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Research paper

Immune response phenotype induced by controlled immunization of neonatal pigs varies in type 1:type 2 bias

J. Schmied, K. Hamilton, P. Rupa, S.-Y. Oh, B. Wilkie*

Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON, N1G 2W1 Canada

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ABSTRACT

Immune response (IR) of pigs varies by litter and by individual such that ratios of type-1 and type-2 IR differ. Estimates of heritability for antibody and cell-mediated IR suggest that genotype and the environment contribute approximately 20% and 80% to this variation. It is hypothesized that the IR phenotype of outbred neonatal pigs is immature and variable progressing with age from type-2 bias to a more balanced phenotype. To test this, pigs were IR phenotyped by a standardized protocol using two intramuscular injections of the combined type-1 and type-2 antigens (Ag) *Candida albicans* (CA) and hen egg white lysozyme (HEWL). Immune response was measured by wheal and flare reaction to HEWL and double skin fold thickness (DSFT) response to each Ag injected intradermally at 35 days of age. Blood was collected at 14 and 35 days of age to measure immunoglobulin IgG₁, IgG₂ and IgE isotype-relatedness of antibody (Ab) to CA and HEWL. Comparison was made between two different groups of pigs (A) and (B), from the same herd tested separately at an interval of two and a half years. An unexpected group difference in IR bias was observed. Bias in IR was not consistently toward type-2. Increase in DSFT to CA, an indicator of type-1 IR, was greater in A while frequency of wheal and flare to injection of HEWL, a type-2 IR correlate, was greater in B. Frequency of individuals with positive serum Ab activity to both Ags was greater in B than A for most isotypes. Ratios of Ab activity by type-1 and 2 isotypes and DSFT to type-1 and 2 Ags indicate diminished type-1 relative to type-2 biased IR response in B. We conclude that in normal neonatal pigs under standard husbandry IR bias is not invariably toward type-2. Phenotype varied between groups in type-1:type-2 bias with implications for protective and immunopathogenic IR. While the etiology was not pursued it is possible that unidentified environmental variables may have induced this change in IR phenotype.

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1. Introduction

Immune response (IR) of pigs measured after standardized induction of antibody (Ab) and cell-mediated IR varies by litter and by individual with heritability of approximately 20% such that relative expression of type-1 and type-2 IR differ. This allows genetic selection for IR and variation may influence response to vaccination, infectious

disease and/or to allergy (Mallard et al., 1992, 1998; Wilkie and Mallard, 1999).

Acquisition of mature IR phenotype in neonates is a critical developmental event. Immune response phenotype in the transition from intra-uterine to extra-uterine environments reflects immune system ontogeny, maternal immune regulators (Nguyen et al., 2007) and early neonatal environmentally derived stimuli (Garn and Renz, 2007). The fetus is an allograft and normally allografts are rejected by a type-1 IR, therefore, to maintain pregnancy, the maternal immune system may be regulated to type-2 IR function by production of type-2 cytokines (Lin et al., 1993). Consequently, it is thought that neonates are type-2 IR biased

* Corresponding author. Tel.: +1 519 824 0632x54760; fax: +1 519 824 5930.

E-mail address: bwilkie@uoguelph.ca (B. Wilkie).

(Nguyen et al., 2007). The environment acting on pregnant dams may influence IR phenotype of the offspring. When pregnant mice are treated with the type-2 skin sensitizer toluene diisocyanate, pups were born with type-2 skewed cytokine profiles and increased susceptibility to asthma (Lim et al., 2007). Pups of mothers treated with the type-1 skin sensitizer dinitrochlorobenzene, were not susceptible to asthma. Moreover, mice immunized as neonates mount type-2 dominant memory responses when re-exposed to the antigen (Ag) as adults (Adkins et al., 2004). Pigs have an epitheliochorial placenta which prevents the transfer of maternal antibodies or lymphocytes (Butler et al., 2006). However, maternal cytokines (Nguyen et al., 2007), lymphocytes (Bandrick et al., 2008), and immunoglobulins (Crawley and Wilkie, 2003) are known to be transferred in colostrum and milk and may influence the IR bias of neonatal pigs.

Although the theory of type-2 IR bias in neonates is widely accepted, recent studies of pigs and humans examining cytokine production during pregnancy and in neonates may indicate otherwise. Immune response bias of neonatal piglets indicated by measurement of mitogen-induced, blood monocyte-derived type-1 and type-2 cytokines was found to be variable by individual with the production of both types of cytokines increasing with age (deGroot et al., 2005). Furthermore, in human maternal, paternal, neonatal triads, cytokine production has been shown to be regulated toward maintenance of a balance between type-1 and type-2 cytokines, which differs or corresponds between individuals depending upon unshared or shared environment (Halonen et al., 2009). The dominant environmental effect is consistent with the hypothesis that type-2 IR bias is attributed to lack of appropriate environmental stimuli derived from ubiquitous microbiota as proposed and confirmed epidemiologically and experimentally under the hygiene hypothesis (Strachan, 1989; Garn and Renz, 2007).

Type-2 IR bias can be detrimental to health as it may increase risk of developing certain infectious diseases, allergy, or inappropriate response to neonatal vaccination

and therefore, in livestock husbandry, significant economic loss (Morein et al., 2007). Enhanced understanding of the IR phenotype of neonatal animals could guide possible beneficial IR biasing interventions.

In this study, we set out to confirm the IR phenotype of neonatal piglets as observed previously (Hamilton, 2008), before pursuing possible IR biasing interventions. It was hypothesized that two different groups of neonatal pigs, would have similar IR bias as indicated by response to a standard IR phenotyping protocol. Comparison was made between two different groups of pigs (A) and (B), from the same herd tested separately at an interval of two and a half years. Results indicate significant individual and group difference in relative expression of type-1 and type-2 IR.

2. Materials and methods

2.1. Experimental design and animals

Two different groups of neonatal Yorkshire pigs from the Arkell Swine Research Station (Specific Pathogen Free, SPF) at the University of Guelph were used. Group A included three litters of 12 pigs/litter and Group B, six litters of 12 pigs/litter. None of the litters in Group A shared a dam or sire. In Group B none of the litters shared a dam; however two of the total four sires were used twice thus producing two half sibling litters for each of these sires. Pigs were managed, bred, housed and fed according to standard operating procedures at the Arkell Swine Research Station. Piglets were weaned at 28 days of age (experimental days = days of age) and were not vaccinated or given antibiotics. Dam or sire IR were not quantified as a breeding criterion. Both groups were IR phenotyped using a standardized protocol (Fig. 1) to quantify and compare type-1 and type-2 IR correlates within and between groups. Piglets in group A were phenotyped first, two and a half years later a new group of piglets, Group B, were similarly phenotyped. Animal use was approved by the University of Guelph Animal Care Committee under guidelines of the Canadian Council for Animal Care.

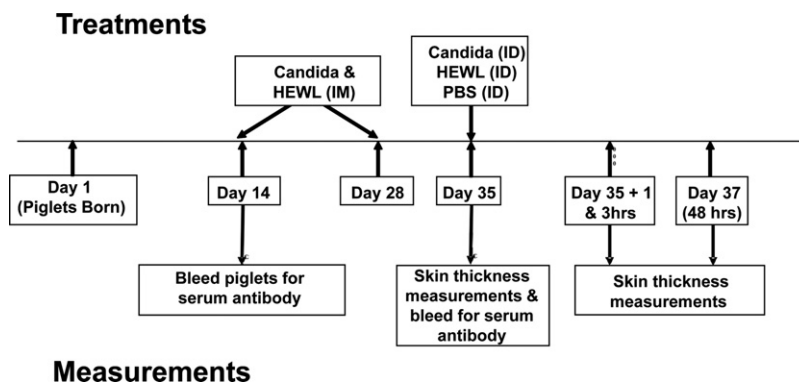


Fig. 1. Immune response phenotyping experimental protocol. Nine litters of Yorkshire pigs (12 pigs/litter) were immune response (IR) phenotyped using this protocol. Pigs were given intramuscular injections of *C. albicans* (CA) and hen egg white lysozyme (HEWL) all combined with Quil A on days 14 and 28. Blood was collected from the retro-orbital sinus on days 14 and 35 to assess serum antibody activity. Intradermal injections of CA and HEWL were made at separate sites on the medial aspect of the thigh, as well as a negative control injection of phosphate buffered saline. Skin test sites were observed for wheal and flare (15 min post injection) and change in double skin fold thickness at 1 and 3 h on day 35, and at 48 h on day 37. Days of experiment are equal to days of age.

As a primary SPF facility the herd is free of mange, lice, atrophic rhinitis, and swine dysentery. The herd is also free of *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, and *Actinobacillus pleuropneumoniae*. The herd has had porcine reproductive and respiratory syndrome (PRRS), but clinical signs have not been observed for the past decade. Vaccination, although recently stopped, was practiced at the time of the present study. Pigs used in the experiment were not vaccinated for PRRS. *Actinobacillus suis*, *Streptococcus suis* and ETEC were present, prevalence was unknown, but not noted to be a problem at the time of study. Porcine Circovirus 2 (PCV2) was first detected in the herd between experimentation with groups A and B, vaccination for PCV2 was instituted, but not practiced in the pigs on trial.

2.2. Induction of immune response

Candida albicans was selected as a type-1 Ag as resistance to infection with CA is associated with T-helper 1 immunity critical to the production of type-1 cytokines necessary to activate and maintain the phagocytic cells required to control the pathogen. Furthermore, DTH reactions are associated with protection against CA in immunocompetent individuals (Romani, 2000). Primary immunization (Fig. 1) began at 14 days and consisted of simultaneous intramuscular (IM) injection of the type-1 Ag killed *C. albicans* (500 µg, crude whole cell, Greer Laboratories Inc., NC, USA) and the type-2 Ag hen egg white allergen, lysozyme (HEWL, 10 µg, Sigma Aldrich, Oakville, ON, Canada) (Fremont et al., 1997; Heriazon et al., 2011) all combined with Quil A adjuvant (1 mg, Cedarlane Laboratories, Hornby, ON, Canada) to a total volume of 1 ml in phosphate buffered saline (PBS). A secondary IM injection was given on day 28. Blood was collected from the retro-orbital sinus on days 14 (pre-immunization) and 35 (post secondary-immunization) to measure CA and HEWL-specific serum Ab activity by enzyme linked immunosorbent assay (ELISA).

2.3. Immune response phenotyping

2.3.1. Skin test reactions: wheal and flare and double skin fold thickness

To measure the skin test reactivity to each Ag, intradermal (ID) injections of HEWL (100 µg), CA (100 µg) and PBS (negative control) were made at three marked sites on the medial aspect of the thigh of each pig. Type-1 hypersensitivity, a type-2 IR criterion, was assessed by the appearance of wheal and flare. Immediate-type hypersensitivity (type-2 IR), intermediate hypersensitivity (type-2 IR) and DTH (type-1 IR) were measured by change in double skin fold thickness (DSFT). The appearance of wheal and flare at HEWL injection sites was determined as positive or negative approximately 15 min after ID injection by consensus between three observers regarding positivity.

Measurements of DSFT were made using calipers immediately prior to (time 0) and at 1 h (immediate hypersensitivity), 3 h (intermediate hypersensitivity) and 48 h (DTH) after ID injection. Measurements were by the same individual to control for consistency. The DSFT measurements were repeated 3 times at each site and the mean

calculated. Percent increase in DSFT was calculated as: % increase in DSFT = [(DSFT at time x – DSFT at time 0)/DSFT at time 0] × 100, and was used to assess the magnitude of the response

2.3.2. Enzyme linked immunosorbent assay

To measure CA and HEWL-specific Ab activity, ELISA assays were established following standard methods, for immunoglobulin (Ig)G heavy and light chain specific (H + L; Sigma Aldrich), IgG₁ (type-2, AbD Serotec, Raleigh, NC 27604, USA), IgG₂ (type-1, AbD Serotec) and IgE (type-2, Rupa et al., 2008)-related Ab. *C. albicans*-specific IgE Ab activity was not measured. 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 3 times (ELX 405 automatic plate washer; Biotek Instruments, Winooski, VT, USA) with 200 µl 0.05% Tween PBS (PBST 0.01 M, pH 7.4 per well). Wells were blocked with 3.0% PBST (pH 7.4), 200 µl 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 µl per well in triplicate. Controls included wells without sera and negative (pooled serum from pre-immunized piglets with low Ab activity) and positive sera (pooled serum from post-immunized piglets with high Ab activity). 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 CA and HEWL-specific IgG 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 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 the controls. Protocol for the HEWL-specific IgE ELISA was similar to that outlined by Rupa et al. (2009). Individual serum 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.

2.3.3. Calculating change in skin fold thickness, positive antibody activity, and ratios of Ig isotype-related antibody activity

Positive change in DSFT was calculated as: [(% increase in DSFT at CA or HEWL injection site at time x) – (mean% change in DSFT at PBS injection site at time x)] > 0. This was used to determine the frequency of pigs within a group that had a positive change in DSFT. Since the various ELISA tests were not standardized to read out as concentration of Ab,

to enable comparison of Ab activity between tests individuals were categorized as positive or negative for antibody activity associated with each isotype. Individual positive or negative sample status was determined as $[(\text{mean}\% \text{ of positive control value on day 35}) - (\text{mean}\% \text{ of positive control value on day 14} + 2 \text{ SD})] > 0$. Ratios of antibody activity related to Ig isotypes were calculated as follows: $(\text{mean}\% \text{ of positive control Ab activity for isotype } x \text{ on day 35}) / (\text{mean}\% \text{ of positive control Ab activity for isotype } y \text{ on day 35})$.

2.4. Statistical analysis

For skin test and Ab activity data Fisher's exact test (GraphPad Instat, GraphPad, San Diego, CA, USA) was used to compare frequency of positive tests by experiment. Significance of difference between means was assessed using the unpaired Student's *t*-test for normally distributed data. Welch's correction was applied for non-normally distributed data as determined by the *F*-test. Data are reported as means $\pm$ the 95% confidence interval and $p \leq 0.05$ was taken to indicate significance. All calculations were performed with GraphPad Prism software 4.0 (GraphPad).

3. Results

3.1. Change in double skin fold thickness after intradermal injection of HEWL and CA

A significant increase in DSFT 1 and 3 h after ID injection of HEWL occurred in pigs from groups A and B indicative of immediate and intermediate-type hypersensitivity responses reflecting type-2 IR bias consistent with the type-2 antigen status of HEWL (Fig. 2). There was no significant difference in the frequency or magnitude of increase in DSFT after 1 h between pigs from the two groups (Fig. 2i, $p = 0.4438$). Significant increase in DSFT after 3 h occurred in pigs from both groups after ID injection with HEWL indicating an intermediate-type reaction. There was no significant difference in frequency or magnitude of the response observed after 3 h between the experimental groups (Fig. 2i, $p = 0.1555$). A significant increase in DSFT 48 h after ID injection with CA occurred in pigs of groups A and B indicative of a DTH response consistent with the type-1 antigen status of CA (Fig. 2). The magnitude of the DTH response to CA was greater in pigs of group A (Fig. 2ii, $p < 0.0001$) although there was no significant difference in the frequency of positive change in DSFT observed between pigs in groups A and B. The lesser magnitude of DSFT in response to CA of pigs in B indicated less type-1-associated IR. This may be accompanied by an increased response to type-2 associated Ags, such as wheal and flare in response to HEWL.

3.2. Wheal and flare response to intradermal injection of HEWL

There were significantly more pigs in group B (49%, $p = 0.0007$) which had a wheal and flare response after ID injection of HEWL. Only 25% of pigs in A had a positive wheal and flare response to this Ag. Therefore, pigs in B had a greater ability to respond to a type-2 associated Ag.

This may be further reflected in different frequency in Ab response and in bias of Ig isotype association of Ab to CA and HEWL.

3.3. Frequency of pigs with positive CA and HEWL-specific antibody activity

All Ig isotype-related CA and HEWL-specific Ab activity for IgG, IgG₁, IgG₂ and IgE measured on day 35 (post-immunization) was significantly increased from day 14 (pre-immunization, $p < 0.0001$). The frequency of pigs with positive HEWL-specific IgG, IgG₁ and IgG₂ Ab activity was greater in group B than A (Fig. 3i, $p = 0.0075$, $p = 0.0022$ and $p < 0.0001$, respectively). There was no significant difference between groups in the frequency of pigs with positive HEWL-specific IgE Ab activity. For CA-specific Ab activity the pigs in B had a higher frequency of positive IgG ($p < 0.0001$). The frequency of pigs with positive IgG₂ Ab activity was greater in group A ($p < 0.0001$). There was no significant difference between groups in the frequency of pigs positive for IgG₁ Ab activity (Fig. 3ii). *C. albicans*-specific IgE Ab activity was not measured. Taken together these results suggest that group B is in greater type-2 IR bias.

3.4. Immunoglobulin-isotype bias of antibody reflects group difference in relative type-1:type-2 immune response phenotype

To compare Ab activity associated with IgG₁, IgG₂ and IgE, Ab isotype ratios were calculated for day 35 (Fig. 4). For the ratios of HEWL-specific Ab, IgG₁:IgG₂ and IgE:IgG₂ were significantly greater in B ($p = 0.0002$ and $p < 0.0001$, respectively) indicative of greater type-2 IR bias. The IgE:IgG₁ ratio was significantly greater in A ($p = 0.0052$). No difference was found for the CA-specific IgG₁:IgG₂ ratio, which was 1.27 for pigs in group A and 1.23 for pigs in B.

4. Discussion

The research tests the hypothesis that two different groups of neonatal pigs (A and B), from the same herd would have a similar IR phenotype as indicated by response to a standard IR phenotyping protocol when group A was tested first and B two and a half years later. A difference in IR phenotype was observed between pigs in groups A and B with more type-2 bias in pigs from B. Most of the IR parameters measured confirmed this type-2 bias, such as increased frequency of wheal and flare to the type-2 Ag HEWL and increased Ab activity to both Ags accompanied by a decreased DTH response to CA and greater use of type-2 Ig isotypes IgG₁ and IgE when compared to pigs in A. It is known that the IR of pigs varies by litter and individual such that the ratios of type-1 and type-2 IR differ. Based on estimated heritability of IR of approximately 20% the environment and gene by environment interactions contribute greatly to this variation (Wilkie and Mallard, 1999).

Although change in DSFT to ID injection of HEWL was not significantly different between pigs in groups A and B, the Group A DTH response to CA was significantly greater in magnitude (Fig. 2). Also, further suggesting type-2 IR

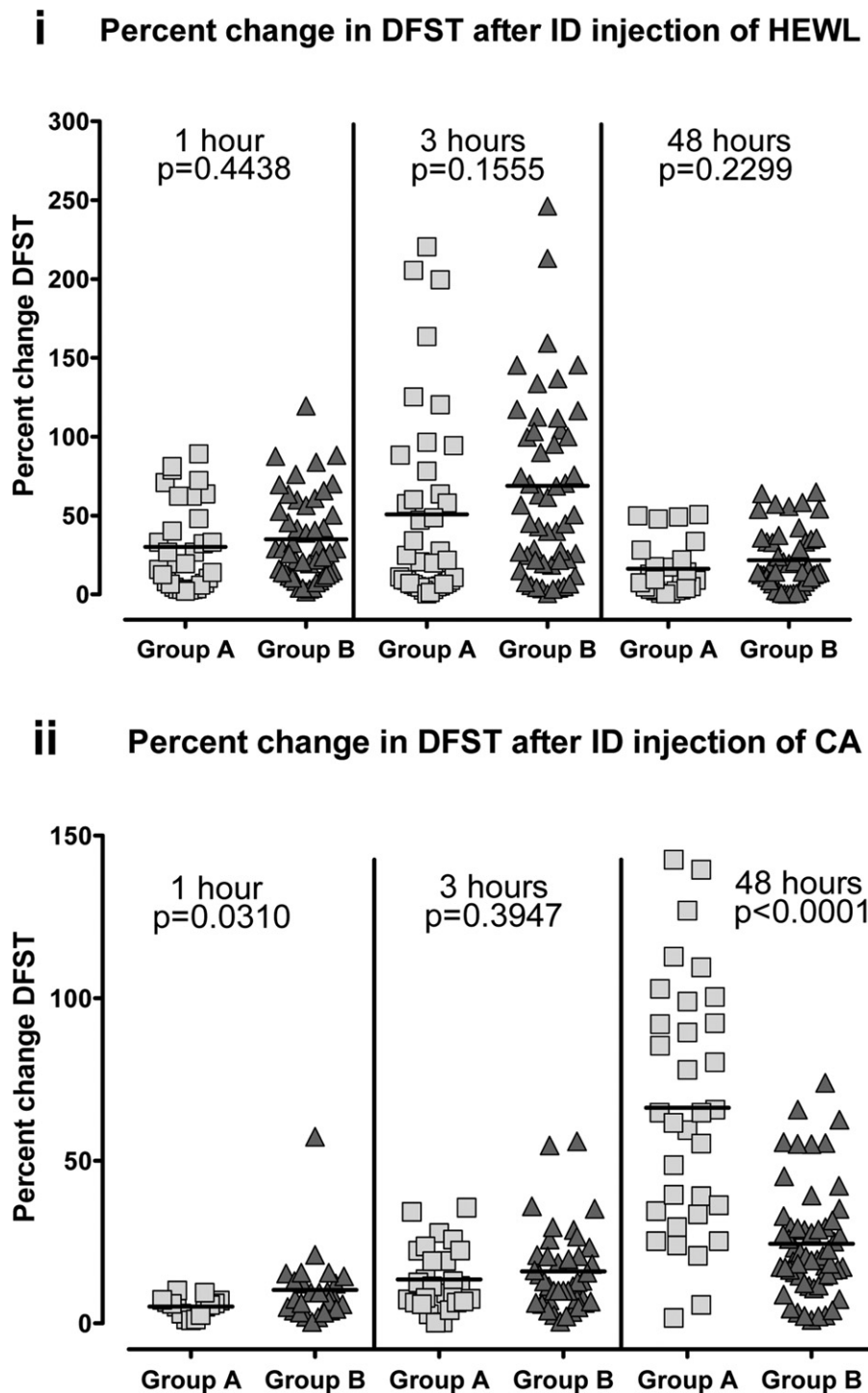


Fig. 2. Percent change in double skin fold thickness after intradermal injection of hen egg white lysozyme (i) or *C. albicans* (ii). Double skin fold thickness (DSFT) was measured over time beginning on day 35 at separate intradermal (ID) injection sites of hen egg white lysozyme (HEWL), *C. albicans* (CA) and phosphate buffered saline (negative control). A single injection of each antigen was made per pig. Measurements of DSFT were taken in triplicate at each site using calipers immediately prior to (time 0) and at 1, 3, and 48 h after ID injection to obtain mean DFST per pig, per time point. Percent increase in DSFT was calculated as: % increase in DSFT = [(DSFT at time (h) – DSFT at 0 h)/DSFT at 0 h] × 100. Significance was assumed at $p \leq 0.05$. Groups A (3 litters or 12 pigs/litter) and B (6 litters or 12 pigs/litter) were immunized with HEWL and CA by the same protocol, B approximately two and a half years after A.

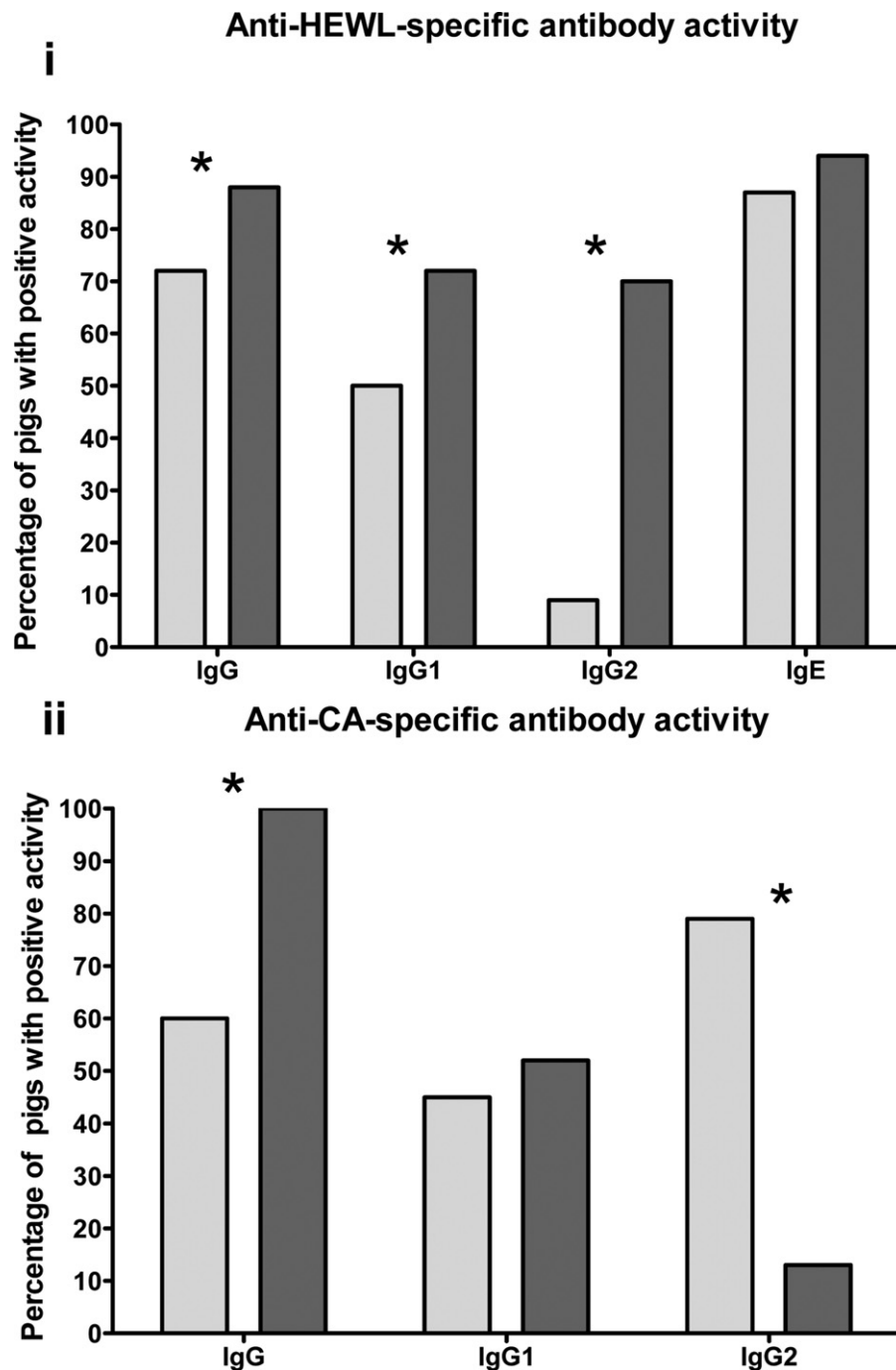


Fig. 3. Frequency of pigs with positive serum antibody activity for hen egg white lysozyme (i) and *C. albicans* (ii) associated with IgG (H + L), IgE, IgG₁ and IgG₂. Blood was taken on days 14 and 35 pre and post-immunization and hen egg white lysozyme (HEWL) and *C. albicans* (CA)-specific serum antibody (Ab) activity was measured by enzyme-linked immunosorbent assay from triplicates of each sample. Data are represented as percentage of pigs with positive antibody activity per group, determined as [(percent of positive control on day 35) – (mean percent of positive control on day 14 + 2 SD)] > 0. Percent of positive control was calculated as (optical density (OD) of test serum)/(OD of positive control serum – OD of negative control [pre-sensitized sera]) × 100%. Significance was taken at $p \leq 0.05$. *indicates significant difference between groups. Light gray bars represent group A, dark gray bars group B. *C. albicans*-specific IgE Ab activity was not measured. Groups A (3 litters or 12 pigs/litter) and B (6 litters or 12 pigs/litter) were immunized with HEWL and CA by the same protocol, B approximately two and a half years after A.

Ratio of isotype associated HEWL-specific antibody activity

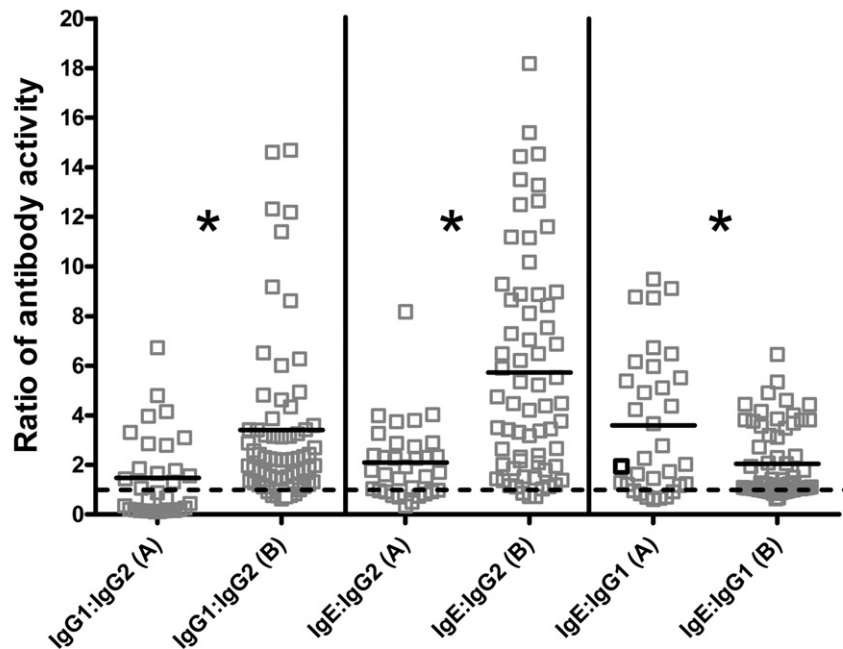


Fig. 4. Isotype ratios of hen egg white lysozyme-specific antibody activity. Serum IgG₁:IgG₂, IgE:IgG₂ and IgE:IgG₁ ratios of antibody (Ab) to hen egg white lysozyme (HEWL) were measured to compare Ig isotype bias of Ab activity in pigs from groups A (3 litters or 12 pigs/litter) and B (6 litters or 12 pigs/litter). Blood was collected post-immunization and serum-Ab activity was measured by enzyme-linked immunosorbent assay, and immunoglobulin isotype-related ratios of Ab-activity were calculated. Significance was assumed at $p \leq 0.05$. * indicates significant difference between groups. Groups A and B were immunized with HEWL and CA by the same protocol, B approximately two and a half years after A.

bias, the frequency of dermal wheal and flare in response to ID injection of HEWL was greater in pigs from group B. This may indicate that piglets in A had a more balanced IR. It was not expected that pigs in B would have less DTH response to ID injection of CA. Since the expression of DTH is associated with protection against CA (Romani, 2000) the diminished response may suggest compromised cell-mediated defense against infectious disease. Piglets in Group B had an increased frequency of positive IgG, IgG₁ and IgG₂ HEWL-specific Ab activity; however the frequency of IgE Ab activity was not different from that of pigs in Group A. Although porcine IgG₂ and IgG₁ are relatively type-1 and type-2-associated isotypes respectively (Crawley and Wilkie, 2003), an overall increase in Ab activity may be considered characteristic of a type-2 biased IR. The lack of difference in HEWL-specific IgE between pigs in A and B is counter intuitive as pigs in A had significantly less IgE-dependent cutaneous wheal and flare positivity. Other research here (Wilkie et al., 2011) and this experiment show that allergen-specific IgE Ab activity and wheal and flare positivity are not positively correlated suggesting that regulatory mediators, such as blocking Abs and T-regulatory cells, may control the development of wheal and flare (Rupa et al., 2011). Furthermore, IgG isotypes may not be exclusively blocking and may positively contribute to expression of immediate type hypersensitivity (Tkaczyk et al., 2002). There is a possibility that pigs in A had a greater frequency of such mediators and therefore displayed less wheal and flare activity in the presence of HEWL-specific

IgE. Ratios of HEWL-specific Ab activity further illustrate the type-2 bias of pigs in B as the IgG₁:IgG₂ and the IgE:IgG₂ ratios were significantly greater in this group, although the IgE:IgG₁ ratio, both type-2 isotypes, was greater in A.

When measuring the IR bias of pigs with respect to the type-1 Ag CA it was observed that pigs in group B were more frequently positive for IgG Ab activity while in A there was a higher frequency of CA-specific IgG₂ (Fig. 3). These results indicate that pigs in A responded to a type-1 Ag with a type-1-associated Ab isotype. Although pigs in B had a greater CA-specific IgG response, the frequency of CA-specific IgG₁ was not different between the two groups. Pigs have six putative IgG isotypes (Butler et al., 2009), of which the biological functions of only two have been identified (Crawley and Wilkie, 2003). Therefore, another yet to be classified type-2 IR-associated IgG-isotype(s) may have mediated Ab response to CA in pigs of group B.

The cause of IR bias differences between groups A and B are not apparent and were not sought, as phenotyping was intended to confirm a baseline in IR bias from which to conduct experimentation into the possible ability of treatments with microbial cells or their components to alter IR bias. Pigs were housed in the same facility using the same personnel and management practices, thus reducing potential influence of the most probable environmental variables. However, between the experimental periods of A and B, PCV2, a virus known to alter IR bias, was detected in the herd. This virus suppresses CpG ODN induced type-1 IFN and TNF-alpha production by plasmacytoid dendritic

cells by interfering with sensing of danger signals, this may have consequences for the development of IR (Vincent et al., 2005). Although pigs in either group were not obviously diseased, it is possible that sub-clinical infection or other mechanisms affecting IR bias operated differentially in the population over time.

There is abundant evidence for the effects of environment on IR. The hygiene hypothesis states that declining family size, urbanization and improved household and family hygiene have reduced opportunity for infection within families and have led to a decrease in microbial exposure and a subsequent increase in atopic disease (Strachan, 1989). Further, it is known that the intestinal microbiome is critical to protection against infection and the maturation of the immune system (Marques et al., 2010). The “disappearing microbiota” hypothesis (Blaser and Falkow, 2009), states that, along with the aforementioned, environmental changes, widespread use of antibiotics in children, an increase in the number of children born by caesarian section and a decrease in breast-feeding rates have affected the transmission and maintenance of the indigenous microbiota. These phenomena are not exclusive to humans and have been observed experimentally in pigs. Mulder et al. (2009) have shown that early life environment influences microbial composition in the adult gut lumen as well as maturation of the mucosal innate immune system. Piglets were housed outdoors or indoors with sow or indoors in isolator units without sow, receiving daily antibiotic treatments. Those housed outdoors had a greater abundance of beneficial lactobacilli in their gut microbiota than the other piglets. Lactobacilli are associated with conferring beneficial health effects (Ramirez et al., 2010).

In the present study, alteration of microbial exposure in the barn may have influenced the development of the neonatal piglet immune system such that the IR of pigs in B had a type-2 bias. Research here has shown that daily IM pre-treatments of newborn pigs with heat-killed *Escherichia coli* (Rupa et al., 2009) or orally administered live *Lactococcus lactis* (Rupa et al., 2011) prevent clinical signs of allergy in pigs subsequently sensitized to the egg white protein allergen ovomucoid and orally challenged with egg white in a neonatal pig model of food allergy. Since food allergy is a type-2 biased disease this indicates that it is possible to direct the neonatal pig immune system from type-2 IR bias to a more balanced IR.

Although we did not measure environmental triggers for IR bias change, variable results of replicate IR phenotyping suggest that environment by time effects alter IR phenotype with implications for protective and immunopathogenic IR. It may also influence IR phenotyping as a tool in selective breeding and investigations of diverse correlations of IR (Wilkie and Mallard, 1999). Repeating standard IR-inducing protocols may result in significantly different phenotypic outcomes and neonatal pigs are not invariably in type-2 IR bias.

Conflict of interest

None of the authors are in conflict of interest.

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