

Research paper

Immune response phenotype of allergic *versus* clinically tolerant pigs in a neonatal swine model of allergy

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ABSTRACT

The prevalence of childhood food allergy and the duration of these allergies, particularly those considered to be transient, like egg and milk allergy, are increasing. The identification of allergic individuals using minimally invasive, non-anaphylaxis-threatening methods is therefore of increasing importance. In this experiment, correlates were sought of an allergic immune response (IR) phenotype in pigs. Using pigs pre-treated with heat-killed bacteria or bacterial components before allergic sensitization with the egg white protein ovomucoid (Ovm), differences were determined in IR phenotype of pigs in the categories treated-allergic, treated-tolerant, control-allergic (CA) and control-tolerant. Phenotype was established by measuring immunoglobulin (Ig)-associated antibody activity (AbA), cytokine profiles and the proportion of blood T-regulatory cells (T-regs) and observing late-phase allergen-specific skin tests (ST). Although 100% of pigs became sensitized to Ovm, only 33% of pigs had clinical signs of allergy after oral challenge with egg white. Pigs without clinical signs were classified as clinically tolerant. Sixty-seven percent of allergic pigs had a positive, late-phase ST classified as very strong or strong, while 84% of clinically tolerant pigs did not have late-phase ST. Treated-allergic pigs and CA pigs had greater total antibody IgG (H+L), IgE and IgG₁ AbA than clinically tolerant pigs. Cytokine profiles of allergic pigs and the proportion of circulating T-regs, did not differ significantly between allergic and clinically tolerant pigs. Therefore, measurement of allergen-specific IgG, IgG₁ and/or IgE activity and evaluation of late-phase ID ST may be useful in identifying allergic IR phenotypes in swine models of food allergy, which may be extended toward human use.

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1. Introduction

The prevalence of children with food allergy in the United States is estimated to have increased from 4–6% to 8%. Peanuts are the most common food allergen, followed by milk, shellfish, tree nuts and eggs (Gupta et al., 2011). Children are now less likely to outgrow allergies once considered to be universally transient, such as milk and eggs, with these allergies persisting into adolescence and sometimes adulthood (Prescott and Allen, 2011).

In an allergic reaction, type-2 cells produce cytokines (interleukin [IL]-4, IL-5, IL-13) that promote B lymphocyte immunoglobulin (Ig) isotype-switching from IgG to IgE, thus increasing IgE-related allergen-specific antibody (Ab) (Sashihara et al., 2006). Antibody associated with IgE mediates type-1 hypersensitivity reactions by sensitizing mast cells, platelets and basophils for allergen-induced release of vasoconstrictive and smooth muscle-activating mediators such as histamine and others. Although there are allergen-specific treatments available for food allergy, including specific immunotherapy (Vickery et al., 2010; Burks et al., 2008), allergen avoidance and access to emergency medication is still the most effective way to manage food allergy (Scurlock et al., 2010). In the absence of suitable correlates, the gold standard test to confirm

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successful treatment of food allergy is the double-blind placebo-controlled food challenge (Diéguez and Cerecedo, 2009) which involves ingestion of the inciting allergen and may induce anaphylaxis. Therefore alternative *in vivo* or *in vitro* tests to diagnose, indicate risk of allergic response and test treatment effects for food allergy are desirable.

Immediate wheal and flare reactions to skin prick tests and detection of allergen-specific serum IgE have been routinely used to identify food allergic children with varying success, usually dependent upon the allergen. Of children identified as sensitive to peanuts based on skin prick tests and serum IgE-related Ab activity, only 22% were clinically allergic (Nicolaou et al., 2010). Using multi-spot microplex microarray to determine IgE binding to specific allergens to compare sera of peanut allergic and tolerant subjects revealed that response to the allergen Ara h 2 was the most important predictor of peanut allergy (Nicolaou et al., 2010). Skin prick test and serum IgE Ab to other peanut allergens were not predictive. However a study of the predictive value of skin prick tests in diagnosing egg allergy determined that 95% of children with positive skin prick tests to egg, measuring more than 5 mm in diameter, were reactive to oral challenge with egg (Dieguez et al., 2008).

Intradermal (ID) skin testing (ST) has been used here to monitor sensitization to the egg white allergen ovomucoid (Ovm) in a neonatal pig model of food allergy (Rupa et al., 2010, 2009, 2008a). In approximately 100% of all pigs treated with Ovm and the adjuvant cholera toxin given intraperitoneally, ID injection of Ovm induced wheal and flare reaction, confirming sensitization. Nearly all sensitized pigs also have serum Ovm-specific IgG and IgE Ab, but positive immediate ST and serum Ab are not sufficient to predict allergic status (Wilkie et al., 2012). Pigs which do not develop wheal and flare, and are therefore not sensitized, never display clinical signs of allergy. During the sensitization process, which occurs over a period of 45 days, ID STs are conducted to monitor sensitization status and have induced vomiting and cutaneous erythema when tested on both the day of the last sensitizing IP injection with Ovm and 10 days later, 24 h before oral challenge with egg white (Rupa et al., 2010). Therefore, in this model, ID ST is not without risk.

Currently, allergy, defined as immediate type hypersensitivity, is thought to be an IgE-mediated disease, however, isotypes of IgG are also capable of eliciting a similar anaphylactic response, although the mechanisms, effector cells and chemical mediators involved are distinct (Ishikawa et al., 2010). In a mouse model of active systemic anaphylaxis using bovine serum albumin (BSA), anaphylaxis occurred in wild-type (WT) mice, mediated by neutrophils and basophils, but not in Fc receptor (FcR)- γ knock-out mice (Bruhns, 2012). Furthermore, anaphylaxis was abolished in WT mice which received injections of blocking antibody specific for activating FcR- γ prior to challenge with BSA, indicating these receptors are responsible for anaphylaxis, even in the presence of antigen (Ag)-specific IgE. The specific isotypes of IgG responsible were not identified, although IgG₁ has been shown to induce anaphylaxis

in mice (Miyajima et al., 1997). Conversely, serum IgG₄ Ab activity is inversely correlated with persistence of cow's milk allergy in children. Children with low serum IgG₄ are less likely to become tolerant to milk than children with high IgG₄, which is considered to be a marker of tolerance, together with an increase in T-regulatory (T-reg) cells and IL-10 production (Savilahti and Savilahti, 2012). Furthermore, IgG₄ may bind to more epitopes of cow's milk in tolerized than in allergic children, consistent with its function as a blocking antibody that reduces opportunity for allergen to bind to cell-associated, allergenic IgE and IgG isotypes (Wang et al., 2010).

In pigs there are putatively six to eight isotypes of IgG (Butler and Wertz, 2006), only two of which, IgG₁ and IgG₂, have been functionally characterized (Crawley and Wilkie, 2003). Pig IgG₂ is type-1 and IgG₁ is type-2 associated. Although the possible function of IgG-isotypes in allergy of pigs is unknown, it was previously found here that pigs pre-treated orally with live *Lactococcus lactis* in a neonatal swine model of food allergy had increased allergen-specific IgG₂ Ab activity (AbA) compared with PBS controls and was associated with decreased sensitization to and clinical signs of, Ovm allergy (Rupa et al., 2010). Due to their similarity in size, organ development, physiology, disease progression and outbred genetic background, pigs are useful animals in which to model human health (Lunney, 2007). Therefore, it may be more advantageous to model human food allergy in pigs rather than in mice (Hein and Griebel, 2003). Indeed, the clinical signs of allergy in pigs are similar to those in humans and several studies have been reported (Thomas et al., 2011; Rupa et al., 2010, 2009, 2008a; Helm et al., 2002; Fornhem et al., 1995).

Previously, we have shown the effect of pre-treatments with heat-killed bacteria and bacterial components prior to allergic sensitization of pigs with Ovm on the immune response (IR) phenotype and frequency of allergy (Schmied et al., 2012b). The present experiment utilized these animals in an extended study to evaluate possible differences in IR phenotype of allergic: treated-allergic (TA), control-allergic (CA) and Tolerant: treated-tolerant (TT), control-tolerant (CT) pigs as potential biomarkers of clinical allergy. It was hypothesized that allergic and tolerant pigs have different IR phenotypes, as reflected by changes in Ig isotypes of AbA, cytokine profiles, proportion of circulating T-regs and late-phase allergen-specific ST.

2. Materials and methods

2.1. Animals and design

Nine litters of newborn Yorkshire pigs, 12 pigs per litter, 3 litters with six pigs each per treatment group (Schmied et al., 2012b) were studied. The pigs were housed and maintained under normal husbandry conditions at the Arkell Swine Research Station (University of Guelph). During the experimental period, vaccinations and antibiotics were withheld from all pigs. Animal use was approved by the Animal Care Committee of the University of Guelph under guidelines of the Canadian Council for Animal Care.

2.2. Treatments with heat-killed *Escherichia coli*, lipopolysaccharide or muramyl dipeptide

Pre-treatment of pigs with bacteria or bacterial components were as outlined (Schmied et al., 2012b). Briefly, three of nine litters of pigs were assigned to each of three groups, six pigs in each litter were treated with either: heat-killed *E. coli* (pigs completing trial $n=9$, BL21 Star cells, 10^9 cfu/ml, Invitrogen, Burlington, ON), LPS (pigs completing trial $n=15$, from *E. coli* 055:B5, $10 \mu\text{g/ml}$, Sigma–Aldrich, Oakville, ON) or MDP (pigs completing trial $n=16$, from *Staphylococcus aureus*, $10 \mu\text{g/ml}$, Sigma–Aldrich), the other pigs were treated with phosphate buffered saline (PBS, negative control, $n=49$, pooled across all litters). Each pig received an intramuscular injection of 1 ml of the designated treatment on each of the first 7 days of life, split into two equal volumes injected into opposite lateral cervical areas. Days of age are equivalent to experimental days.

2.3. Sensitization to ovomucoid

All pigs were sensitized to the egg white allergen Ovm according to a previously established protocol (Rupa et al., 2008a). Beginning at 14 days of age and again on days 21 and 35, pigs received an IP injection of $100 \mu\text{g}$ of purified Ovm (prepared in lab, Rupa et al., 2008a) adjuvanted with $10 \mu\text{g}$ of cholera toxin (List Biologicals, Campbell, CA). On day 45, pigs were fasted for 18 h before oral challenge on day 46 with 100 ml of egg white mixed with yogurt in a 3:2 (v/v) ratio. The egg white/yogurt mixture was delivered to each pig using a Kendall Monoject 60cc Toomey tip syringe (Covidien, Mansfield, MA) with an attached polypropylene tube to simulate suckling.

2.4. Cutaneous hypersensitivity to ovomucoid

To monitor the sensitization response to Ovm, ID STs were performed prior to the primary IP sensitization on day 14, as well as on days 21, 35 and 45. Two separate injection sites were marked on the medial aspect of opposite thighs, one for the test Ag Ovm and another for the PBS control. Injections of $100 \mu\text{l}$ of Ovm ($10 \mu\text{g/ml}$) or PBS were made using a tuberculin syringe with a 25-gauge needle (Covidien) and monitored for the appearance of wheal and flare, approximately 10–15 min after injection. Wheal and flare reactions were evaluated by three separate observers and scored using a scale to indicate severity: very strong positive (+++), strong positive (++), weak positive (+) or negative (–). Late phase ST responses, on day 46 only, were also evaluated. On day 46, 24 h after ID injection on day 45, prior to oral challenge with egg white, presence and severity of sustained erythema was recorded. Severity of erythema was scored using the same scale as indicated above for wheal and flare (Fig. 1a).

2.5. Clinical signs and classification as allergic and clinically tolerant

Following oral challenge, pigs were observed for at least 2 h by three researchers in a blinded fashion and

monitored for the occurrence of clinical signs. Each pig was assessed individually and assigned a score as outlined in Table 1. In order to be classified as allergic, pigs had to exhibit cutaneous erythema and/or gastrointestinal signs such as vomiting or diarrhea and therefore, have a minimum clinical score of 1. The clinical signs scratching, sneezing, isolation and lethargy and respiratory difficulty had to occur in combination with one of the aforementioned systemic or gastrointestinal signs to be considered signs of the allergic response and were not sufficient by themselves to identify a pig as allergic (Table 1). Thus, signs were additive and clinical score indicative of the severity of the allergic reaction. In severe responses epinephrine was administered to effect. Pigs which were sensitized, as indicated by ID ST, but did not develop clinical signs of allergy were considered to be clinically tolerant. Once pigs had been identified as allergic or clinically tolerant, they were further classified into one of four groups: treated-allergic ($n=14$), TT ($n=30$), CA ($n=12$) and CT ($n=37$). For example, all pigs treated with *E. coli*, LPS or MDP that developed clinical signs were classified as TA and grouped together.

2.6. Measurement of immunoglobulin isotype-specific antibody activity to ovomucoid by enzyme linked immunosorbent assay

Sera obtained on day 45 and stored at -20°C were used to determine the Ig isotype-specific AbA to Ovm by enzyme linked immunosorbent assay (ELISA). Anti-pig isotypes IgG heavy and light chain specific (rabbit polyclonal, H + L; Sigma–Aldrich), IgG₁, IgG₂ (mouse monoclonal, AbD Serotec, Raleigh, NC) and IgE (rabbit polyclonal, Rupa et al., 2008b) were used to quantify-related antibody activity. Optimal Ag-coating conditions were determined using polystyrene, flat-bottomed, Immulon 2HB, 96 well plates (Dynex Technologies Inc., VWR International, Mississauga, ON) and 0.05 M carbonate–bicarbonate coating buffer (pH 9.6). Plates were washed three times (ELX 405 automatic plate washer; Biotek Instruments, Winooski, VT) with $200 \mu\text{l}$ 0.05% Tween PBS (PBST 0.01 M, pH 7.4 per well). Wells were blocked with 3.0% PBST (pH 7.4), $200 \mu\text{l}$ per well, and incubated for 1 h at room temperature (RT). Washing was repeated and sera diluted in 0.05% PBST were added at $100 \mu\text{l}$ per well in triplicate. Controls included wells without sera and negative (pooled serum from pre-immunized piglets with low AbA) and

Table 1
Clinical score assessment for systemic and local signs of allergy.

Clinical signs	Score awarded
Isolation and lethargy	1
Repeated scratching	0.5
Repeated sneezing	0.5
Cutaneous erythema ^a	Isolated reddening – 1 Partly confluent – 2 Confluent – 3
Emesis ^a	One incident – 1 Repeated episodes – 2
Diarrhea ^a	One incident – 1 Repeated episodes – 2
Respiratory difficulty	2

^a Pigs must display at least one of these signs to be considered allergic.

Positive correlation between late skin test response and clinical signs

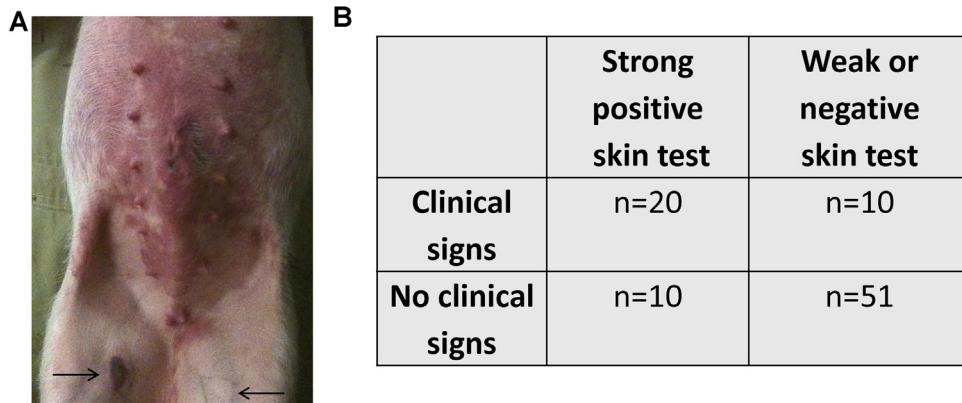


Fig. 1. (A) Representative image of a very strong late skin test response and allergic cutaneous erythema. All pigs were administered 100 µg of ovomucoid (Ovm) via intradermal injection on day 45 and the occurrence of wheal and flare was monitored. All pigs were positive for wheal and flare on day 45, indicating sensitization to Ovm. Twenty four hours later, immediately before oral challenge on day 46, the occurrence and severity of the "late skin test reaction" at the wheal and flare site was recorded and visually graded as: very strong positive (+++), strong positive (++), weak positive (+) or negative (–). Depicted here is a pig with a very strong positive late skin test (ST) reaction, as indicated by the left arrow, cutaneous erythema on the abdomen elicited by oral challenge with egg white is also evident. The right arrow indicates a negative skin test response to PBS control. (B) Positive correlation between late skin test response and clinical signs of allergy after oral challenge with egg white. Pigs with very strong or strong late responses (as described above) were more likely to develop clinical signs of allergy than those with a weak or negative late response. Sixty seven percent of allergic pigs had a very strong or strong late ST response whereas 84% of pigs with weak or negative late ST responses were not allergic ($p < 0.0001$, Fisher's exact test, significance taken at $p < 0.05$).

positive sera (pooled serum from post-immunized piglets with high AbA). Plates were incubated for 2 h at RT and washed. Alkaline phosphatase (Alk-phos)-conjugated rabbit anti-swine IgG (H+L), diluted in 0.05% Tween Tris buffered saline (TTBS, pH 7.4), for Ovm-specific IgG (H+L) activity, or unconjugated monoclonal mouse anti-swine IgG₁ or IgG₂ for the isotype-specific ELISAs, were added at 100 µl/well and incubated for 1 h at RT. Plates were washed and for IgG 100 µl p-nitrophenol phosphate substrate (Sigma–Aldrich), dissolved in 10% diethanolamine (pH 9.8) to a concentration of 1.0 mg/ml, was added to each well, and incubated at RT in the dark. For IgG₁ or IgG₂ isotype-specific ELISAs Alk-phos-conjugated goat-anti-mouse IgG (H and L specific, Sigma–Aldrich) diluted in TTBS was added at 100 µl/well after washing and incubated for 1 h. Substrate was added after washing, as for IgG ELISA, and incubated at RT in the dark. Optical density (OD) of test sample wells was measured at 405 nm (EL 808, Biotek Instruments) when the positive control OD reached 1.0. Mean values of OD were obtained from triplicates of test serum and from the controls. The protocol for Ovm-specific IgE activity was similar to that outlined by Rupa et al. (2008b). For individual sera, antibody activity of triplicate means were expressed as percent of the positive control values for each plate as: % of positive control activity = (mean test serum OD/mean positive control serum OD) × 100.

2.7. Measurement of type-1, type-2 and pro-inflammatory cytokine concentrations

Blood collected on day 45 was used to isolate BMCs for culture and stimulation with the T-cell mitogen phytohemagglutinin-P (PHA-P, Sigma–Aldrich).

Concentration of type-1, 2 and pro-inflammatory cytokines were measured in culture supernatant using capture ELISA.

Blood mononuclear cells were isolated from whole blood using density gradient centrifugation on Histopaque (Sigma–Aldrich) according to manufacturer's guidelines. To summarize, approximately 3 ml of blood was collected from each pig in an EDTA-coated 5 ml Vacutainer tube (Becton Dickinson, Mississauga, ON). Blood was diluted with 3 ml of sterile PBS and under-layered with 6 ml of Histopaque at RT. The samples were then centrifuged at 400 × g for 30 min at RT after which the layer containing BMCs was removed and transferred to a new, sterile tube and washed twice at RT in PBS. Resulting BMCs were re-suspended in complete Dulbecco's modified eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum (Invitrogen), L-glutamine (2 mM, Invitrogen) and penicillin (100 U/ml)/streptomycin (100 µg/ml). Cells were counted using an automatic cell counter (Bio-Rad T-C10 Cell Counter, Mississauga, ON) and diluted with DMEM to 2.5×10^6 cells/ml, cultured in triplicate on 24 well plates (Costar, Sigma–Aldrich) with 1 ml of medium and 10 µg of PHA-P per well. After 96 h of incubation cell culture supernatant was removed, aliquoted and stored at –80 °C.

The type-1 cytokines interferon (IFN)-γ and IL-12p40, the type-2 cytokines IL-4 and IL-10 and the pro-inflammatory cytokine IL-17A were measured in cell culture supernatant using porcine quantitative sandwich ELISA kits for each cytokine exactly as outlined by the manufacturers' instructions (Invitrogen [IFN-γ, IL-4 and IL-10], Kingfisher, St. Paul, MN, [IL-17A] R&D Systems, Minneapolis, MN [IL-12p40]). In summary, plates were coated with mouse monoclonal anti-porcine IFN-γ, IL-4 or IL-10 overnight at 4 °C, and IL-12p40 overnight

at RT. Mouse monoclonal anti-porcine IL-17A was pre-coated on the assay plate. Culture supernatant from PHA-P-stimulated and unstimulated BMCs and standards of known cytokine concentration were added in duplicate to wells of flat-bottomed, Immulon 2HB, 96 well plates (Dyrex Technologies Inc.), and were incubated as specified by manufacturer per assay. After washing, 50 μ l of biotinylated anti-cytokine antibody was added to each well and incubated for 1 h. Plates were then washed and 100 μ l of streptavidin-peroxidase was added. After a final wash, color was developed with 100 μ l substrate solution (tetramethylbenzidine; Sigma–Aldrich) for 30 min at RT in the dark. Stop solution (1.8 N sulfuric acid) was added, and OD was measured at 450 nm using a microplate reader. Optical density was directly proportional to the concentration of cytokine. Linear standard curves were plotted for concentrations using GraphPad Prism Software and used to estimate cytokine concentration in test samples. For all cytokines assayed, if a test sample OD was below the sensitivity of the test to detect positivity, the lowest concentration detected on the standard curve was assigned to that sample.

2.8. Determination of proportion of T-regulatory cells in lymphocytes of whole blood by flow cytometry

Blood collected on day 45 was also used to measure the proportion of blood T-regs from all pigs by flow cytometry, on the basis of CD25 and Forkhead Box P3 (FoxP3) double positivity (Käser et al., 2008). Using standard methods, 100 μ l of blood from each pig was used for double staining with mouse anti-porcine CD25 (VMRD, Pullman, WA) and R-phycoerythrin (PE)-conjugated anti-mouse FoxP3 (eBiosciences, San Diego, CA, 14). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG₁ (Invitrogen), was used as a secondary antibody to detect anti-CD25. Isotype and unstained controls were included to confirm specific staining of anti-CD25 and anti-Foxp3 and to adjust compensation. The antibody isotype controls used were mouse IgG₁ negative control (AbD Serotec) for CD25 and PE-conjugated rat IgG_{2a} for FoxP3. Unstained samples were treated with wash buffer, PBS with 0.1% bovine serum albumin (PBA). Permeabilization buffer (0.1% saponin in PBS) was used to enable intracellular staining.

Samples were stored at 4 °C for approximately 18 h before proportion of T-regs was measured by flow cytometry using a Becton Dickinson FacScan flow cytometer (Becton Dickinson) and FACStation software (BD Biosciences). Percentage of T-regs was determined by gating on lymphocytes using forward and side scatter and identification of double positive cells for the markers CD25 and FoxP3. Resulting data were visualized using FCS Express version 4.0 software (De Novo Software, Los Angeles, CA) and analyzed using GraphPad Prism Version 5.0.

2.9. Statistical analyses

All data were analyzed using GraphPad InStat Version 3.05 or GraphPad Prism software Version 5.0. Significance of difference in percent positive control (PPC) of AbA, ratios of PPC AbA, concentration of cytokines

and proportion of T-regs were all determined using the unpaired Student's *t*-test for normally distributed data. When necessary, Welch's correction was applied for non-normally distributed data as determined by the *F*-test. All data were pooled according to the clinical outcome within the treatment and control groups. The correlation between presence of late ST and expressed clinical signs was calculated using Fisher's exact test. Significance was taken at $p < 0.05$ and trends at $p < 0.10$.

3. Results

3.1. Severity of late-phase skin test response prior to oral challenge is indicative of allergic status

All pigs, in the treatment and control groups, were sensitized to Ovm as 100% of pigs had positive wheal and flare on day 45. Late-phase ST responses, 24 h after ID injection of Ovm, were positively associated with allergic status after oral challenge (Fig. 1a, $p < 0.0001$). Sixty seven percent of allergic pigs (20 of 30), pooled across treatment and control groups, had +++ or ++ late-phase ST. Only 33% of pigs with similar ST were clinically tolerant (10 of 30). Of the pigs with negative or weak late phase ST, 84% were clinically tolerant (51 of 61), only 16% developed clinical signs (10 of 61, Fig. 1b).

3.2. Antibody activity associated with immunoglobulin G (H + L), E and G₁ is associated with clinical signs in treated and control allergic pigs

Immunoglobulin G (H + L) AbA, or total AbA, was higher in allergic than in clinically tolerant pigs (Fig. 2a). Specifically, TA pigs had greater total AbA compared to TT ($p = 0.0424$) and CT ($p = 0.0223$) pigs. Control-allergic pigs had greater total AbA than TT ($p = 0.0004$) and CT ($p < 0.0001$) pigs. There was no difference in total AbA for TA and CA pigs. The AbA of the type-2 isotype, IgE was greater in TA pigs than in TT ($p = 0.0002$) and CT ($p < 0.0001$) pigs. There was a trend indicating higher IgE activity in CA pigs compared to CT pigs ($p = 0.0625$, Fig. 2b). Similarly, IgG₁-associated AbA was greater in TA pigs than in CT pigs ($p = 0.0493$) and TT pigs, although this was a trend ($p = 0.0629$). Control-allergic pigs had more IgG₁ AbA than both TT ($p = 0.0460$) and CT ($p = 0.0276$) pigs (Fig. 2c). Unexpectedly, type-1-associated IgG₂ AbA trended toward greater activity in CA pigs compared to TA ($p = 0.0830$), TT ($p = 0.0600$) and CT ($p = 0.0819$) pigs (Fig. 2d). Ratios of AbA were calculated, but were generally uninformative. A trend toward an increased ratio of IgG₁:IgG₂ in TA pigs over CA pigs ($p = 0.0747$) was found in addition to a similar trend in the ratio of IgE:IgG₂ for the same groups ($p = 0.0557$).

3.3. Concentration of type-1, 2 and pro-inflammatory cytokines do not correlate with allergy and clinical tolerance

Although the concentration of the type-1 cytokine IL-12p40, the type-2 cytokines IL-4 and IL-10 and the pro-inflammatory cytokine IL-17A were measured, no difference between any of the groups, TA, TT, CA or CT were

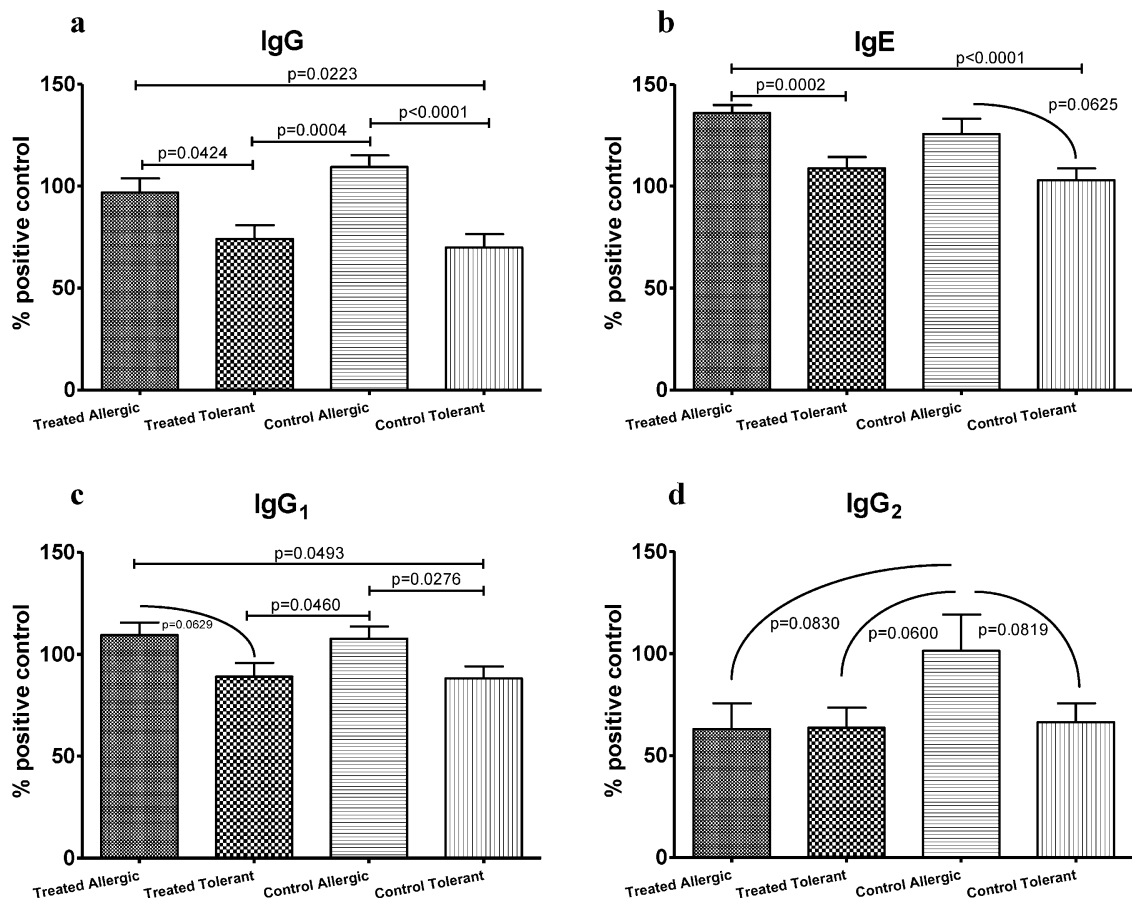


Fig. 2. Immunoglobulin isotype-related antibody activity in allergic and clinically tolerant pigs. Serum immunoglobulin isotype-specific antibody activity (AbA) to ovomucoid (Ovm), in Ovm sensitized pigs, was measured by enzyme linked immunosorbant assay 24 h before oral challenge with egg white (day 45). Immunoglobulin G (H + L) AbA to Ovm was found to be greater in control-allergic (CA, $n = 12$) compared to treated and control-tolerant (TT, $n = 30$, CT, $n = 37$) pigs. The same was true for treated-allergic (TA, $n = 14$) pigs. In TA pigs, IgE-associated AbA was greater than in TT and CT pigs. There was a trend toward greater IgE-activity in CA pigs than in CT. Similarly, TA pigs had increased IgG₁ AbA compared to CT pigs. For IgG₂, CA pigs trended toward increased AbA compared to all other groups (unpaired *t*-test, Welch's correction applied when necessary, significance taken at $p < 0.05$, trends at $p < 0.10$).

found. Interferon-gamma was undetectable in all samples and IL-4 minimally detectable when found in measurable concentrations. Measurable amounts of all cytokines were detected in pigs from each of the groups.

3.4. Proportion of T-regulatory cells does not correlate with allergy and clinical tolerance

The proportion of blood T-reg was measured for pigs in each group, however, contrary to the hypothesis, no significant difference or trend was found. The proportion of T-reg was relatively constant between the four groups: TA = 5.03% (SEM = 0.9288), TT = 3.75% (SEM = 0.5000), CA = 4.15% (SEM = 1.247), CT = 3.89% (SEM = 0.4011).

4. Discussion

Immune response phenotype of allergic and clinically tolerant animals differed such that allergic pigs had greater IgG (H + L), IgE and IgG₁ AbA than clinically tolerant pigs. Cytokine profiles of allergic pigs and the proportion of T-regs, contrary to the hypothesis, did not differ significantly

from those of clinically tolerant pigs. The severity of late-phase ST response was in high positive correlation with allergic outcome.

Skin prick testing is often used to predict allergy in humans to food allergens, with varying degrees of success (Johannsen et al., 2011; Konstantinou et al., 2010; Diegues et al., 2008), which may depend on the criteria used to determine a positive result. This is most often evaluated by measuring the diameter of wheal and flare produced and assigning appropriate cut-offs to identify positives from negatives (Ford et al., 2012). Often, when skin prick tests are negative, ID STs are conducted, the efficacy of which is also debatable (Oppenheimer and Marshall, 2009), these authors argue that ID STs do not provide additional information when testing against potent allergens, such as cat and grass, but may be helpful when testing weaker allergens. In the present and other studies conducted here, ID ST of Ovm sensitized pigs with Ovm yielded approximately 100% positive results for wheal and flare, indicating all pigs were sensitized, however, less than half of these pigs were allergic on oral challenge with egg white (Wilkie et al., 2012; Rupa et al., 2010, 2009, 2008a). In the present report,

the predictive value of late-phase ID ST was however significantly positive. Late phase type-1 hypersensitivity responses involve recruitment of T-helper 2 cells to sites of mast-cell activation where they release type-2 cytokines and induce recruitment of additional inflammatory cells, including neutrophils, basophils and eosinophils (Squaiella et al., 2008). In a study of allergen dose effects on the early-phase and late-phase cutaneous responses in cynomolgus monkeys, it was found that at optimal doses of allergen, late phase cutaneous responses characterized by the appearance of erythema and edema at the injection site, could be observed 24 h after intradermal injection of *Ascaris suum* protein extract (Tomkinson et al., 2009). The authors propose that evaluating the effect of anti-allergic treatments using this late phase response would be useful in determining efficacy of anti-allergic therapy. Here, we report the value of monitoring late-phase STs, 24 h after ID injection, to predict the outcome of exposure to allergen and expression of allergic disease. Sixty-seven percent of allergic pigs had a strong or very strong late skin test response, whereas 84% of non-allergic pigs did not have such a response. This test was better at predicting the outcome of oral allergen challenge than were immediate wheal and flare reactions, which were positive in all sensitized pigs and hence had no positive predictive value.

In this report, serum IgE AbA was higher in TA pigs than in TT and CT and there was a strong trend showing CA pigs having greater IgE AbA compared to CT pigs. There was no difference between IgE AbA in TA and CA pigs (Fig. 2b). Therefore, as hypothesized, allergic pigs have greater IgE AbA than clinically tolerant pigs and thus IgE activity may be useful as part of an IR phenotyping index to predict allergy. Differences between allergic and tolerant pigs were also noted for total (IgG [H + L]) and IgG₁ AbA. Food allergy typically occurs immediately after exposure to the inciting food allergen and is mediated by the activation of sensitized tissue mast cells and blood basophils through cross-linking of FcR-ε-bound allergen-specific IgE (Sicherer, 2002). Activation releases vasoconstrictive and smooth muscle-activating mediators such as histamine and others (Sashihara et al., 2006), resulting in signs and symptoms of allergy. Increased serum IgE has long been a hallmark of allergic disease and its elevated activity in serum, both allergen-specific and nonspecific, is often used as a criterion to diagnose allergy (Dehlink et al., 2010; Johannsen et al., 2011).

Here, serum IgG and IgG₁ AbA, which on the basis of low complement activation and opsonization is a type-2-associated Ab in pigs (Crawley and Wilkie, 2003), was elevated in CA compared to TT and CT pigs and in TA compared to CT pigs, with a trend toward greater activity in TA pigs compared to TT for IgG₁ (Fig. 2a and c). However, for the type-1 isotype IgG₂, AbA trended toward greater activity in CA pigs compared to TA, TT and CT animals (Fig. 2d). Ratios of AbA comparing IgG₁:IgG₂ and IgE:IgG₂ trended toward decreased relative expression of IgG₂ in TA animals compared to CA animals and may reflect bias in IR of treated animals (Schmied et al., 2012b), such that relatively greater type-2-associated AbA was required to elicit an allergic response. No other difference in ratio of AbA was found between the groups. These results indicate the

possible utility of measuring total antibody activity and/or IgG₁ activity with IgE AbA, as suggested above, or alone, in a phenotyping index to monitor allergic status. Non-IgE-mediated allergic response as in children with cow's milk allergy, is usually mediated by IgG isotypes (Kumar et al., 2012). Elevated serum IgG₁, type-1-associated and IgG₄, type-2-associated antibodies have been found in children with multiple food allergies but not in non-allergic children and adults. It is proposed that IgG₄, which is non-complement activating with poor ability to bind to Fc-γ receptors, in contrast to IgG₁, may block IgE sensitization of mast cells by competing for binding to epitopes on allergens (Scott-Taylor et al., 2010). Furthermore, the ratio of IgG₁:IgG₄ appears to be important in controlling tolerance to allergens, such that decreasing ratios, due to relative increase in IgG₄, correlate with induction of tolerance.

The concentration of type 1, type 2 and pro-inflammatory cytokines were compared between allergic and tolerant pigs. There were no differences in IL-17A, IL-10 or IL-12 concentrations. Therefore, cytokines do not enhance the prediction of allergic status based on late-phase ST response and allergen-specific AbA. As highlighted by HayGlass (2011), measurement of cytokines to distinguish between allergic and non-allergic humans is complicated by the redundancy of the immune system, population diversity and the difficulty to replicate results across varying environments and ages, which is also the case for pigs (Schmied et al., 2012a). Chemokines, which are present at 10–50-fold greater concentrations in serum than cytokines, are an attractive alternative in the quest to identify a useful allergic biomarker (HayGlass, 2011). Unfortunately, chemokines were not measured in the present study.

Although natural and inducible, and therefore allergen-specific, T-regs are critical to maintain a healthy immune response to allergens (Haribhai et al., 2011), in the present study data did not indicate higher proportion of blood T-regs in clinically tolerant versus allergic pigs. This was surprising as in a previous experiment T-regs in pigs receiving pre-treatment of heat-killed *E. coli*, lipopolysaccharide or muramyl dipeptide before allergic sensitization to Ovm were correlated with the allergic outcomes and IR bias associated with each treatment group (Schmied et al., 2012b).

It is reported here that allergic pigs, either pre-treated or not with heat-killed bacteria or bacterial components before allergic sensitization with Ovm, differ in IR phenotype compared to treated or control pigs that are clinically tolerant. Although cytokine concentration and proportion of circulating T-regs did not provide any differential data, total AbA (IgG [H + L]), IgE and IgG₁-associated AbA were elevated in both TA and CA pigs in comparison to TT and CT animals. Very strong or strong late-phase ST responses were correlated with clinical signs of allergy and were evident in 67% of allergic pigs. Therefore, measurement of allergen-specific IgG, IgG₁ and/or IgE activity and late-phase ID ST may be useful markers of allergic IR phenotypes in swine models of food allergy, which may indicate utility in diagnosis of allergy in humans.

Conflict of interest

The authors confirm no conflict of interest in this report.

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