Direct experimental evidence that early-life farm environment influences regulation of immune responses

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Abstract

Background: In mammals, early-life environmental variations appear to affect microbial colonization and therefore competent immune development, and exposure to farm environments in infants has been inversely correlated with allergy development. Modelling these effects using manipulation of neonatal rodents is difficult due to their dependency on the mother, but the relatively independent piglet is increasingly identified as a valuable translational model for humans. This study was designed to correlate immune regulation in piglets with early-life environment.

Methods: Piglets were nursed by their mother on a commercial farm, while isolator-reared siblings were formula fed. Fluorescence immunohistology was used to quantify T-reg and effector T-cell populations in the intestinal lamina propria and the systemic response to food proteins was quantified by capture ELISA.

Results: There was more $CD4^+$ and $CD4^+CD25^+$ effector T-cell staining in the intestinal mucosa of the isolator-reared piglets compared with their farm-reared counterparts. In contrast, these isolator-reared piglets had a significantly reduced $CD4^+CD25^+Foxp3^+$regulatory T-cell population compared to farm-reared littermates, resulting in a significantly higher T-reg-to-effector ratio in the farm animals. Consistent with these findings, isolator-reared piglets had an increased serum IgG anti-soya response to novel dietary soya protein relative to farm-reared piglets.

Conclusion: Here, we provide the first direct evidence, derived from intervention, that components of the early-life environment present on farms profoundly affects both local development of regulatory components of the mucosal immune system and immune responses to food proteins at weaning. We propose that neonatal piglets provide a tractable model which allows maternal and treatment effects to be statistically separated.

Keywords: early life; environment; mucosa; tregs; weaning

In the intestine, immune responses against 'harmless' antigens are associated with autoimmunity, inflammatory disease and allergy, and there is increasing evidence that such responses are controlled by regulatory T-lymphocytes expressing CD4, CD25 and the transcription factor Foxp3 (1). There is clear indication that expansion of a competent immune system in neonates depends on the composition of the intestinal microbiota and therefore exposure to environmental microbes (2). However, the mechanisms linking early-life environment to later development of allergy are only just starting to be clarified. In adult mice, numbers and function of $Foxp3^+$ T-cells in both the intestinal mucosa and periphery can be affected by diet and microbiota (1). However, manipulating the neonatal environment of rodents in a manner relevant to human infants is demanding.

Similarities in digestive physiology and immunology between pigs and humans (3) make neonatal piglets a valuable intermediate model between rodent studies and clinical trials in human infants. Large-scale genomic studies have demonstrated fewer differences between humans and pigs than between humans and rodents (4), and intra-individual stability and

inter-individual variability of the microbiota are more similar between humans and pigs than between humans and mice (5). Furthermore, as in human infants, the newborn piglet has few immunologically relevant cells present in the gut mucosa and the gut-associated lymphoid tissues are underdeveloped. In response to antigenic challenge, especially from the intestinal microbiota, myeloid and lymphoid cells begin to infiltrate the lamina propria in an ordered and predictable manner (6). Like human infants, young piglets mount systemic antibody responses to components of their diet.

By rearing piglets under different conditions, we have already demonstrated significant effects of environment on the intestinal microbiota, on host cells associated with the immune system and on gene expression. Given the important role of regulatory T-cells in rodents, we hypothesized that their numbers would be altered by the conditions generated in our low/high hygiene model and that these differences would be reflected functionally in antibody responses to novel food protein. The results presented here confirm this hypothesis and further demonstrate that neonatal piglets are a tractable model for human infants, in which maternal and treatment effects can be statistically separated.

Materials and methods

Animal model

Animal housing and experimental procedures were all performed according to local ethical guidelines: all experiments were performed under a UK Home Office License and were approved by the local ethical review group. To examine the effects of rearing environment, 12 piglets from six litters were matched into two equal groups 24 h after birth. One group

(high hygiene) was removed to an SPF facility (positive-pressure, HEPA-filtered air), disinfected in Virkon®, individually housed and automatically fed hourly with a commercial, bovine milk formula (Piggimilk, Parnutt Feeds, UK). Litter-matched siblings were left on the farm and were nursed by the mother (low-hygiene). These piglets were killed at 28 days. In a parallel study, two litter-matched groups of piglet were established similarly except that at 28 days, these piglets were mixed together and weaned onto a soya-based diet. Serum samples were obtained at 28 and 42 days, and the animals were killed at 42 days. Adult samples were taken from 5-month old conventionally reared animals killed in a commercial abattoir.

Fluorescence immunohistology

The jejunal samples (avoiding Peyer's patches) were obtained from 28-day-old piglets and adult animals and were snap frozen, sectioned and fixed in acetone. Non-specific binding sites were blocked using 10% goat and pig serum, and the samples were then stained. For the analysis of $Foxp3^+$ T-cell numbers, the following monoclonal antibodies were used: anti-porcine CD4 (clone MIL17), anti-mouse Foxp3 (clone FJK-16s) and anti-porcine CD25 (clone K231.3B2). Binding was detected with the following: goat anti-mouse IgG_{2b} TRITC (Southern Biotechnology, Birmingham, Alabama, USA), goat anti-rat IgG FITC (Stratech, Newmarket, Suffolk, UK) and biotinylated goat anti-mouse IgG_1 (Southern Biotechnology), detected with AMCA-Avidin D (Vector Laboratories, Southgate, Peterborough, UK). Image capture and proportional area of CD4 and CD25 staining was analyzed using an in-house macro and ImageJ version 1.44 (http://rsbweb.nih.gov) (7). Briefly, 10 × 16-bit images were captured for each piglet, and the proportion of positive pixels in each colour channel was measured using a specifically developed inhouse macro. This allowed quantification of

positive staining (and co-staining) by the primary antibodies. Because expression of Foxp3 was nuclear, numbers of $CD4^+CD25^+Foxp3^+$ were analyzed using the cell counter plugin. The density of $CD4^+$ staining was such that it was not possible to determine cell numbers; instead, the proportion of area which stained positive for CD4 (or $CD4^+CD25^+$ co-expression) was measured and the values logged to achieve normal distributions, as verified by Inman et al. (7).

Antibody assay

Serum samples were analyzed for anti-soya and anti-β-lactoglobulin IgG_1 and IgG_2 antibodies by indirect ELISAs. Target antibodies were detected using isotype-specific monoclonal antibodies; anti-pig IgG_1 (clone K139.3C8) and anti-pig IgG_2 (clone K68.1G2) (Serotec, UK) and relative concentrations were determined by interpolation of samples onto the reference standard serum (obtained from pigs hyperimmunized with the soya protein 11S, or from a piglet previously shown to have responded to β-lactoglobulin).

Statistical analysis

Results were analyzed by general linear modelling (SPSS) using treatment and litter as factors. Statistical analysis was carried out only on the piglet groups; adult was not included in the model. The values represent the means ± SEM. A value of $p < 0.05$ was considered to be significant.

Results

The effect of early-life environment on intestinal $CD4^+$ T-cell populations

Consistent with our previous results demonstrating development of the T-cell component of the intestinal mucosa with age, there were significantly higher proportions of CD4 (Fig. 1a) and $CD4^+CD25^+$ (Fig. 1b) staining in the adult animals ($p < 0.007$). However, whilst the area of $CD4^+$ and $CD4^+CD25^+$staining was increased in the jejunal mucosa from piglets in the SPF environment compared with their siblings left with the mother ($p = 0.001$, and $p = 0.028$ respectively), there were fewer $CD4^+$ cells expressing Foxp3 in the SPF isolator-reared animals ($p = 0.001$, Fig. 1c–e). When the number of $CD4^+CD25^+Foxp3^+$ cells was expressed as a proportion of the total area of CD4 staining, the level in the SPF piglets was similar to that seen in the adult animals, indicating that the proportion of $CD4^+$ cells which were regulatory was significantly increased in the farm-reared piglets ($p < 0.001$, Fig. 1f). This is consistent with a specific increase in $CD4^+CD25^+Foxp3^+$ T-cells in the farm group rather than a general increase in all effector (Th1, Th2, Th17) $CD4^+$ T-cells.

Maternal effects on intestinal $CD4^+$ T-cell populations

The results of statistical analysis indicated an extremely strong effect of litter on all the subsets measured ($p < 0.001$). In fact, the effects of treatment were only significant when litter was included as a factor in the analysis, and this occurred for the total proportion of $CD4^+$ and $CD4^+CD25^+$ positive area and when $CD4^+CD25^+Foxp3^+$ cells were counted as a proportion of $CD4^+$ total area. Interestingly, the inclusion of litter in the model for predicting total numbers of $CD4^+CD25^+Foxp3^+$ cells was not necessary in order for significance to be achieved, but the overall significance was increased ($p = 1.7 \times 10^{-3}$ to $p = 7.7 \times 10^{-8}$).

Serum antibody response to novel oral protein at weaning

To determine whether the effects of the rearing environment on regulatory T-cells, observed in 4-wk-old animals, were associated with functional differences in responses to food, piglets were weaned at this age. Piglets kept in isolator conditions prior to weaning produced increased systemic, soya-specific IgG_1 ($p = 0.0016$) and IgG_2 ($p = 0.01$) in response to novel dietary 11S soya protein in the weaning diet (Fig. 2a,b), compared with their farm and sow-reared siblings. However, there was no difference in the level of systemic antibody to bovine β-lactoglobulin between isolator-reared and farm-reared siblings either at 4 or 6 wk (data not shown).

Discussion

Epidemiological data from humans have shown that exposure to farm environments correlates with protection against the development of allergy in childhood (8). Although causal links are difficult to establish in humans, an increase in Foxp3 transcripts in cord blood of infants born to mothers exposed to farm environments has been recorded (9). Similarly, in rodents, reduction in $CD4^+CD25^+Foxp3^+$ T-cells has been correlated with an increase in allergy symptoms in rodents (10). In mice, colonization of germ-free animals with single strains can trigger expansion of a range of $CD4^+$ T-cell subsets (11), but the influence of complex environments, such as a farm, is difficult to study. The observed effects of environment on regulatory T-cells in the intestinal mucosa of a species other than humans or rodents indicate that this is a generally applicable biological phenomenon. In addition, our results indicate that complex factors in the farm environment result in expansion of the mucosal effector T-cell populations ($Foxp3^-$), consistent with the idea that a balance between effector and regulatory cells is critical.

The ability to litter-match treatment groups in this outbred animal model also allows the maternal and treatment effects to be statistically separated using relatively small numbers of animals. The observation of strong effects of litter on mucosal regulatory and effector $CD4^+$ T-cells, over and above the effects of environment, is of considerable relevance in regard to human infants, where genetic and environmental predisposition to allergy has been identified.

In conclusion, we provide the first direct evidence, derived from intervention, that the farm environment profoundly affects both local development of regulatory components of the mucosal immune system and immune responses to food proteins at weaning. Clearly, many factors other than bacterial exposure differed between our farm and isolator piglets including diet (sow's milk or bovine milk formula), social interactions and aerial contaminants, but the influence of these different factors can be determined by direct intervention using our piglet model in future experiments. In fact, recent studies have suggested that unpasteurized milk may be an important component of the farm effect on allergy in human infants (12). The large litter size and relative independence of neonatal piglets provide a tractable model for human infants in which the effects of genetics, microbiota and diet on the mucosal immune system and on development of allergic responses can be further dissected.

Acknowledgments

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References

1. Barnes MJ, Powrie F. Regulatory T-cells reinforce intestinal homeostasis. *Immunity* 2009: 31: 401–11.
2. Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001: **108**: 516–20
3. Lunney JK. Advances in swine biomedical model genomics. *Int J Biol Sci* 2007: **3**: 179–84.
4. Jorgensen FG, Hobolth A, Hornshoj H, Bendixen C, Fredholm M, Schierup MH. Comparative analysis of protein coding sequences from human, mouse and the domesticated pig. *BMC Biol* 2005: doi: 10.1186/1741-7007-3-2.
5. Thompson CL, Hofer MJ, Campbell IL, Holmes AJ. Community dynamics in the mouse gut microbiota: a possible role for IRF9-regulated genes in community homeostasis. *PLoS ONE* 2010: doi: 10.1371/journal.pone.0010335.
6. Rothkotter HJ, Ulbrich H, Pabst R. The postnatal-development of gut lamina propria lymphocytes - number, proliferation, and T-cell and B-cell subsets in conventional and germ-free pigs. *Pediatr Res* 1991: **29**: 237–42
7. Inman CF, Rees LEN, Barker E, Haverson K, Stokes CR, Bailey M. Validation of computer-assisted, pixel-based analysis of multiple-colour immunofluorescence histology. *J Immunol Methods* 2005: **302**: 156–67.
8. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* 2010: **10**: 861–8.
9. Schaub B, Liu J, Hoppler S, *et al.* Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol* 2009: **123**: 774–82.e5.
10. van Esch BC, Schouten B, de Kivit S, *et al.* Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3(+) regulatory T-cells in the mesenteric lymph nodes. *Pediatr Allergy Immunol* 2011: **22**: 820–6
11. Gaboriau-Routhiau V, Rakotobe S, Lécuyer E, *et al.* The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T-cell responses. *Immunity* 2009: **31**: 677–89
12. Waser M, Michels KB, Bieli C, *et al.* Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* 2007: **37**: 661–70.

Figure 1. Quantitative analysis of three colour fluorescence immunohistology in the lamina propria of frozen sections of the distal Jejunum from 28 day old neonatal piglets, reared in either high-hygiene (isolator reared and formula-fed) or low hygiene (farm reared and suckled by their sow), and adult animals. (a) The proportion of the area which stained positively for CD4 was increased in the adult gut compared to the neonates, and was also increased in the isolator-reared piglets compared to the farm-reared piglets ($p < 0.001$). (b) Similar results were observed for the area of CD4 co-expressed with CD25 ($p = 0.028$, isolator vs. farm piglets). (c, d), CD4 (red), CD25 (blue) and Foxp3 (green) in distal jejunum of an isolator reared piglet (c) and its littermate which was farm-reared (d). The farm-reared littermate had reduced proportion of $CD4^+$ staining in the lamina propria of the villus overall, but an increased number of $CD4^+CD25^+$ cells expressing intercellular Foxp3, indicated by the arrows. (e) The number of $CD4^+CD25^+Foxp3^+$ cells/mm^2 was not significantly different between the farm-reared piglets and adult animals, and significantly reduced in the isolator-reared piglets ($p = 0.001$). (f) The $CD4^+CD25^+Foxp3^+$cells were a higher proportion of the total area stained positive for CD4 in the farm–reared animals compared to those reared in the isolator ($p < 0.001$) which had proportions of $CD4^+CD25^+Foxp3^+$ cells comparable to those in the adult animals. Results for (a), (b), (e) and (f) are the means of log-transformed data ± SEM (n = 6).

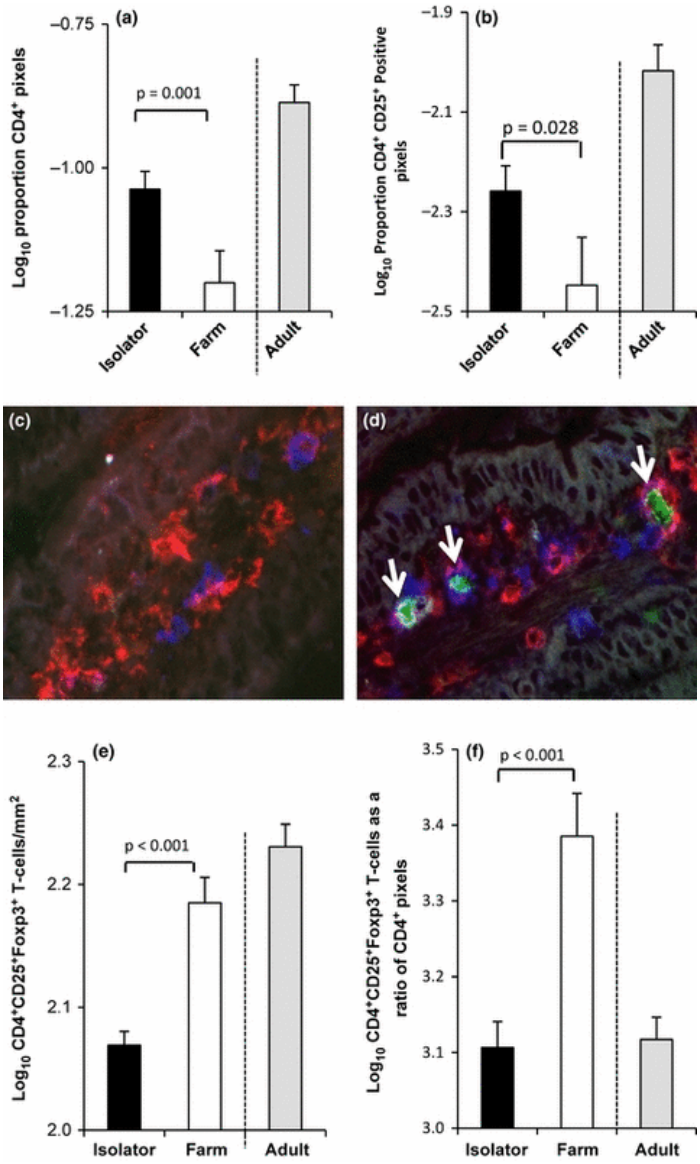

Figure 2. Serum anti-11S-soya antibody after weaning onto a soya-based diet at 28 days of age. Fold change in IgG1 (a) and IgG2 (b) antibody

$$\left(\frac{\text{antibody at 42 days}}{\text{antibody at 28 days}}\right)$$

in serum of piglets reared initially under SPF conditions and fed milk formula (Isolator, n = 5) and of litter-matched, farm-reared piglets (Farm, n = 6) (p = 0.0016 and p = 0.01 respectively). Each point represents the results from a single piglet.