



Induction of interleukin-4 production in neonatal IgE⁺ cells after crosslinking of maternal IgE

Bettina Wagner^{a,*}, Tracy Stokol^a, Dorothy M. Ainsworth^b

^a Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States

^b Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States

ARTICLE INFO

Article history:

Received 30 October 2009

Received in revised form 30 November 2009

Accepted 1 December 2009

Available online 16 December 2009

Keywords:

Basophil

Cytokine

Horse

Passive transfer

Cell differentiation

ABSTRACT

Transfer of maternal IgE antibodies to the neonate with the colostrum has been described in different mammalian species. Previous work in horses has shown that IgE bound to the surface of neonatal basophils is solely of maternal origin. However, the functional role of the maternal IgE transfer remained unclear. We hypothesized that maternal IgE mediates the onset of innate IL-4 production in equine neonatal basophils. Intracellular IL-4 production was measured in PBMC of newborn and older foals by flow cytometric analysis. A small population of IL-4⁺ cells was observed in the peripheral blood at days 3–5 after birth. Phenotyping of the IL-4⁺ cells showed that they were IgE⁺/MHCII^{low}/CD4⁻ cells. Magnetic cells sorting of the IgE⁺/MHCII^{low} cells identified them as basophils. Anti-IgE stimulation *in vitro* induced IL-4 in IgE⁺/MHCII^{low} basophils, but not in MHCII⁺ cells or cells collected before colostrum ingestion. In conclusion, stimulation via maternal IgE antibodies mediated innate IL-4 production in neonatal basophils which might provide a paragenetic mechanism to promote the development of adaptive T-cell responses in the neonate after birth.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Colostrum transfer of maternal IgE antibodies to the neonate has been described in humans [1], cattle [2] and horses [3,4]. While antibodies can be transmitted during pregnancy in humans, the structure of the equine placenta (placenta epitheliochorialis) [5] inhibits the transfer of maternal immune components such as antibodies, immune cells or cytokines to the fetus during the intrauterine development [6,7]. Consequently, maternal passive immunity is solely transferred to the equine neonate with the colostrum after birth [8]. This also includes maternal IgE antibodies which are detectable in foal serum and on peripheral blood cells after colostrum ingestion [3]. Previous work has also shown that equine neonates neither produce endogenous IgE at birth nor for the subsequent 6–12 months of life [3,4]. Consequently, IgE is not detectable on neonatal cells before colostrum uptake [3]. This led to the conclusion that IgE detected on neonatal cells is of maternal origin only. Cell sorting of equine IgE⁺ PBMC indicated that these cells represented a mixed population of basophils, monocytes and lymphocytes [9] and flow cytometric analysis showed that foal and adult IgE⁺ cells are composed of similar cell populations [3].

The functional role of maternal IgE antibodies in the neonate is not yet known. In human colostrum the levels of IgE were believed

to be too low to have a significant effect on the neonatal immune system [1]. In cattle, the kinetics of maternal and endogenous IgE followed those of IgG antibodies and it was suggested that maternal IgE provides early protection from disease, especially against intestinal parasites [2]. However, the onset of IgE production in horses clearly differed from that in cattle. A gap of several months was observed in foals after maternal IgE antibodies disappeared from PBMC and the circulation and before the endogenous IgE production became detectable [3]. Thus, maternal IgE antibodies may provide passive immunity against parasite antigens during the first weeks of life but it is rather unlikely that this function is maintained until foals develop their own IgE response.

In this article, we focused on the immune mechanisms induced by maternal IgE antibodies after binding to neonatal cells. We hypothesized that maternal IgE induces IL-4 production in neonatal basophils, thereby providing a potential trigger for the onset of Th2 development in the newborn foal. Here, we investigated the first part of our hypothesis and determined the production of IL-4 by neonatal IgE⁺ cells and the influence of maternal IgE antibodies on the neonatal IL-4 secretion.

Human and rodent basophils and mast cells were shown to produce IL-4 in response to crosslinking of their high affinity receptor for IgE (FcεRI) [10–15]. More recent publications also showed that basophils are the primary IL-4 producing cells during parasitic infections [16–18] and allergic responses [19,20]. Basophils, mast cells and eosinophils constitutively expressed

* Corresponding author. Tel.: +1 607 253 3813; fax: +1 607 253 3440.
E-mail address: bw73@cornell.edu (B. Wagner).

IL-4 and IL-13 transcripts in amounts that are sufficient for rapid cytokine production after stimulation. Out of these cell types basophils were the most potent IL-4 producers [21]. In addition to FcεRI crosslinking, IgE-independent pathways such as activation through TLRs [22], leukocyte immunoglobulin-like receptors [23], or CD200R-like protein, resulted in production of IL-4 by human basophils [24,25]. Today, various groups have shown increasing evidence that basophils have important immunoregulatory functions for the induction of innate and adaptive immune responses including the development of Th2 immunity and the regulation of humoral immunity in adult individuals or experimental rodent models [17,20,24–27].

2. Materials and methods

2.1. Animals and blood samples

The foal ($n = 30$) and adult horse groups ($n = 15$) consisted of Warmbloods, Thoroughbreds, and Thoroughbred crosses. All adult horses were mares between 8 and 22 years of age (median 14 years). Foals were born and raised at the Cornell University Equine Park during the spring of 2007, 2008 and 2009. All animals were clinically healthy throughout the study. The foals suckled colostrum ad lib. On day 1 after birth, a blood IgG quantification was performed by the Snap Foal IgG test (IDEXX laboratories, Westbrook, ME). All foals had serum IgG levels of >800 mg/dl indicating sufficient passive transfer of maternal immunoglobulins.

Blood samples were obtained from the V. jugularis using the BD Vacutainer system (Becton Dickinson, Franklin Lakes, NJ). A total of 56 samples were taken from the 30 foals at various time points after birth ranging from 12 to 144 h. Blood samples were also obtained from 15 of these foals at 6 and 12 weeks of age. Additional samples from 10 foals were collected at day 5 after birth to perform the IgE stimulation and cell sorting experiments. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee and were in accordance with the guidelines established by the NIH.

2.2. Isolation and culture of PBMC

PBMC were isolated from heparinized blood by density gradient centrifugation (Ficoll-Paque™ Plus, GE Healthcare, Piscataway, NJ). A total of 3×10^6 cells/ml were cultured in cell culture medium (DMEM containing 10% (v/v) FCS, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μg/ml gentamycin). For intracellular staining of cytokines, the secretion of proteins from the cells was blocked by adding Brefeldin A (10 μg/ml; Sigma, St. Louis, MO) to the culture medium. After incubation for 4 h at 37 °C in 5% CO₂, the cells were washed in PBS and fixed in 2% formaldehyde for 20 min at room temperature.

2.3. Intracellular staining, phenotyping of PBMC and flow cytometric analysis

Intracellular staining was performed with anti-equine IL-4 monoclonal antibody [28] in saponin buffer (PBS, supplemented with 0.5% (w/v) BSA, 0.5% (w/v) saponin and 0.02% (w/v) NaN₃). A murine IgG1 isotype control was included in the intracellular staining procedure using an aliquot of the PBMC. For cell surface staining, monoclonal antibodies to equine CD4 (HB61A; VMRD, Pullman, WA), equine CD8 (CVS8; kindly provided by Dr. Paul Lunn, Colorado State University) to label T-cells, or to equine IgM (anti-IgM 1–22) [29] for detection of B-cells, equine MHC class II (cz.11 [30]), equine IgE-176 [9], and equine CD14 (Wagner et al., unpublished) were used. All antibodies were conjugated to Alexa

fluorochromes A647 or A488 (Molecular Probes, Invitrogen, Eugene, OR) according to the protocol provided by the supplier. In addition a FITC conjugated anti-bovine IFN-γ antibody (MorphoSys, AbD Serotec, Oxford, UK) which was previously found to crossreact with equine IFN-γ [31,32] was included as a control for the intracellular staining procedure. After antibody incubation for intracellular staining, the cells were washed twice with saponin buffer. For cell surface staining, the fixed cells were incubated simultaneously with two of the directly labeled antibodies in PBS/BSA (PBS, supplemented with 0.5% (w/v) BSA and 0.02% (w/v) NaN₃) and were washed once in PBS/BSA after incubation. All samples were measured by flow cytometry using either a FACSCalibur or a FACSCantoII flow cytometer (both BD Biosciences, San Diego, CA).

2.4. Magnetic cell sorting

Cell sorting was performed using the MACS system (Miltenyi Biotech, Auburn, CA). The sorting procedure was performed on PBMC from foals at day 5 after birth and on those from adult horses. Approximately 5×10^7 PBMC were first stained for MHC class II followed by incubation with rat anti-mouse IgG1-beads (Miltenyi Biotech, Auburn, CA). The cells were separated over a LD column for negative selection. The MHC class II depleted fraction typically contained less than 5% MHC class II positive cells. The MHC class II depleted fraction was then stained with anti-IgE-134 [9] followed by another incubation with anti-mouse IgG1-beads. The cells were separated on a LS column to positively select IgE⁺ cells. Some contamination of MHC II⁺ cells that were not depleted in the first sorting step was carried over to the IgE⁺ cell enrichment using this method. However, it could be used to clearly enrich and phenotype the IgE⁺ cells. The magnetic cell sorting procedure was strictly performed at 4 °C to avoid activation of the IgE⁺ cells during the sorting process. All fractions were measured by flow cytometric analysis. To determine the enriched cell types in the IgE⁻/MHC II^{low} and IgE⁺/MHC II^{low} fractions, cytospin smears were prepared in a cytocentrifuge (Cytospin 3, Shandon, Pittsburgh, PA) at $113 \times g$ for 10 min and stained with Wright's stain. Differential cell counts were then performed by a single blinded observer.

2.5. Stimulation with anti-IgE and detection of IL-4 in cell culture supernatants

PBMC from neonates after birth and before colostrum ingestion, PBMC from 5-day-old foals, and aliquots of the MHCII⁺, IgE⁻/MHC II^{low} and IgE⁺/MHC II^{low} magnetic cell-sorted fractions were cultured to detect secreted cytokines in the cell culture supernatants. A total of 6×10^5 cells/ml were incubated in cell culture medium alone or stimulated in medium supplemented with 5 μg/ml anti-equine IgE-134 antibody [9], or for control 5 μg/ml anti-equine IgG1 (CVS45; kindly provided by Dr. Paul Lunn, Colorado State University). Both anti-equine antibodies were murine IgG1 isotypes. After 48 h, the supernatants were harvested and IL-4 concentrations were determined by an equine bead-based multiplex assay [33]. The analytical sensitivity of the IL-4 assay was 40 pg/ml.

2.6. Statistical analysis

A Shapiro–Wilk normality test was performed on each data set and showed that several variables were not Gaussian distributed. Thus, non-parametric tests were used for data analysis. For the analysis of IgE⁺ and IgE⁺/MHCII^{low} cells, a Mann–Whitney test was used. Wilcoxon signed rank tests were used for the cytokine analysis of individual treatments and cell populations. All tests were performed 1-tailed and with 95% confidence intervals. The

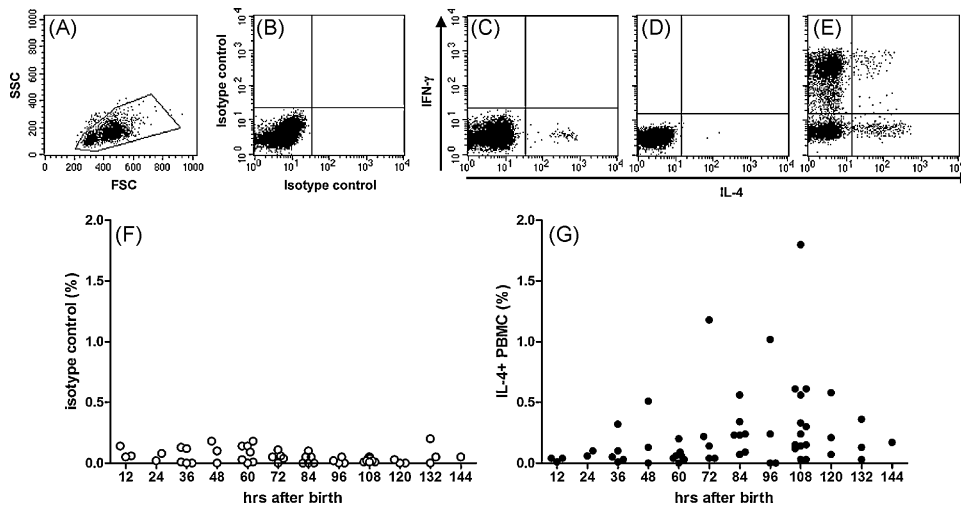


Fig. 1. IL-4 producing cells occur in the circulation of neonates between days 2 and 5 after birth. IL-4 production was measured in neonatal PBMC by flow cytometric analysis. Foal PBMC were isolated on day 5 after birth and were incubated in cell culture medium with the secretion blocker Brefeldin A for 4 h. Afterwards, the cells were fixed and intracellular staining was performed. (A) For the analysis a gate was set on PBMC; (B) intracellular staining of neonatal PBMC using isotype controls or (C) antibodies to IL-4 and IFN- γ ; (D) the same antibody pair was used to stain PBMC from an adult horse incubated in medium with Brefeldin A alone or (E) after stimulation with PMA and ionomycin. (F and G) Blood samples ($n = 56$) were obtained from healthy neonates at 12–144 h after birth. Aliquots of the cells were stained with (F) an isotype control or (G) with anti-equine IL-4.

statistical calculations were performed using the GraphPad Prism program, version 5.01.

3. Results

3.1. IL-4 producing cells in the peripheral blood of neonates

To investigate whether IL-4 producing (IL-4⁺) cells can be found in the circulation of neonates, non-stimulated PBMC samples from 56 neonatal foals (12–144 h after birth) were analyzed by intracellular staining and flow cytometry. In many of these PBMC samples a small population of IL-4 producing (IL-4⁺) cells was detected (Fig. 1C). This was in clear contrast to other cytokines, such as IFN- γ , which could neither be detected in PBMC from neonates (Fig. 1C) nor in PBMC from adult horses in the absence of *in vitro* stimulation (Fig. 1D). In PBMC from healthy adult horses, cytokine producing cells were only detected after mitogen or PMA stimulation (Fig. 1E).

The analysis of all 56 neonatal PBMC samples showed that the neonatal IL-4⁺ cells peaked between days 3 and 5 after birth (Fig. 1G). Although the IL-4⁺ cells represented a small population in neonatal PBMC, they could clearly be detected compared to the corresponding isotype controls (Fig. 1B and F). At day 5, the median percentage of neonatal IL-4⁺ cells cultured in medium was 0.24% (0.03–1.8%).

To identify the phenotype of the neonatal IL-4⁺ cells, they were stained with different cell surface markers (Fig. 2). The flow cytometric analysis showed that neonatal IL-4⁺ cells were surface IgE⁺ and did not express cell surface markers of T- or B-cells and

only low levels of MHC class II molecules. The percentage of IL-4⁺ cells in the IgE⁺ cell population ranged from 4 to 94% (median 83%) at day 5 of life.

3.2. Characterization of IgE⁺ cells

In peripheral blood of adult horses, IgE⁺ cells were previously characterized as a mixture of basophils, lymphocytes and monocytes [34]. Markers selectively detecting equine basophils are not yet available. To further characterize equine IgE⁺ cells we used various available surface markers for equine cells. Flow cytometric analysis showed that IgE⁺ cells can be distinguished by staining for IgM, CD14 and MHC II. Although IgE⁺ B-cells can generally be identified by additional staining for IgM and MHC II in adult horses (IgE⁺/IgM⁺/MHCII⁺ cells, data not shown), IgE⁺/IgM⁺ B-cells were rarely detected in foals during the first 5 days after birth (Fig. 3A). Most of the neonatal IgE⁺ cells were also negative for MHC class II expression (Fig. 3A) or, in some foals, they were composed of a MHC II^{low} and a MHC II⁺ population (Fig. 3B). In these foals, IgE⁺/MHCII⁺ cells were also positive for surface CD14 expression and were thus identified as monocytes, while the IgE⁺/MHCII^{low} cells were negative for CD14 (Fig. 3B). We concluded that the IgE⁺/IgM⁻/CD14⁻/MHCII^{low} cells are likely to be basophils and that these cells showed the same phenotype (IgE⁺/IgM⁻/MHCII^{low}; Fig. 2) as the neonatal IL-4⁺ cells.

To find additional evidence that the neonatal IL-4⁺ cells are basophils, magnetic cell sorting was performed and the IgE⁺/MHCII^{low} cells were isolated using PBMC from adult horses ($n = 5$)

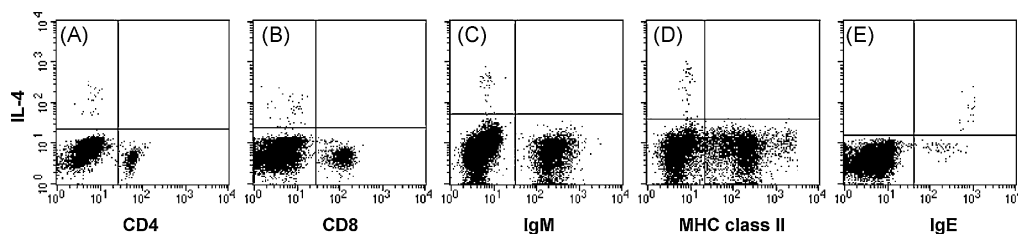


Fig. 2. Neonatal IL-4⁺ cells are IgE⁺/MHCII^{low}/non-T/non-B-cells. Flow cytometric analysis of neonatal IL-4⁺ cells on day 5 after birth. PBMC were cultured in medium containing Brefeldin A for 4 h. The cells were fixed and stained for intracellular IL-4 production and with various cell surface markers for phenotyping: (A) anti-CD4; (B) anti-CD8; (C) anti-IgM; (D) anti-MHC class II; and (E) anti-IgE.

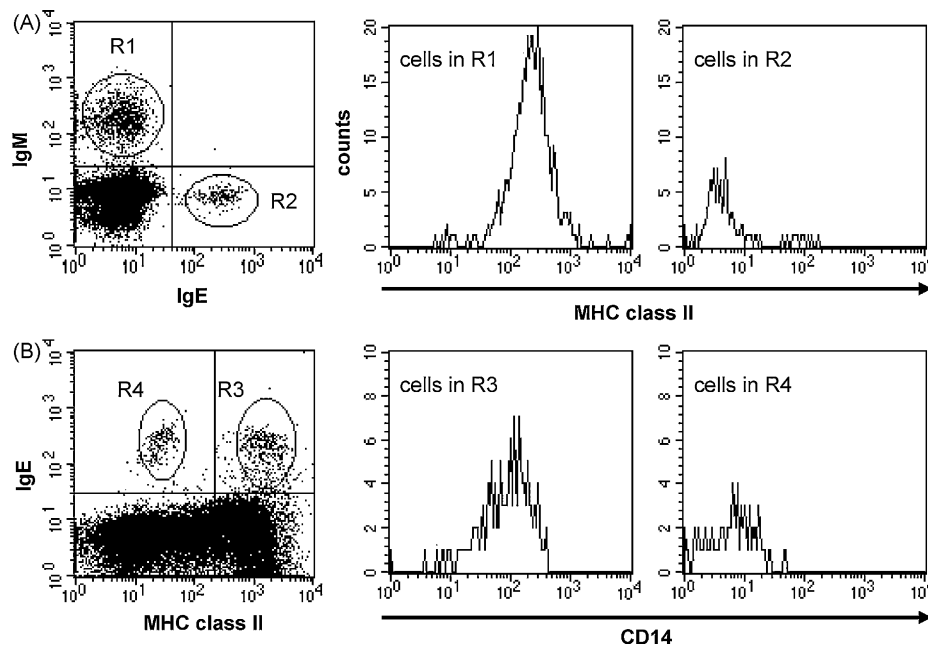


Fig. 3. IgE^+ cells in neonatal PBMC can be distinguished into $\text{MHCII}^{\text{low}}$ and $\text{MHCII}^{\text{high}}$ populations. Phenotyping of IgE^+ cells in PBMC from two foals at day 5 after birth using cell surface staining and flow cytometric analysis. (A) Foal 1: Staining for cell surface IgE and IgM; histograms showing MHC class II staining of cells in R1 ($\text{IgM}^+/\text{IgE}^-$) and of cells in R2 ($\text{IgE}^+/\text{IgM}^-$); (B) Foal 2: cells stained for IgE and MHC class II; histograms showing cells in R4 ($\text{IgE}^+/\text{MHCII}^{\text{low}}$) and cells in R3 ($\text{IgE}^+/\text{MHCII}^{\text{high}}$) stained for CD14.

and 5-day-old foals ($n = 5$) (Fig. 4). The initial depletion step of MHCII^+ cells resulted in the removal of most lymphocytes and monocytes from the PBMC fraction (Fig. 4B). The $\text{MHCII}^{\text{low}}$ fraction was then used for positive selection of IgE^+ cells. Depending on the individual, magnetic cell sorting enriched the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells from around 0.5% in PBMC (see below) to up to 80% in the sorted fraction (Fig. 4D). Differential staining of the sorted cell populations indicated that basophils were highly enriched in the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ fraction (Fig. 4F). Basophil cell counts ranged from 20 to 80% in the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cell fractions. The remaining cells were composed of lymphoid cells, monocytes, and in some samples neutrophils, none of which showed a clear enrichment in the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ fraction. Differences in the cell sorting results between foals and adult horses were not observed. Because of the clear enrichment of basophils in all samples, we concluded that the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ are basophils.

In agreement with the previously reported findings [3], the number of IgE^+ cells in neonates peaked between days 2 and 5 of life and decreased constantly afterwards. At 6 or 12 weeks of age, IgE^+ cells were almost undetectable in the circulation of the foals (Fig. 5A). The same kinetics was observed for $\text{IgE}^+/\text{MHCII}^{\text{low}}$ basophils (Fig. 5B). The higher percentage of total IgE^+ cells in adults compared to neonates ($p = 0.0164$) was the result of a clear population of IgE^+ B-cells in adult horses ($\text{IgE}^+/\text{IgM}^+$ cells; 1.91% (0.34–5.14)) that was reduced in neonates (0.13% (0.02–0.39)). Adult horses and neonates at 2–5 days after birth had similar percentages of $\text{IgE}^+/\text{MHCII}^{\text{low}}$ basophils in their circulation ($p = 0.305$). At 6 and 12 weeks of age the IgE^+ and $\text{IgE}^+/\text{MHCII}^{\text{low}}$ basophils were significantly decreased compared to young foals ($p < 0.0001$). We also analyzed IL-4^+ cells in non-stimulated PBMC from foals at 6 and 12 weeks of life and from adult horses (Table 1). Compared to neonates at days 2–5, significant less IL-4^+ cells were observed at 6 weeks ($p = 0.0011$) and 12 weeks of life ($p = 0.0004$). In adult horses, IL-4^+ cells were not observed in the absence of stimulation ($p < 0.0001$) (Fig. 5C). The similar kinetics of $\text{IgE}^+/\text{MHCII}^{\text{low}}$ and IL-4^+ cells suggested that the production of IL-4 by neonatal basophils was dependent on maternal IgE antibodies on their surface.

3.3. IL-4 secretion in neonatal PBMC and $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells after IgE crosslinking

To investigate whether IL-4 secretion can be induced by crosslinking of maternal IgE antibodies bound to neonatal cells, PBMC from neonates were cultured and stimulated with anti- IgE . IL-4 secretion was detected in supernatants of PBMC from 6 out of 12 neonates at day 5 of age but not in neonatal PBMC obtained before colostrum uptake or cells incubated in medium (Fig. 6A). At day 5 and after binding of maternal IgE to the neonatal cells, IL-4 secretion increased significantly in anti- IgE stimulated PBMC compared to cells in medium ($p = 0.0156$). Anti-equine IgG1 antibodies which were of the same mouse isotype as the anti-equine IgE reagent failed to induce detectable IL-4 secretion in neonatal PBMC.

As described above, basophils are a rare cell population in equine PBMC and we concluded that the anti- IgE induced IL-4 secretion might not have reached detectable concentrations in all samples. To increase the number of neonatal basophils in the samples and to confirm that they are the source of the anti- IgE induced IL-4 , anti- IgE stimulation was performed on enriched $\text{IgE}^+/\text{MHCII}^{\text{low}}$ basophils and MHCII^+ cells from neonates at day 5 after birth (Fig. 6B). Significantly higher IL-4 concentrations were found in the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ basophil fraction than in the $\text{IgE}^-/\text{MHCII}^{\text{low}}$ cell fraction with or without anti- IgE stimulation ($p = 0.002$). IL-4 was also not induced in the stimulated or non-stimulated MHCII^+ fractions.

Aliquots of the sorted $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells from four of the neonates were cultured for 4 h in the presence of Brefeldin A. Afterwards, staining for intracellular IL-4 and cell surface IgE was performed and the cells were measured by flow cytometry (Fig. 6C). The percentages of the enriched $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells ranged from 25 to 56% in these samples. Approximately 20–40% of the enriched IgE^+ cells produced IL-4 in response to anti- IgE stimulation.

In summary, our findings show that crosslinking of maternal IgE antibodies induced IL-4 secretion in neonatal $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells. Flow cytometric analysis, combined with magnetic cell sorting and

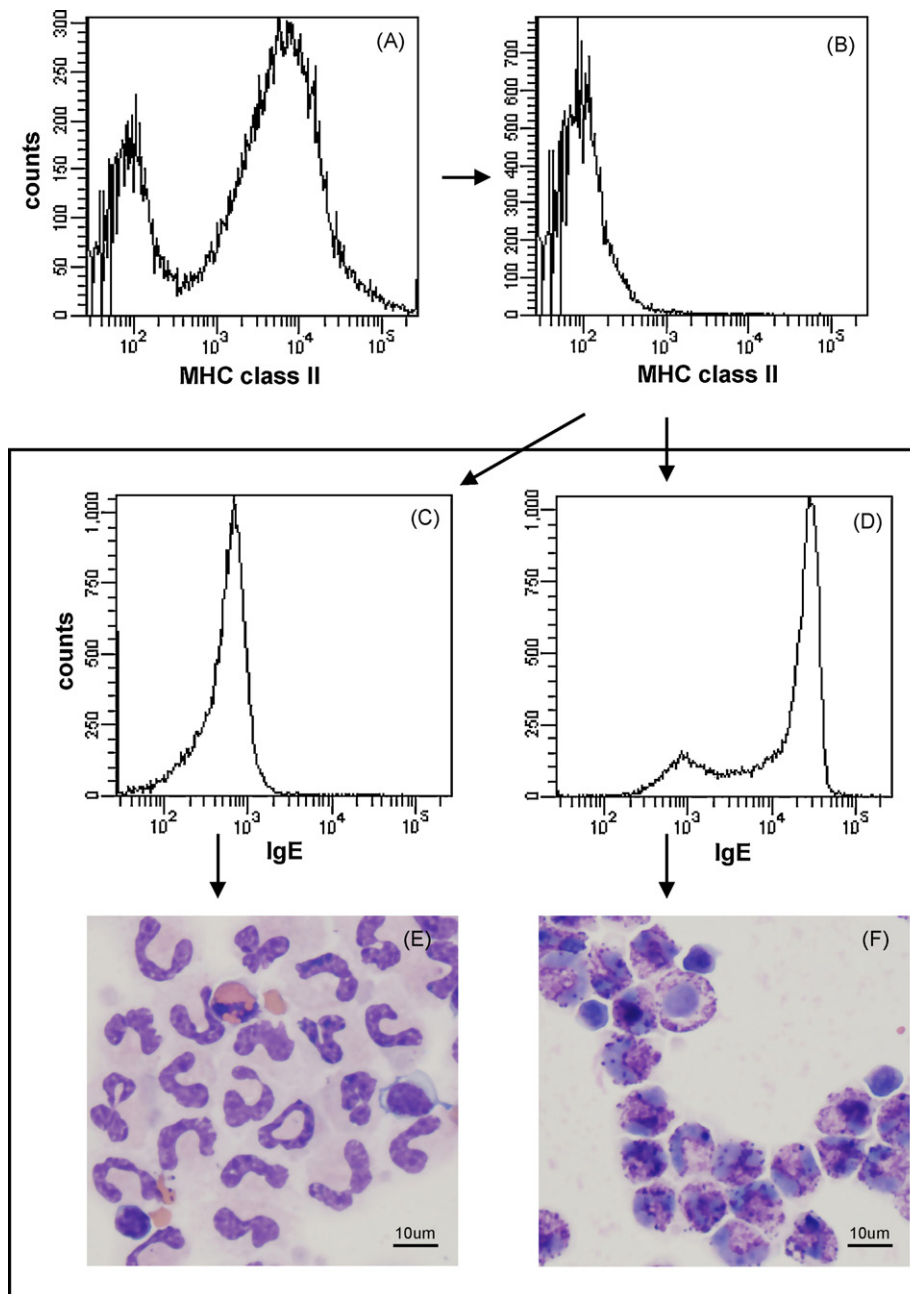


Fig. 4. $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells are basophils. Magnetic cell sorting of $\text{IgE}^+/\text{MHCII}^{\text{low}}$ PBMC. The cell sorting was performed in two steps. First, a depletion of MHC class II positive cells was performed. The MHC class II depleted cell fraction was then used for positive selection of IgE^+ cells. (A) Histograms of equine PBMC stained for MHC class II before cell sorting; (B) MHC class II depleted cell fraction; (C) $\text{IgE}^-/\text{MHCII}^{\text{low}}$ cell fraction; and (D) $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells. (E) Differential cell staining of $\text{IgE}^-/\text{MHCII}^{\text{low}}$ cells; and (F) $\text{IgE}^+/\text{MHCII}^{\text{low}}$ basophils. The sorting was repeated 10 times with PBMC from individual horses. The figure shows a representative result from one horse.

differential staining suggested that the $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells are likely to be basophils.

4. Discussion

IL-4 has multiple immunoregulatory effects on B-cells, T-cells, monocytes, dendritic cells, and non-immune cells including endothelial cells and fibroblasts [35,36]. It is a characteristic cytokine of Th2-cells [37]. In humans and mice, IL-4 was found to be the predominant regulatory cytokine inducing immunoglobulin class switching to IgE [38,39]. Together with its functional synergist IL-13 [34], IL-4 has a major role in the pathogenesis of allergic diseases [10,24] and in the development of protective host immune responses during nematode infection [40].

The general concept of various pattern recognition receptors that are activated by bacterial, viral or fungal pathogens and subsequently result in the induction of Th1 or Th17 immune responses is widely accepted in immunology. The differentiation of naïve T-cells into Th1- or Th17-phenotypes is regulated by dendritic cells producing IL-12 or IL-1 β and IL-6, respectively [41,42]. In contrast, the innate mechanisms and cellular sources leading to the development of Th2-type responses was less well understood. Recent reports provided increasing evidence that basophils are important initial inducers of Th2-cell differentiation during allergic conditions and responses to parasites by providing the early IL-4 that triggers polarization of the Th2 phenotype [24,17,20,25–27,43].

Here, we found that $\text{IgE}^+/\text{MHCII}^{\text{low}}$ cells produce IL-4 early in neonatal development. These IL-4 $^+$ cells were observed *ex vivo* in

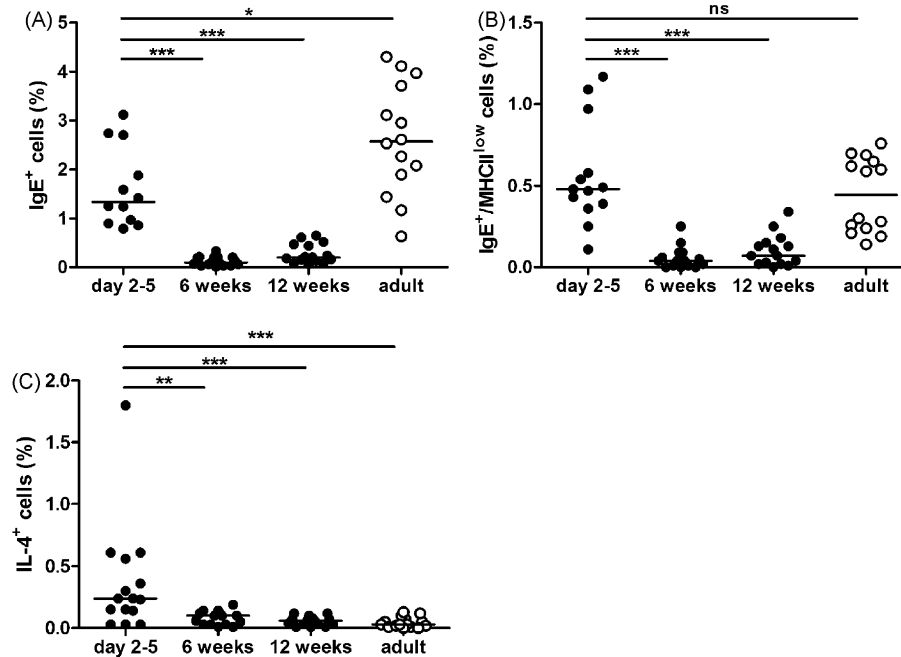


Fig. 5. IgE⁺/MHCII^{low} basophils and IL-4⁺ cells can be detected in PBMC after birth and decline in older foals. IgE⁺ cells were detected in PBMC from neonates, foals and adult horses by cell surface staining and flow cytometric analysis. Neonatal and foal PBMC were obtained at 2–5 days after birth and at 6 and 12 weeks of age. The cells were stained for cell surface bound IgE and for MHC class II expression. (A) Percentages of total IgE⁺ cells and (B) IgE⁺/MHCII^{low} basophils. (C) IL-4⁺ cells which were also found to have an IgE⁺/MHCII^{low} phenotype showed a similar kinetics in neonates and foals but were not detected in adult horses. The bars within the data points represent medians. **p* < 0.05; ***p* < 0.01; ****p* < 0.0001; ns = not significant.

Table 1

IL-4 producing cells (%) from neonates, foals and adults horses measured by intracellular staining and flow cytometric analysis of PBMC.^a

	Foals				Adults
	Birth ^b	Day 5	6 weeks	12 weeks	
<i>N</i>	15	15	15	15	15
Isotype control	0 (0–0.08)	0.04 (0.01–0.05)	0.01 (0–0.27)	0 (0–0.13)	0.01 (0–0.11)
IL-4 ⁺	0.04 (0.01–0.30)	0.24 (0.03–1.80)	0.1 (0.01–0.19)	0.06 (0.01–0.12)	0.03 (0–0.13)

All data represent medians (ranges) of the respective group.

^a The PBMC were cultured in medium with the secretion blocker Brefeldin A for 4 h. Then, they were fixed and intracellular staining was performed.

^b Samples were obtained 12–60 h after birth.

the absence of additional stimulation and appeared in the circulation between days 3 and 5 of life. IL-4⁺ cells were rarely observed in non-stimulated peripheral blood cells of older foals or adult horses. Data reported here and by others supported that the neonatal IgE⁺/MHCII^{low} IL-4 producing cells are likely to be basophils:

First, equine IgE⁺ cells were previously described to be a mixed cell population consisting of basophils, monocytes and lymphocytes [9].

Second, out of these cell populations basophils constitutively expressed the high affinity IgE receptor [12,15,44]. Although antibodies to detect the equine FcεRI are not yet available, it is likely that this receptor is involved in the binding of passively transferred maternal IgE antibodies to neonatal basophils [3]. Third, we identified a IgE⁺/MHCII^{low} cell population in equine PBMC. Out of the equine IgE⁺ cells populations mentioned above, equine lymphocytes and monocytes expressed high levels of MHC class II antigens on their surfaces [30,45,46]. Compared to B-cells or APCs, human and murine basophils expressed low levels of MHC class II. However, these were sufficient for antigen internalization, processing and presentation and the MHC class II-dependent activation of CD4⁺ T-cells [47,48]. Here, we observed a small population of around 0.5% IgE⁺/MHCII^{low} cells in PBMC of neonates and adult horses.

Magnetic cell sorting of the IgE⁺/MHCII^{low} cells clearly enriched basophils to up to 80% in the sorted fraction. Furthermore, the percentages of basophils in the enriched fractions identified by differential staining corresponded to those of IgE⁺ cells detected by flow cytometry (data not shown). Taken all this information together, we concluded that equine IgE⁺/MHCII^{low} cells in peripheral blood are basophils.

Mast cells, basophils and eosinophils were considered to be programmed for IL-4 expression early in ontogeny and IL-4 transcript expression was initiated in a common eosinophil/basophil progenitor in the bone marrow [49]. Murine basophils constitutively expressed IL-4 and IL-13 transcripts. However, IL-4 and IL-13 secretion in basophils required stimulation [21]. We hypothesized that neonatal IL-4⁺ basophils were triggered to produce IL-4 through an IgE-dependent mechanism. Stimulation of neonatal IgE⁺/MHCII^{low} basophils with anti-IgE confirmed that crosslinking of IgE induced IL-4 production by these cells. Nevertheless, the finding was not restricted to neonatal basophils. As expected, treatment of IgE⁺/MHCII^{low} basophils from adult horses with anti-IgE also resulted in IL-4 production by these cells (data not shown).

As mentioned above, IL-4⁺ basophils were also observed *ex vivo* in non-stimulated neonatal PBMC a few days after birth. This suggested that some of the basophils were activated *in vivo* during

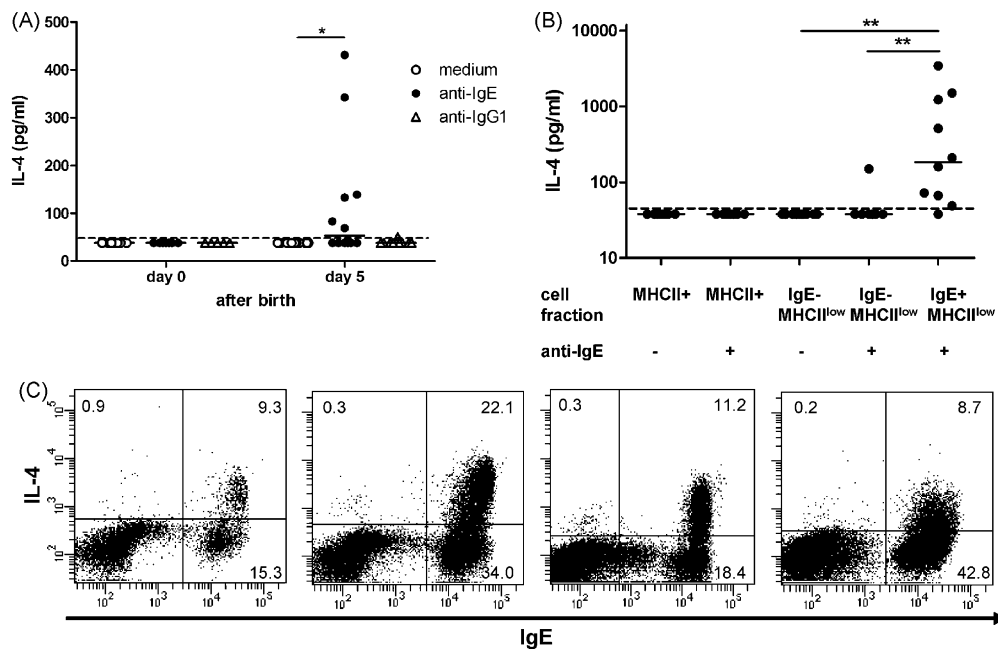


Fig. 6. Stimulation with anti-IgE induces IL-4 production by neonatal basophils. IL-4 production by neonatal PBMC and IgE⁺/MHCII^{low} cells was detected in the cell culture supernatants using a bead-based multiplex assay or by intracellular staining and flow cytometric analysis. (A) PBMC were obtained directly after birth and before colostrum uptake (day 0; $n = 7$) or at 5 days of age (day 5; $n = 12$) and were kept in medium, stimulated with anti-IgE or incubated with an isotype control (anti-equine IgG1) for 48 h. IL-4 was determined in the cell culture supernatants. The bars within the data points represent median values. The dotted line shows the lower limit of detection for the IL-4 assay. $p < 0.05$. (B) IL-4 secretion from neonatal IgE⁺/MHCII^{low} cells. The cell fractions were obtained by magnetic cell sorting of PBMC from neonates at day 5 of life ($n = 10$). The different cell populations were incubated with or without anti-IgE for 48 h. IL-4 secretion was detected in the cell culture supernatants. The bars show medians and the dotted line the lower limit of detection. $**p < 0.01$. (C) IgE⁺/MHCII^{low} cells from four neonates (each of which represents one plot) were cultured for 4 h in the presence of the secretion blocker Brefeldin A. The cells were fixed, stained for intracellular IL-4 production and cell surface IgE antibodies, and measure by flow cytometry. The number indicates percentages in the different quadrants.

the first days of life resulting in the appearance of IL-4⁺ basophils in the circulation. Neonatal basophils are solely equipped with IgE of maternal origin and equine neonates do not produce endogenous IgE for several months [3]. By binding of passively transferred maternal IgE antibodies, neonatal basophils become equipped with the accumulated acquired IgE repertoire of the mare. Thus, a wide variety of antigens could crosslink the maternal IgE on neonatal basophils including parasite or environmental antigens or allergens that previously induced an IgE response in the mare. After birth, the neonate can be exposed to many of these antigens which could subsequently induce IL-4 production in neonatal basophils or mast cells via IgE-mediated FcεRI signaling. If this hypothesis is correct, it also suggests that neonatal basophils equipped with maternal IgE to allergen can provide a paragenetic mechanism to promote the development of allergy in the offspring by induction of the early IL-4 production which then triggers the onset of allergen-specific Th2-cell differentiation. However, the confirmation of this hypothesis requires further experiments and the investigation of the allergen-specific IL-4 response in offspring from allergic mares.

Notably and in contrast to neonates, IL-4⁺ cells were not observed in PBMC from adult horses in the absence of *in vitro* stimulation. This suggested that differences exist in the responsiveness of neonatal and adult basophils to environmental antigens. A recent finding on anti-IgE-mediated histamine release in neonatal and adult basophils showed an impaired mediator release in neonatal cells [50]. This, together with the increased IL-4 production in neonatal compared to adult basophils, suggests to age-dependent differences in the responsiveness of basophils to IgE-mediated activation.

Horses provide a unique opportunity to study the influences of maternal IgE antibodies on Th2-cell differentiation and the development of allergen-specific immune responses in neonates.

In the horse, maternal immunity is exclusively transferred with the colostrum and all activating or regulatory immune mechanisms induced by maternal immune components are taking place after birth. This is different from many other species, such as humans, non-human primates or rodents, where the transfer of maternal immunity starts during pregnancy. In humans, most atopic disorders develop in childhood and several studies have described the onset of immune responses to environmental antigens *in utero* [51–53]. This also suggested that maternal non-genetic factors may influence the susceptibility to allergy during the gestational and neonatal period.

Maternal antibodies are well known as important mediators of passive immunity providing the offspring with protection against infections until its own immune system produces sufficient amounts of antibodies [54]. In addition, maternal IgG antibodies have various active immunostimulatory functions which have inductive effects on the neonatal immune system. Although the direct effects of maternal IgG antibodies are restricted to a short period early in the neonatal life, their determinative influences are believed to last life-long [55]. Many of the effects of maternal IgG antibodies were found to be mediated by the formation of anti-idiotypic antibodies in the neonate, influencing the development of the neonatal T- and B-cell repertoire, the magnitude of immune responses, and antimicrobial protection in the offspring [56–59]. Similarly, the early IL-4 production mediated by maternal IgE antibodies in neonates may contribute to resistance against parasites or susceptibility to allergic diseases later in life.

In summary, this is the first report on the induction of IL-4 in neonatal basophils by maternal IgE. Stimulation of neonatal basophils equipped with maternal IgE antibodies is likely to provide the innate IL-4 signal that promotes the differentiation of Th2-cells early in life.

Conflict of interest

The authors have no financial or commercial conflicts of interest.

Acknowledgements

We would like to thank Ms. Carol Collyer, the crew of the Equine Park at Cornell University and Dr. Alexandra Burton for their help with obtaining the samples for this project. We also thank Ms. Julie Hillegas, Bronwen Childs, and Esther Kabithe for excellent technical assistance. Funding for this project was provided by the Dean's Fund for Clinical Research and the Harry M. Zweig Fund for Equine Research at Cornell University.

References

- [1] Duchon K, Bjorksten B. Total IgE levels in human colostrum. *Pediatr Allergy Immunol* 1996;7:44–7.
- [2] Thatcher EF, Gershwin LJ. Colostral transfer of bovine immunoglobulin E and dynamics of serum IgE in calves. *Vet Immunol Immunopathol* 1989;20:325–34.
- [3] Wagner B, Flaminio JBF, Hillegas J, Leibold W, Erb HN, Antczak DF. Occurrence of IgE in foals: evidence for transfer of maternal IgE by the colostrum and late onset of endogenous IgE production in the horse. *Vet Immunol Immunopathol* 2006;110:269–78.
- [4] Marti E, Ehrensperger F, Burger D, Ousey J, Day MJ, Wilson AD. Maternal transfer of IgE and subsequent development of IgE responses in the horse (*Equus caballus*). *Vet Immunol Immunopathol* 2009;127:203–11.
- [5] Allen WR, Stewart F. Equine placentation. *Reprod Fertil Dev* 2001;13:623–34.
- [6] Jeffcott LB. Passive immunity and its transfer with special reference to the horse. *Biol Rev* 1972;47:439–64.
- [7] McGuire TC, Crawford TB, Hallowell AL, Macomber LE. Failure of colostral immunoglobulin transfer as an explanation for most infections and death of neonatal foals. *J Am Vet Med Assoc* 1977;170:1302–4.
- [8] Sheoran AS, Timoney JF, Holmes MA, Karzenski SS, Crisman MV. Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. *Am J Vet Res* 2000;61:1099–105.
- [9] Wagner B, Radbruch A, Rohwer J, Leibold W. Monoclonal anti-equine IgE antibodies with specificity for different epitopes on the immunoglobulin heavy chain of native IgE. *Vet Immunol Immunopathol* 2003;92:45–60.
- [10] Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255–81.
- [11] Seder RA, Paul WE, Dvorak AM, Sharkis SJ, Kagey-Sobotka A, Niv Y, et al. Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. *Proc Natl Acad Sci USA* 1991;88:2835–9.
- [12] Turner H, Kinet JP. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* 1999;402(6760 Suppl):B24–30.
- [13] MacGlashan Jr D, Schroeder JT. Functional consequences of Fc epsilonRIalpha up-regulation by IgE in human basophils. *J Leukoc Biol* 2000;68:479–86.
- [14] Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol* 2000;12:624–31.
- [15] Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2002;2:773–86.
- [16] Haisch K, Schramm G, Falcone FH, Alexander C, Schlaak M, Haas A. A glycoprotein from *Schistosoma mansoni* eggs binds non-antigen-specific immunoglobulin E and releases interleukin-4 from human basophils. *Parasite Immunol* 2001;23:427–34.
- [17] Min B, Prout M, Hu-Li J, Zhu J, Jankovic D, Morgan ES, et al. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J Exp Med* 2004;200:507–17.
- [18] Mitre E, Taylor RT, Kubofcik J, Nutman TB. Parasite antigen-driven basophils are a major source of IL-4 in human filarial infections. *J Immunol* 2004;172:2439–45.
- [19] Gibbs BF. Human basophils as effectors and immunomodulators of allergic inflammation and innate immunity. *Clin Exp Med* 2005;5:43–9.
- [20] Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced Thelper type 2 responses. *Nat Immunol* 2008;9:310–8.
- [21] Gessner A, Mohours K, Mohours M. Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. *J Immunol* 2005;174:1063–72.
- [22] Bieneman AP, Chichester KL, Chen YH, Schroeder JT. Toll-like receptor 2 ligands activate human basophils for both IgE-dependent and IgE-independent secretion. *J Allergy Clin Immunol* 2005;115:295–301.
- [23] Sloane DE, Tedla N, Awoniyi M, Macglashan Jr DW, Borges L, Austen KF, et al. Leukocyte immunoglobulin-like receptors: novel innate receptors for human basophil activation and inhibition. *Blood* 2004;104:2832–9.
- [24] Falcone FH, Zillikens D, Gibbs BF. The 21st century renaissance of the basophil? Current insights into its role in allergic responses and innate immunity. *Exp Dermatol* 2006;15:855–64.
- [25] Kojima T, Obata K, Mukai K, Sato S, Takai T, Minegishi Y, et al. Mast cells and basophils are selectively activated in vitro and in vivo through CD200R3 in an IgE-independent manner. *J Immunol* 2007;179:7093–100.
- [26] Mack M, Schneider MA, Moll C, Cihak J, Brühl H, Ellwart JW, et al. Identification of antigen-capturing cells as basophils. *J Immunol* 2005;174:735–41.
- [27] Oh K, Shen T, Le Gros G, Min B. Induction of Th2 type immunity in a mouse system reveals a novel immunoregulatory role of basophils. *Blood* 2007;109:2921–7.
- [28] Wagner B, Hillegas JM, Antczak DF. A monoclonal antibody to equine interleukin 4. *Vet Immunol Immunopathol* 2006;110:363–7.
- [29] Wagner B, Glaser A, Hillegas JM, Erb HN, Gold C, Freer H. Monoclonal antibodies to equine IgM improve the sensitivity of West Nile virus-specific IgM detection in horses. *Vet Immunol Immunopathol* 2008;122:46–56.
- [30] Barbis DP, Bainbrigde D, Crump AL, Zhang CH, Antczak DF. Variation in expression of MHC class II antigens on horse lymphocytes determined by MHC haplotype. *Vet Immunol Immunopathol* 1994;42:103–14.
- [31] Pedersen LG, Castelruiz Y, Jacobsen S, Aasted B. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Vet Immunol Immunopathol* 2002;88:111–22.
- [32] Wagner B, Robeson J, McCracken M, Wattrang E, Antczak DF. Horse cytokine/IgG1 fusion proteins – mammalian expression of biologically active cytokines and a system to verify antibody specificity to equine cytokines. *Vet Immunol Immunopathol* 2005;105:1–14.
- [33] Wagner B, Freer H. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. *Vet Immunol Immunopathol* 2009;127:242–8.
- [34] Kuperman DA, Schleimer RP. Interleukin-4, interleukin-13, signal transducer and activator of transcription factor 6, and allergic asthma. *Curr Mol Med* 2008;8:384–92.
- [35] Chomarat P, Banchereau J. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int Rev Immunol* 1998;17:1–52.
- [36] Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 2005;23:749–86.
- [37] Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;7:145–73.
- [38] Siebenkotten G, Esser C, Wabl M, Radbruch A. The murine IgG1/IgE class switch program. *Eur J Immunol* 1992;22:1827–34.
- [39] Stavnezer J, Amemiya CT. Evolution of isotype switching. *Sem Immunol* 2004;16:257–75.
- [40] Finkelman TD, Shea-Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden KB, et al. Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu Rev Immunol* 1997;15:505–33.
- [41] Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003;3:133–46.
- [42] Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin-17 producing human T helper cells. *Nat Immunol* 2007;8:630–8.
- [43] Min B, Le Gros G, Paul WE. Basophils: a potential liaison between innate and adaptive immunity. *Allergol Int* 2006;55:99–104.
- [44] Mukai K, Matsuoka K, Taya C, Suzuki H, Yokozeki H, Nishioka K, et al. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 2005;23:191–202.
- [45] Crepaldi T, Crump A, Newman M, Ferrone S, Antczak DF. Equine T lymphocytes express MHC class II antigen. *J Immunogenet* 1986;13:349–60.
- [46] Lunn DP, Holmes MA, Duffus WP. Equine T-lymphocyte MHC II expression: variation with age and subset. *Vet Immunol Immunopathol* 1993;35:225–38.
- [47] Yoshimoto T, Yasuda K, Tanaka H, Nakahira M, Imai Y, Fujimori Y, et al. Basophils contribute to Th2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol* 2009;7:706–12.
- [48] Perrigoue JG, Saenz SA, Siracusa MC, Allenspach EJ, Taylor BC, Giacomin PR, et al. MHC class II-dependent basophil-CD4+ T-cell interactions promote Th2 cytokine-dependent immunity. *Nat Immunol* 2009;7:697–705.
- [49] Cyr MM, Denburg JA. Systemic aspects of allergic disease: the role of the bone marrow. *Curr Opin Immunol* 2001;13:727–32.
- [50] Wagner B, Miller WH, Erb HN, Lunn DP, Antczak DF. Sensitization of skin mast cells with IgE antibodies to Culicoides allergens occurs frequently in clinically healthy horses. *Vet Immunol Immunopathol* 2009;132:53–61.
- [51] Jones AC, Miles EA, Warner JO, Colwell BM, Bryant TN, Warner JA. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. *Pediatr Allergy Immunol* 1996;7:109–16.
- [52] Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses towards the Th2 cytokine profile. *J Immunol* 1998;160:4730–7.
- [53] Warner JA, Jones CA, Jones AC, Warner JO. Prenatal origins of allergic disease. *J Allergy Clin Immunol* 2000;105:S493–8.

- [54] Zinkernagel RM. On natural and artificial vaccinations. *Annu Rev Immunol* 2003;21:515–46.
- [55] Lemke H, Coutinho A, Lange H. Lamarckian inheritance by somatically acquired maternal IgG phenotypes. *Trends Immunol* 2004;25:180–6.
- [56] Martinez C, Toribio ML, De la Hera A, Cazenave PA, Coutinho A. Maternal transmission of idiotypic network interactions selecting available T cell repertoires. *Eur J Immunol* 1986;16:1445–7.
- [57] Elliott M, Kearney JF. Idiotypic regulation of development of the B-cell repertoire. *Ann NY Acad Sci* 1992;651:336–45.
- [58] Montesano MA, Colley DG, Freeman Jr GL, Secor WE. Neonatal exposure to idio type induces *Schistosoma mansoni* egg antigen-specific cellular and humoral immune responses. *J Immunol* 1999;163:898–905.
- [59] Lemke H, Lange H. Generalization of single immunological experiences by idiotypically mediated clonal connections. *Adv Immunol* 2002;80:203–41.