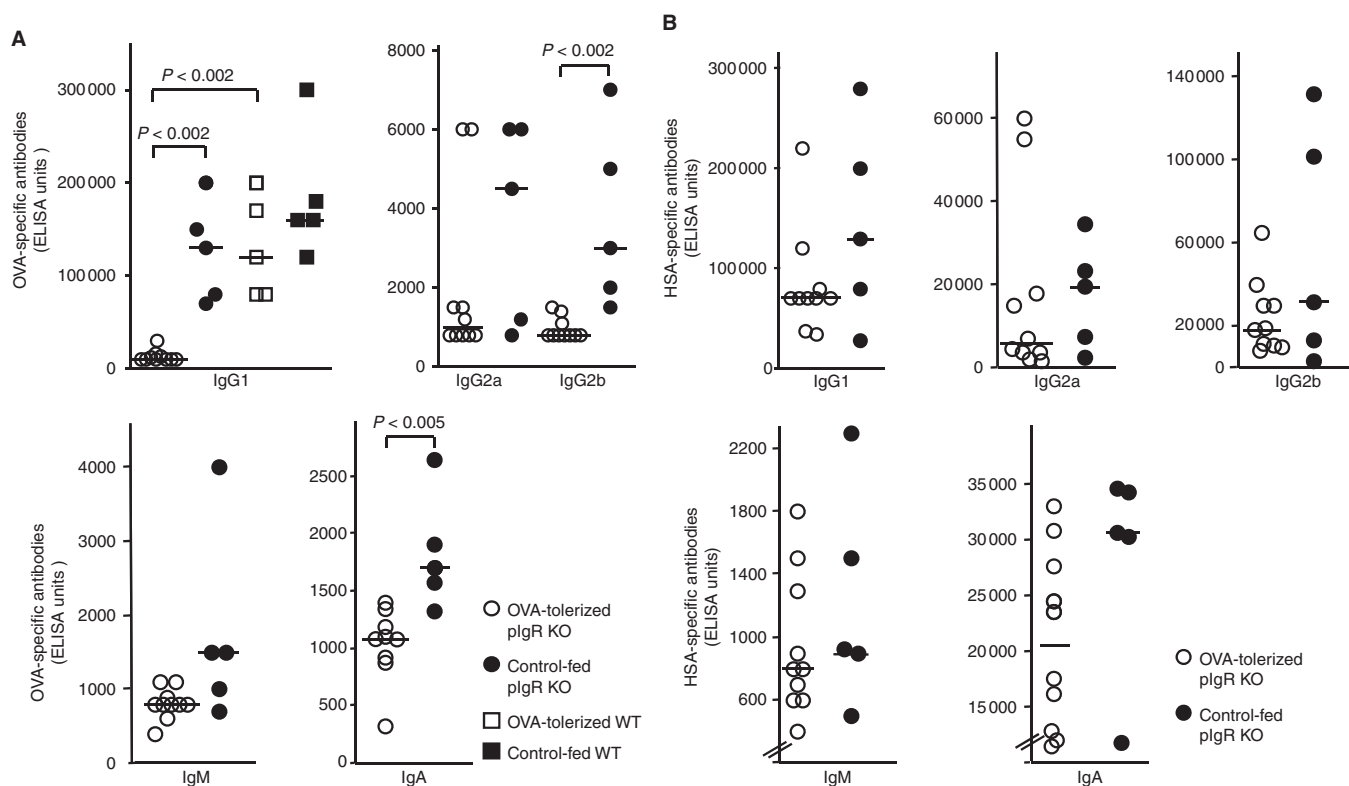


Figure 1. Difference in antigen-specific and bystander suppression of serum antibody levels after oral tolerization with OVA compared with control feeding, followed by parenteral sensitization with a mix of OVA and HSA in pIgR KO mice. The experiment was repeated at three separate time points. Note reduced number of control-fed mice due to lethal anaphylaxis (see Table 1). Two-tailed Mann–Whitney's *U*-test was used for statistical analysis. Horizontal bars indicate medians. (A) Antibody levels to OVA generally tended to be reduced in OVA-tolerized pIgR KO mice, most consistently for IgG1 which was strikingly more suppressed in this strain than in the wild-type (WT) counterpart. (B) Antibody levels to the bystander antigen (HSA) used for joint sensitization (OVA + HSA) tended to be reduced but were not significantly suppressed after OVA tolerization.

mally challenged with the two antigens in different ears to measure the T-cell helper/effector function by DTH reaction revealed as ear swelling (Table 1). After oral tolerization, both pIgR KO and WT mice showed significantly weaker DTH both to OVA and HSA than control-fed mice, particularly when the reaction was normalized in relation to the pre-challenge ear thickness (Fig. 2).

Importantly, the tolerized pIgR KO mice showed much more pronounced suppression of OVA hypersensitivity ($P < 0.01$) than the tolerized WT mice – with an individual difference generally more than 10% – despite a trend towards stronger DTH reaction in control-fed pIgR KO mice than in WT controls (Fig. 2). Conversely, bystander suppression of HSA hypersensitivity after joint antigen sensitization was similar in OVA-tolerized pIgR KO and the WT counterparts (Fig. 2).

However, in the three surviving pIgR KO mice sensitized separately with OVA and HSA in different hind legs (Table 1), the DTH was strikingly different after OVA feeding; while suppression to this antigen was the same as in jointly sensitized (OVA + HSA) animals, the DTH to HSA remained exactly at the level seen in control-fed pIgR KO mice – consistent with the high levels of IgG1 antibodies against HSA and susceptibility to anaphylaxis after separate sensitization with the two antigens (Table 1). Thus, bystander suppression in all

three read-outs appeared to depend on concurrent T-cell encounter with both antigens in lymph nodes draining the sensitizing injection site, making these results of interest despite the small test group.

Oral tolerance in pIgR KO mice involves $CD25^+$ T_{reg} s

To address the mechanism for downregulated T-cell helper/effector activity revealed by reduced DTH (Fig. 2), we performed proliferation assays with splenocytes from OVA-fed pIgR KO mice sensitized to OVA and HSA. When the splenocytes were derived from tolerized mice, the proliferative response to OVA was significantly suppressed, and that to HSA almost reached the level of significance (Fig. 3).

To examine if T_{reg} s had been induced by OVA feeding, we removed $CD25^+$ cells from the splenocytes; this resulted in significantly enhanced proliferation to OVA (Fig. 3, top panel). The conclusion that $CD25^+$ T_{reg} s were involved in the observed tolerization was supported by complete lack of effect on proliferation when splenocytes were obtained from the control-fed pIgR KO mice (Fig. 3, top panel).

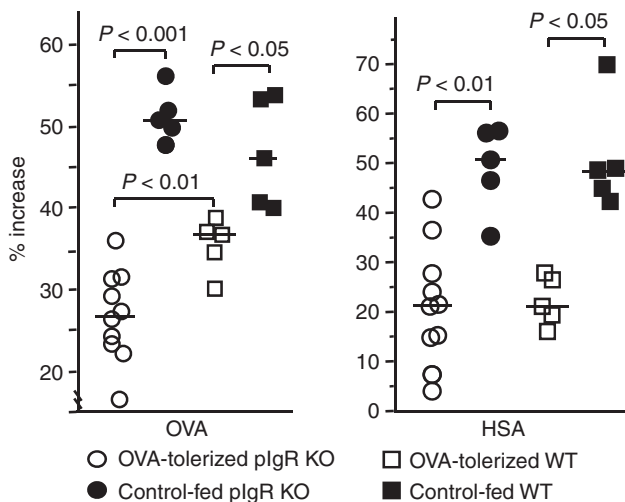


Figure 2. Suppressed *in vivo* tissue immunoreactivity in orally tolerized and systemically sensitized pIgR KO compared with WT mice as determined by DTH reaction (see Table 1). The mice were systemically sensitized by subcutaneous immunization with a mix of OVA and HSA 3 weeks before ear skin challenge. The increase in ear thickness 24 h after separate intradermal injection of OVA and HSA was normalized in relation to the pre-challenge thickness. Note reduced number of control-fed and sensitized pIgR KO mice due to lethal anaphylaxis (see Table 1). The experiment was repeated at three separate time points. Two-tailed Mann–Whitney *U*-test was used for statistical analysis. Vertical bars indicate medians.

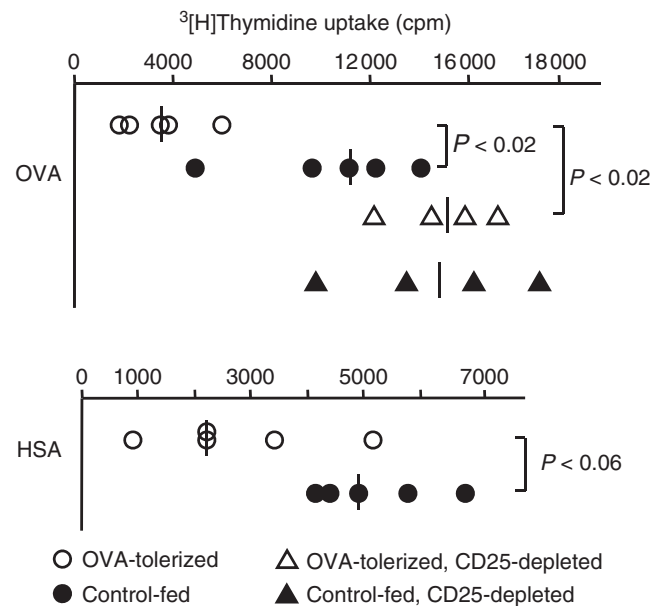


Figure 3. Reduced *in vitro* lymphocyte proliferation mediated by regulatory $CD25^+$ T cells in OVA-tolerized pIgR KO mice. Proliferation (thymidine uptake) of splenic lymphocytes against OVA or HSA was analyzed – against OVA also after depletion of $CD25^+$ cells. All mice were systemically sensitized with a mix of OVA and HSA (see Table 1). The splenocytes were harvested 1 week after the boost and then stimulated in culture with OVA or HSA for 6 days as indicated on the left. Radioactive thymidine (^3H -Tdr) was added for the last 12–16 h, and the cells were analyzed for incorporation of ^3H -Tdr (cpm). The experiment was repeated at three separate time points. Two-tailed Mann–Whitney *U*-test was used for statistical analysis. Vertical bars indicate medians.

Oral tolerance is transferable with CD4⁺ CD25⁺ T cells

We next examined whether CD4⁺ CD25⁺ T cells from OVA-fed pIgR KO mice could transfer tolerance to naïve WT mice. Tolerance in donor mice through the feeding protocol was confirmed by reduced DTH (data not shown). FACS-sorted CD4⁺ CD25⁺ and CD4⁺ CD25⁻ splenic T cells from either OVA- or control-fed pIgR KO mice were transferred to naïve WT mice (Fig. 4A). Delayed-type hypersensitivity to OVA and HSA was then measured in the recipients by ear skin challenge 3 weeks after s.c. sensitization with a mix of the two antigens.

Wild-type mice that had received CD4⁺ CD25⁺ T cells from OVA-tolerized pIgR KO mice showed significant reduction of DTH to OVA ($P < 0.05$) and a strong trend ($P < 0.06$) to reduced DTH to HSA (Fig. 4B). Conversely, CD4⁺ CD25⁻ T cells from the tolerized mice transferred tolerance neither to OVA nor to HSA (Fig. 4B). No difference between the recipient groups was revealed when we determined the levels of specific

serum antibody or tested the *in vitro* proliferation against OVA and HSA (data not shown).

Discussion

The first study that examined a possible involvement of intestinal IgA (coproantibodies) in mucosally induced systemic hyporesponsiveness was published in 1975 by the group of Heremans (20). Since then the involvement of IgA in allergy has been controversial although there is currently a focus on leaky epithelia in several immune-mediated diseases (21); a role of microbial stimulation and early antigen exposure for adequate tolerance induction is under intense debate (22). Here, we examined concurrently in pIgR KO mice how the absence of secretory antibodies influences immunological effector functions and oral tolerance. This mouse strain lacks both SIgA and SIgM and was shown to be predisposed to systemic anaphylaxis after parenteral sensitization, although suppressive mechanisms induced via the gut

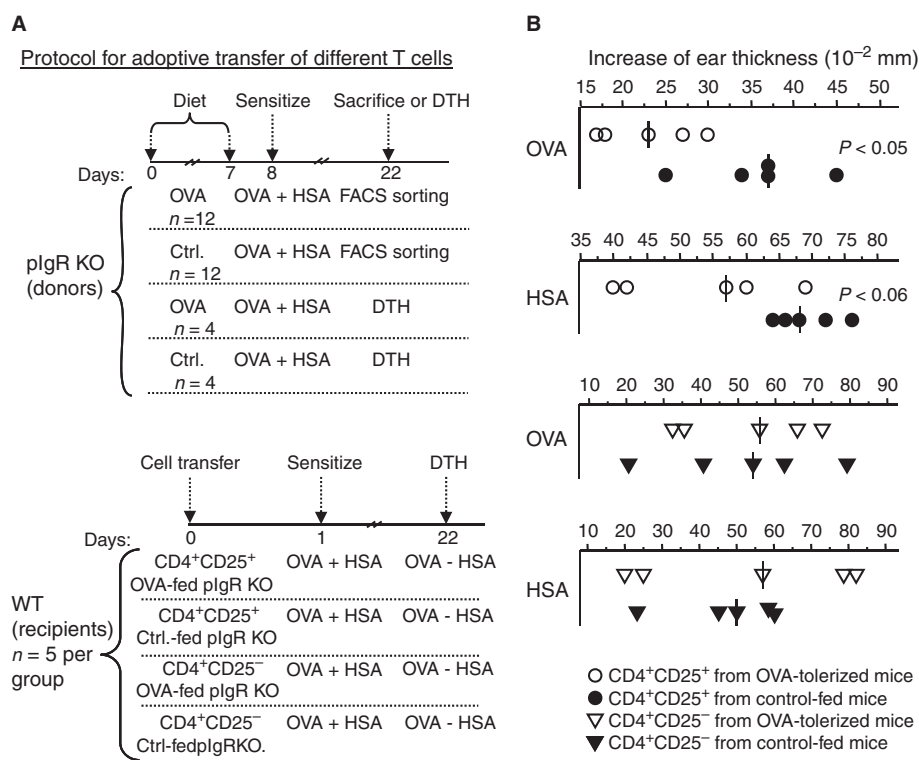


Figure 4. Transfer of tolerance by CD4⁺ CD25⁺ T cells obtained from orally tolerized pIgR KO mice. (A) Schematic overview of experimental protocol for adoptive transfer: induction of oral tolerance by antigen feeding; systemic sensitization by s.c. immunization with a mix of OVA and HSA; and intradermal challenge for DTH testing (see Table 1), T-cell purification by FACS sorting and adoptive transfer of different subsets were performed according to the displayed time lines for donor pIgR KO mice (top) and WT recipients (bottom). Feeding was performed with OVA or ordinary pellets (Ctrl. feed). (B) DTH (ear swelling) observed after intradermal challenge with OVA or HSA as indicated on the left in WT mice following adoptive transfer of splenic CD4⁺ CD25⁺ T cells or CD4⁺ CD25⁻ T cells obtained from OVA-tolerized or control-fed pIgR KO mice. The experiment was performed at two separate time points, and the results were the same after normalization of data in relation to the pre-challenge ear thickness. Two-tailed Mann–Whitney *U*-test was used for statistical analysis. Vertical bars represent medians.

by first feeding the antigen (OVA) strikingly protected against this overreaction.

In our model, tolerance was presumably induced in MLNs where tolerogenic conditioning of antigen-carrying dendritic cells (DCs) takes place (7). But T_{regs} become rapidly disseminated to peripheral lymph nodes by only partially understood homing mechanisms (23). There they can expand in parallel with helper/effector T cells when a continuous supply of cognate antigen is present (24) – which was the case in our model with regard to OVA because of the s.c. FCA depots.

Thus, jointly OVA- and HSA-sensitized – but mucosally OVA-tolerized – pIgR KO mice were completely protected against anaphylaxis caused by intradermal ear challenge with the two antigens. Such challenge with a minute antigen dose (20 µg) caused death in 50% of the sensitized control-fed pIgR KO mice but in none of the sensitized WT mice. Orally tolerized and systemically sensitized pIgR KO mice also showed strong suppression of helper/effector T-cell function as revealed by reduced DTH and downregulated T-cell proliferation against OVA. However, bystander suppression of anaphylaxis, DTH and IgG1 antibody response to HSA was seen only after s.c. injection of both sensitizing antigens in the same site. These consistent results, although observed in a small group of animals, suggested that the action of T_{regs} on helper/effector T cells depended on the concurrent presence of both antigens in skin-draining lymph nodes.

The total IgE level was substantially increased in only four of fifteen of the sensitized pIgR KO mice, as could be expected in the C57BL/6J strain after immunization with CFA. Thus, there was no reason to believe that the observed anaphylaxis could be ascribed to IgE-mediated hypersensitivity, particularly as this would be blocked by IgG antibodies (15, 16). As clearly documented by others, IgG-mediated release of platelet-activating factor from macrophages (Mφs) through activation via their transmembrane $\text{Fc}\gamma\text{RIII}$ (CD16) is the most important reaction pathway for antigen-induced systemic anaphylaxis in mice (15). Notably, we observed that suppression of OVA-specific IgG1 was strong and uniform in the OVA-tolerized pIgR KO mice, in contrast to WT controls. Importantly, the activation of Mφs via CD16 is almost exclusively accomplished by the IgG1 subclass (25).

Antibodies to HSA only tended to be suppressed after OVA feeding and joint sensitization with both antigens (OVA + HSA). This accords with the opinion that oral tolerance controls better cellular than humoral responses (19). It was therefore remarkable that consistent and strong suppression of IgG1 to OVA occurred after OVA feeding in the pIgR KO mice. This probably reflected the strong tolerogenic activity induced against T helper/effector cells in general as suggested by the finding that their DTH to OVA was significantly more reduced than in tolerized WT mice. Increased OVA uptake when secretory antibodies are lacking is a likely explanation (6), as it was recently reported that a mild or transient breach

of the intestinal barrier leads to a dominant T_{reg} response (26).

As a functional readout for T_{regs} we demonstrated that adoptive transfer of the $\text{CD4}^{+}\text{CD25}^{+}$ subset from OVA-fed pIgR KO mice-mediated suppression of DTH to OVA and HSA in WT mice which were naïve before systemic sensitization and intradermal ear challenge with these antigens. This accorded with previous oral tolerance studies reporting induction of $\text{CD4}^{+}\text{CD25}^{+}\text{T}_{\text{regs}}$ both in mice and humans (8, 13). Nevertheless, other suppressive mechanisms were probably also induced as suggested by the fact that adoptive transfer of $\text{CD4}^{+}\text{CD25}^{+}$ T cells mediated a relatively short-lived T-cell suppression which did not affect antibody production. Thus, intestinal epithelial cells may produce tolerogenic elements (tolerosomes) that appear in serum and can transfer tolerance with activation of $\text{CD25}^{+}\text{T}_{\text{regs}}$ in the recipients (27).

A central role of gut microbiota in oral tolerance has been demonstrated both in germ-free mice and after treatment with antibiotics (26, 28). We do not have sufficiently detailed information on the composition of the microbiota in pIgR KO mice (6). It has recently been shown that SIgA directed against a specific intestinal microbial epitope can alter the bacterial characteristics (29). Moreover, a positive effect on tolerance induction has been obtained by mixing fed antigen with lipopolysaccharide (LPS) from gram-negative bacteria (30). Additional factors that might influence T_{reg} induction are production of prostaglandin E2 and indoleamine 2,3-dioxygenase, which contribute to the development of DCs with the ability to induce T_{regs} (31). Finally, the vitamin A derivative retinoic acid released by DCs in the gut and MLNs is essential for local T_{reg} development together with IL-2 and TGF- β (32).

The continuous lack of SIg-mediated reinforcement of the epithelial barrier allows influx of excessive amounts of bacterial products, including LPS from *Escherichia coli* (5, 6). By interacting with pattern recognition receptors on innate immune cells such as Mφs, this probably promotes the immunological hyperreactivity revealed in the systemically OVA-sensitized pIgR KO mice. Experiments with germfree mice monocontaminated with *Bacteriodes thetaomicon* has demonstrated that specific SIgA antibodies directed against a capsular polysaccharide can inhibit activation of innate response markers such as oxidative burst and NF κ B (29). The net effect of mild epithelial leakiness in our pIgR KO strain was nevertheless intensified induction of tolerance when the mice were fed in advance with the sensitizing antigen (Fig. 5). We have obtained further evidence of hyperreactivity in the pIgR KO strain by crossing it with NF- κ B reporter mice (33); substantially increased systemic and intestinal NF- κ B activation as well as increased spleen weight was observed in our pIgR KO mice under specific pathogen-free conditions (Reikvam DH, Carlsen H, Blomhoff R, Brandtzaeg P, Jahnsen FL, Johansen FE, unpublished data). Also, in a recent report we showed how hyperactivation of systemic

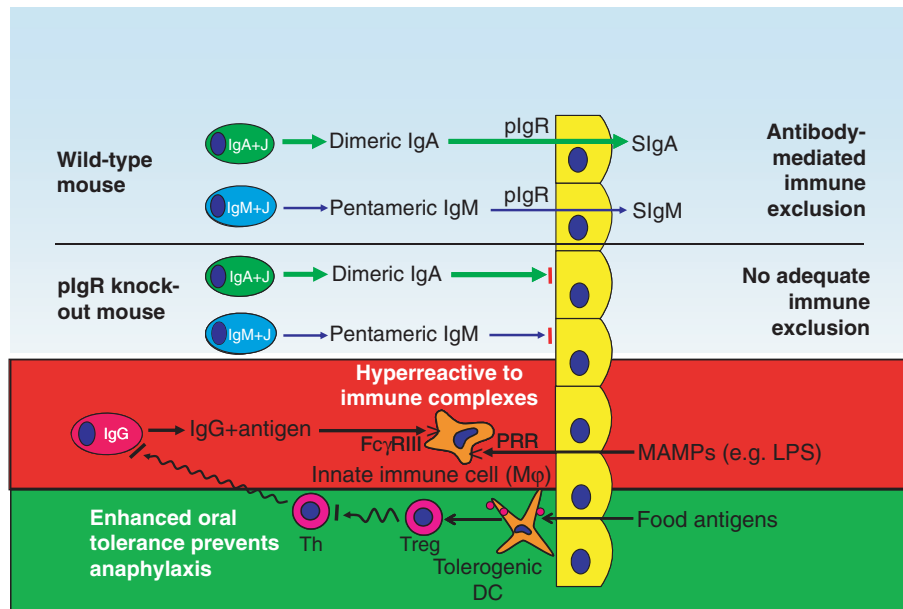


Figure 5. Depiction of experimental model. Lack of secretory antibodies (SIgA and SIgM) in pIgR knockout mice leads to inadequate immune exclusion of microbial products from the gut lumen. Such microbe-associated molecular patterns (MAMPs), e.g. lipopolysaccharide (LPS), will interact with pattern recognition receptors (PRRs) on innate immune cells including macrophages (Mφs), which become hyperreactive. These cells are therefore sensitive to IgG-containing immune complexes interacting with FcγRIII, which renders the mice predisposed to anaphylaxis. However, the deficient epithelial barrier also allows increased uptake of food antigens from the gut lumen (e.g. fed ovalbumin) which enhances oral tolerance induction, providing a net anti-inflammatory effect. Production of IgG antibodies against the same sensitizing antigen will thus be downregulated and the animal is protected against systemic anaphylaxis after parenteral antigen challenge.

IgG immunity reflects dissemination of commensal bacteria when the intestinal barrier function is defect (34).

A contributor to the prominent tolerance induction in pIgR KO mice could be intestinal intraepithelial lymphocytes (IELs), which show numerical increase and cytotoxic activity in this strain (35). In addition to being involved in microbial defense, IELs may possess regulatory properties (36). However, when exposed in the gut to dextran sulfate sodium, a striking difference in the severity of mucosal inflammation was revealed between this strain and IgA KO mice (37). In the latter, the reaction was no more severe than in WT mice, probably because IgA KO mice export compensatory SIgM and also free SC, which both might contribute to the defense of the mucosal barrier (38).

In conclusion, our study demonstrated that mice lacking secretory antibodies are highly susceptible to immunological sensitization but show compensatory tolerance after feeding with the relevant antigen. This homeostatic mechanism is at least in part mediated by

CD4⁺ CD25⁺ T_{regs}. Despite their defective mucosal barrier and hyperreactivity, the pIgR KO mice exhibit no overt pathology in a specific pathogen-free environment, except for a mild protein-losing enteropathy (5). Their capacity for robust oral tolerance apparently controls both systemic DTH and proinflammatory IgG responses, and is critical to prevent lethal anaphylaxis.

Acknowledgments

This work was supported by grants from the University of Oslo, the Norwegian Asthma and Allergy Association, and the Research Council of Norway. Dr. Malin Karlsson was a postdoctoral fellow of the Norwegian Cancer Society while performing this work. We thank Dr. Kathy McCoy for performing the IgE measurements, and the staff at the Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway for excellent help with the experimental animals. Hege Eliassen and Erik Kulø Hagen are acknowledged for their assistance in preparing the manuscript.

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