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Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken

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ABSTRACT

The intestinal mucosa is of major importance for immune development. To further study the ontogeny of avian mucosal immunity, mRNA levels of IgM, IgY and IgA, the polymeric immunoglobulin receptor (pIgR) and a number of cytokines were determined at different ages in jejunum and ileum of non-immunized healthy juvenile layer chickens. Immunoglobulin genes were successively expressed in jejunum and ileum. IgM expression was maximal in week 1, IgY expression peaked in week 5, and IgA expression was most dominant after week 7 post hatch. PIgR gene expression was relatively low in the first 2 weeks post hatch, but increased thereafter. Generally, increased expression levels of IL-1, IL-10, IL-12p40, iNOS and interferon-γ mRNA levels were found between days 14–42 as compared to days 3 and 49–70 post hatch ($p < 0.05$). Correlation was found between IgA and IL-10, TGF-β and IFN-γ expression levels on days 21, 28 and 35. Cytokine mRNA expression levels decreased to basal levels between 49 and 70 days post hatch, whereas IgA reached its maximum levels in this period. Based on the current results, we hypothesize that chicken sIgA, as mammalian sIgA, may contribute to the maintenance of intestinal homeostasis.

1. Introduction

Hatch of chicken, like birth in mammals, is at the centre of a critical window of development. In this window, immunity of newborns transit from dependence on innate and maternal immunity to an increasing dependence on endogenous adaptive immunity (Butler et al., 2006). Studies in germ free and gnotobotic animals show that commensal bacteria and microbial products such as lipopolysaccharide (LPS) and beta-glucans are required for adequate activation of adaptive immune functions (Björksten, 2004; Tlaskalová-Hogenová et al., 2004; Hrncir et al., 2008). The intestinal mucosa plays a central role in this initial immune activation and subsequent maturation of regulation (Hrncir et al., 2008). In addition, epidemiological and experimental studies provide increasing evidence that 'education' of the immune system takes place early in life and is dependent on the composition of the intestinal microbiota (Bedford Russell and Murch, 2006). Disturbance of this bacterial ecosystem early in life, for example by antibiotics, may influence the functionality of the immune system later in life (Bedford Russell and Murch, 2006). Consequently, during the juvenile period the immune system can be modulated by application of immunomodulatory strategies such as pre/probiotics or dietary components. Knowledge of (mucosal) immune development in the juvenile period is a prerequisite for the search for novel immunomodulatory components and strategies.

The intestinal mucosal immune system of higher vertebrates is, among other things, characterized by production of large amounts of secretory IgA (sIgA). In mammals, 80% of all plasma cells are found in the intestinal lamina propria and most of these plasma cells produce polymeric IgA (pIgA) (Johansen and Brandtzaeg, 2004). This antibody isotype is unique for mucosal tissues and is held to be responsible for the defence of the mucosa against pathogenic microorganisms (Macpherson et al., 2008). In addition, sIgA is thought to play a role in the maintenance of mucosal homeostasis (Cerutti and Rescigno, 2008; Johansen et al., 1999; Suzuki et al., 2007) and may also influence development of systemic immunity (Favre et al., 2005). Furthermore, sIgA may also determine the composition of the intestinal microbiota (Suzuki et al., 2007). The major cytokine signal for IgA class switch recombination in mammals is transforming growth factor beta (TGF-β) with contributions from interleukins (IL)-2, -4, -6 and -10 (Macpherson et al., 2008). Other costimulatory molecules for sIgA are nitric oxide and the

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Table 1
Primer sequences, corresponding accession numbers and sizes of the amplification products.

Gene	Access no.	Primer sequences 5′ → 3′	Product size (bp)
28S	DQ018756	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	62
IL-1	AJ245728	F: CAGCAGCCTCAGCGAAGAG R: CTGTGGTGTGCTCAGAATCCA	86
IL-2	AF033563	F: TTCAAAATATCGAAAAGAACCTCAAG R: CGGTGTGATTTAGACCCGTAAGAC	51
IL-4	AJ621249	F: GTGCCCACGCTGTGCTTAC R: AGGAAACCTCTCCCTGGATGTC	82
IL-10	AJ621614	F: CGCTGTCACCGCTTCTTCA R: TCCCGTTCTCATCCATCTTCTC	88
IFN-γ	Y07922	F: GTGAAGAAGGTGAAAGATATCATGGA R: GCTTTGCGCTGGATTCTCA	71
TGF-β4	M31160	F: ACCTCGACACCGACTACTGCTT R: ATCCTTGCGGAAGTCGATGT	86
IgA	S40610	F: GTCACCGTCACCTGGACTACA R: ACCGATGGTCTCCTTCACATC	192
IgY	X07174.1	F: ATCACGTCAAGGGATGCCCG R: ACCAGGCACCTCAGTTTGG	118
IgM	X01613.1	F: GCATCAGCGTCACCGAAAGC R: TCCGCACTCCATCCTCTTGC	98
PIgR	AY233381	F: GGATCCGACGTGCAGATCCAGCTCCTTCGT R: TCACCATCATCGACTTCCCAGAGCAGG	247
INOS	U46504	F: TGGGTGGAAGCCGAAATA R: GTACCAGCCGTTGAAAGGAC	241

tumor necrosis family (TNF) members APRIL (A proliferation inducing ligand) and BAFF (B cell activating factor) (Macpherson et al., 2008).

In mammals, pIgA is actively transcytosed across the mucosal epithelium to external secretions. This active transport is mediated by the polymeric immunoglobulin receptor (pIgR). Upon transport of pIgA the extracellular secretory component (SC) of pIgR is digested and released into the lumen, complexed with IgA. In this format, IgA is thought to be protected against degradation by proteases and pH fluctuations in the gut. Mammalian pIgR has been studied extensively (reviewed by Kaetzel, 2005). PIgR is a glycoprotein expressed on the basolateral surface of secretory epithelial cells of microvilli and crypts of the intestinal tract. In rodents, pIgR is also expressed by hepatocytes, enabling biliary transport of pIgA (Huling et al., 1999). Since endogenous IgA is produced at relatively low levels immediately after birth (Pérez-Cano et al., 2005), sIgA in neonates is obtained from colostrum and milk. PIgR expression is regulated by the cytokines IL-1, IL-4, TNF-α and interferon-γ, and various hormones (Hempen et al., 2002; Kaetzel, 2005). In addition, conserved microbial molecules like lipopolysaccharide (LPS) probably influence pIgR expression via TLR signalling (Schneeman et al., 2005).

The existence of a secretory immune system in aves has been established for long (Watanabe and Kobayashi, 1974). In contrast to mammalian pIgR, chicken pIgR lacks the second immunoglobulin-binding domain, but the conserved immunoglobulin-binding regions in the first domain are present (Wieland et al., 2004). Like in mammals, chicken pIgR bind pIgA, suggesting a similar role for chicken pIgR in mucosal immunity (Wieland et al., 2004, 2006).

Development of intestinal immunity in juvenile chicken has been studied before (Bar-Shira et al., 2003; Jeurissen et al., 1989). However, knowledge of the kinetics of IgA and pIgR production in relation to production of IgM and IgY and concurrent cytokine expression during the juvenile period is limited. Moreover, studies regarding expression of immune related genes in the small intestine are limited to the first 2 weeks post hatch (p.h.) (Bar-Shira et al., 2003). Therefore, this study aimed to extend existing knowledge of mucosal immune development in the chicken small intestine. The results show that the intestinal immune system between 2 and 6 weeks post hatch has a highly dynamic nature.

2. Materials and methods

2.1. Chickens

In this study two different experiments were performed. Experiment 2 was done to confirm and extend the results obtained from experiment 1. For experiment 1, eggs were collected from artificially inseminated hens of a random-bred line of an ISA Brown Warren cross (medium heavy layers) kept at our department for 26 years. The eggs were incubated at 41 °C and 55% humidity. For experiment 2, 1-day-old chicks (Lohmann Brown) were derived from a commercial hatchery and kept in cages in groups of five chicks. Chicks in both experiments obtained ad lib. standard commercial starter feed for laying hens (without antimicrobials) and drinking water. The chicks were not vaccinated. Cross-sections from jejunum (midsection between meckels diverticulum and duodedum) and ileum (midsection between meckels diverticulum and ceco-ileac junction) were collected from 5 chicken per time point on days 3, 7, 14, 28 and 49 p.h. (experiment 1), or from 10 chicken per time point on days 4, 7, 10, 14, 18, 21, 28, 35, 49 and 70 p.h. (experiment 2). At days 3 and 49 also duodenum, liver, heart, thymus and Bursa of Fabricius were collected in experiment 1. Small sections (50–100 mg) of tissue were immediately snap frozen in liquid nitrogen and stored until use at −80 °C.

2.2. Immunohistochemistry

For detection of IgA positive plasma cells in jejunum and duodenum, frozen tissue sections (7 μm) were incubated with 1:500 diluted goat anti-chicken-IgA-HRPO (Bethyl Laboratories, Montogomery, USA). Tissue sections were stained with 3-amino-4-ethyl-carbazole (AEC) in NaAc (pH 4.8) and counter stained with haematoxilin.

2.3. RNA isolation

Total RNA extraction was performed using TRIzol® reagent (Lifetechnologies, Breda, The Netherlands) according to the manufacturer's recommendations. RNA concentrations were determined

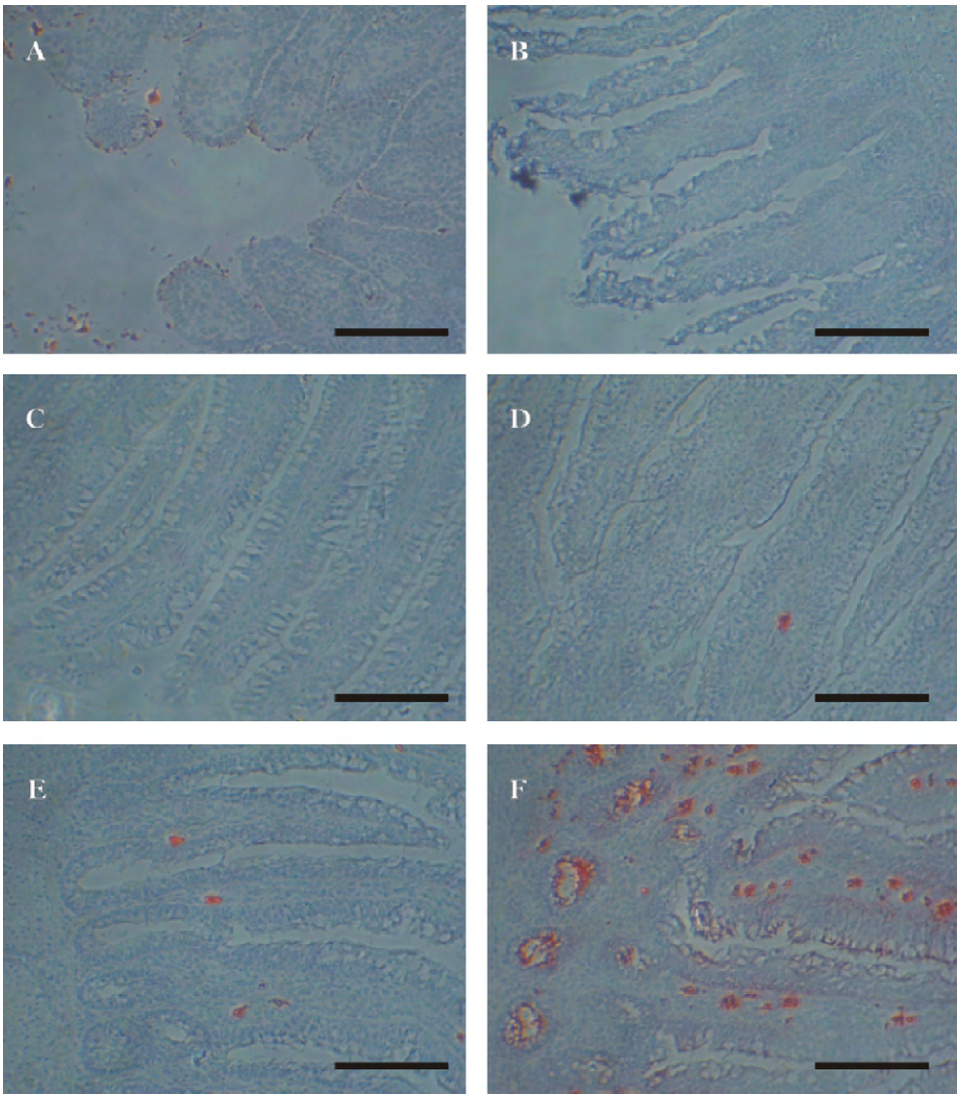

Fig. 1. Immunohistochemical staining of jejunum for IgA positive cells. Frozen sections of jejunum (7 μm thick) of day 18 embryos (A), day 3 (B), day 7 (C), day 14 (D), day 28 (E) and day 49 (F) p.h. were stained with polyclonal HRPO conjugated anti-chicken-IgA serum (1:500 dilution). IgA positive plasma cells are visible as red dots. Bar is 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by spectrophotometric analysis and equalized by dilution in DEPC water. The integrity of the isolated RNA was checked by agarose gel electrophoresis. RNA was stored at −70 °C until use.

2.4. DNase treatment and first strand cDNA synthesis

One microgram of total RNA was treated with 1 μl DNase I (Invitrogen, Breda, The Netherlands, 18068-015), and incubated for 15 min at room temperature in a total volume of 10 μl. DNase was inactivated by adding 1 μl 25 mM EDTA and incubation at 65 °C for 10 min. Next, 300 ng random hexamers, 1 μl 10 mM dNTP mix, 4 μl 5 × first strand buffer, 2 μl 0.1 M DTT and 10 U RNase inhibitor (Invitrogen, Breda, The Netherlands, 15518-012) were added and the mixture was incubated for 10 min at 37 °C. To each sample 200 U Superscript RNase H^- reverse transcriptase (RT; Invitrogen, Breda, The Netherlands, 18053-017) was added and the samples were incubated for 50 min at 37 °C. All reactions were filled up with demineralized water to a total volume of 1 ml and stored at–20 °C for future use.

2.5. Real-time quantitative PCR

For qRT-PCR, 5 μl cDNA and forward and reverse primer (500 nm each) were added to 12.5 μl SYBR® Green PCR Master Mix (Applied BioSystems, Nieuwerkerk ad IJssel, The Netherlands) and filled up with demineralized water to a final volume of 25 μl. The primer sequences used for qRT-PCR are listed in Table 1. All primer sets allowed DNA amplification efficiencies between 94% and 100%. RQ-PCR (10 min 95 °C, 40 cycles of 15 s 94 °C, 30 s 59 °C and 36 s 72 °C, followed by 15 s at 95 °C, 1 min at 59 °C) was carried out on an Applied BioSystems 7500 real-time PCR machine (Applied BioSystems, The Netherlands). After each run, melting curves were collected by detecting the fluorescence from 60 to 90 °C at 1 °C intervals. Amplification efficiencies for the gene of interest and the internal standard (28S) were checked using 10-fold dilutions of the plasmid vector pGEMTeasy containing the gene of interest. In this study, only primer combinations that resulted in amplification efficiencies above 90% were used. Relative expression was expressed as $2^{-\Delta Ct}$ (Applied Biosystems user Bulletin #2; ABI prism 7700 detection system, 2001).

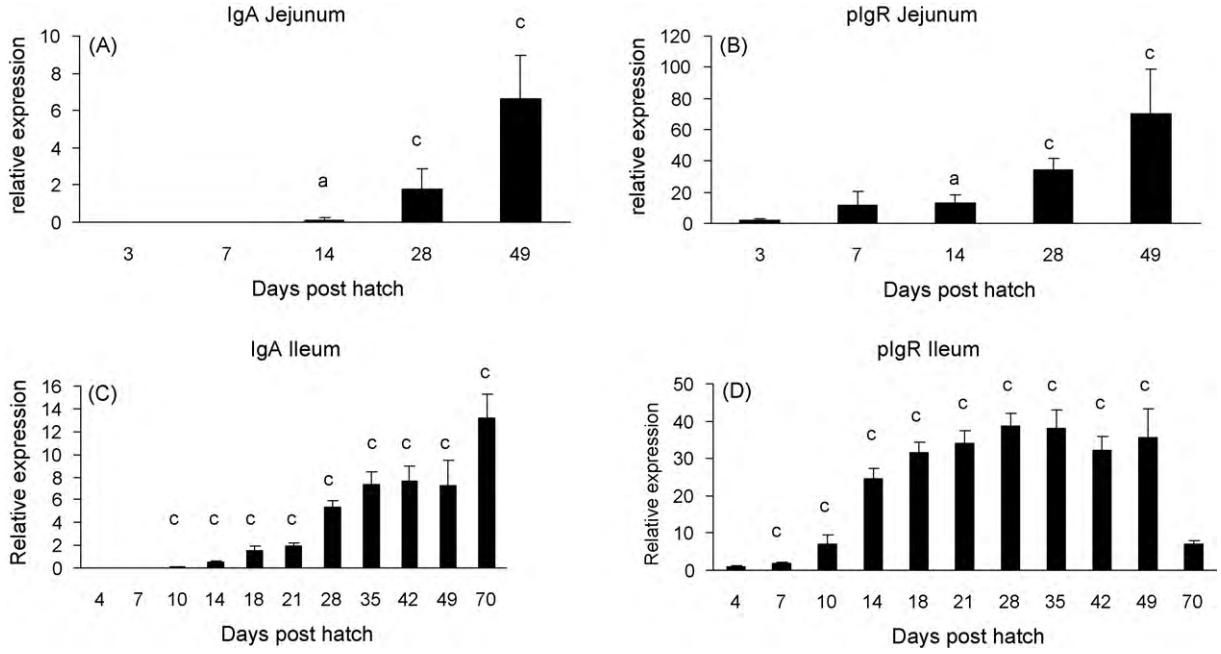

Fig. 2. qRT-PCR results: Relative mRNA expression levels of IgA (A, jejunum and C, ileum) and pIgR (B, jejunum and D, ileum) in juvenile chickens. Results at each time point are the averages of individual animals ($n = 5$ for jejunum and $n = 10$ for ileum). Expression values are standardized using 28S as an internal standard. Within each experimental period, relative expression levels that differ significantly from day 3 are indicated with superscripts above the bars ($a = p < 0.05$; $c = p < 0.001$).

2.6. Statistics

All qRT-PCR data were log transformed to obtain normal distribution of the data. Expression levels were tested ($N = 5$/time point for experiment 1 and $N = 10$/time point for experiment 2) for significant difference from days 3 to 49 (experiment 1) or days 4 to 70 (experiment 2) using the GLM procedure of SAS (SAS Institute, 2004). Mean differences were adjusted with Bonferoni's test for multiple comparisons. Spearman correlations between IgA and cytokine expression levels were calculated for each time point.

3. Results

3.1. Postnatal increase of IgA positive plasma cell numbers in jejunum

Frozen sections of jejunum and duodenum from chicken of experiment 1 were immunohistochemically stained to investigate the appearance of IgA positive plasma cells in the small intestine during the neonatal period. Tissue sections, collected at 18 days of egg incubation and 3, 7, 14, 28 and 49 days p.h., were incubated with HRPO labelled anti-chicken-IgA to visualize IgA positive cells. IgA positive cells were absent in lamina propria of jejunum before 14 days p.h., but cell numbers started to increase from this time point on, with maximal numbers at 49 days p.h. (Fig. 1). The cytoplasm of the positive cells was clearly stained, indicating their nature as IgA producing plasma cells. In addition, the intestinal crypts of 49-day-old chicken were also positively stained using HRPO labelled anti-chicken-IgA antibody. Identical staining patterns were visible in duodenum sections (results not shown). These results indicate that local production of IgA in the chicken small intestine is relatively low before the age of 4 weeks.

3.2. IgA and pIgR mRNA levels in jejunum and ileum increase after 10–14 days post hatch

Quantitative PCR was applied to determine development of IgA and pIgR mRNA expression levels in jejunum during neonatal life (Fig. 2A and B). During this period, we observed a steady increase of the levels of IgA and pIgR, with the levels of pIgR preceding the levels of IgA mRNA. To confirm and extend this initial study, a second experiment was done using more time points and a different location in the small intestine was sampled (Fig. 2C and D). The kinetics of IgA mRNA expression were similar both in jejunum and ileum (Fig. 2A and C). In both intestinal segments expression levels started to increase significantly ($p < 0.05$) between 7 and 14 days p.h. On day 49, relative IgA expression levels increased 2200-fold in jejunum and 3100-fold in ileum compared to expression levels on days 3 and 4, respectively. In ileum, IgA mRNA expression increased further between 49 and 70 days ($p < 0.05$). The results of IgA mRNA expression were in accordance with the immunohistochemical results shown in Fig. 1.

Like IgA expression, average pIgR mRNA levels in jejunum (experiment 1), as well as in ileum (experiment 2) started to increase significantly ($p < 0.05$) between 7 and 14 days post hatch (Fig. 2B and D). The pIgR expression levels on day 49 were almost 30-fold (jejunum) and 45-fold (ileum) increased as compared to expression levels on day 3 or 4, respectively.

Previously, we found with Northern blot experiments that pIgR is also expressed in liver, Bursa of Fabricius and thymus of adult chicken [18]. In the current study, these results are confirmed with qRT-PCR, except for the thymus where only background levels of mRNA were detected (Fig. 3). The pIgR mRNA expression levels in liver and bursa were, like in the intestine, low at day 3, but were strongly increased at 49 days p.h.

3.3. In ileum of juvenile chicken the three different antibody isotypes are successively expressed

Relative mRNA expression levels of IgM, IgY and IgA at different time points are shown in Fig. 4A–C. First, IgM expression was maximal at day 7, decreased strongly until day 21, peaked again at day 35, after which expression levels decreased below 20%. Second, IgY expression was minimal on days 4–10, after which expression increased until day 18 and after a small dip peaked on day 35 and decreased to levels below 10% at 70 days p.h. Finally, IgA expression

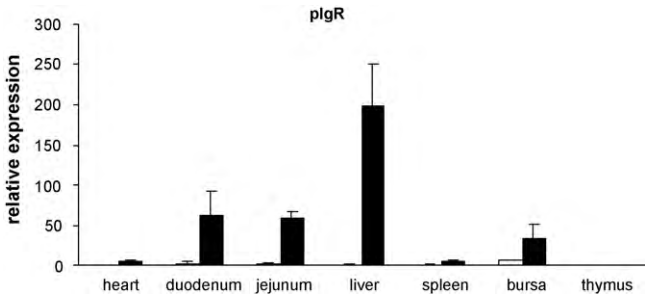

Fig. 3. Relative pIgR expression in different organs at 3 days (white bars) and 49 days (gray bars) p.h. as determined with qRT-PCR.

started to increase after 10 days p.h. and reached maximal levels at 70 days p.h. Expression levels of all three isotypes are at minimal values at 21 days p.h. In Fig. 4D the relative expression as percentage of the maximum expression of that isotype are represented. This figure shows clearly the successive expression in the intestine of the three different isotypes.

To get more insight in the temporary changes in isotype expression in the mucosal tissue, the relative contribution of each isotype (IgM, IgY and IgA) to the total immunoglobulin mRNA expression is shown in Fig. 4E. IgM is the dominant isotype until day 10. From days 14 to 21, IgY mRNA is most abundant. From 21 to 35 days the contribution of IgY and IgA is more or less equal and after 35 days IgA becomes the most dominant isotype, whereas IgM and IgY mRNA expression is minimal during this period.

3.4. Cytokine expression levels are generally enhanced between 2–6 weeks p.h.

A relation between changes in cytokine mRNA expression profiles and dynamics of isotype expression in the small intestine of juvenile chickens was studied. Therefore, the relative expression levels of various cytokines were determined in jejunum and ileum at various time points (Figs. 5 and 6, respectively). In general, cytokine mRNA levels of IL-1, IL-2, IL-4, IL-10, IFN-γ and TGF-β were measured in jejunum and ileum. Additional measurements of iNOS and IL-12p40 were performed with tissue from ileum. At both intestinal sites similar trends in cytokine expression pattern became visible. In both jejunum and ileum, we observed strong changes in cytokine expression, with minimal expression levels before 10 and after 42 days. Between 2 and 6 weeks of age expression levels of IL-1, IL-10, IL-12p40, IFN-γ, iNOS and to a lesser extent IL-2, were generally higher than on day 3 or 4 and 49 and/or 70 p.h. ($p < 0.05$). In contrast to these cytokines, the expression levels of IL-4 and TGF-β were not enhanced in weeks 2–6, except for TGF-β at day 28. The results show that expression of (pro-inflammatory) cytokines returns to basal levels when sIgA expression reached maximal levels (Figs. 5 and 6). A correlation analysis was done to find a possible functional link between IgA and concurrent cytokine expression levels. IgA expression levels correlated positively to IFN-γ expression levels on days 7, 21, 28 and 35 ($p < 0.05$) and to TGF-β expression levels on days 7, 21, 35 and 49 as well ($p < 0.05$). IgA and IL-10 expression levels tended to correlate on days 28 ($p < 0.1$), and 35 ($p \leq 0.05$). Other correlations between IgA and cytokine expression levels were only found on day 7 for IL-1 and IL-2 ($p < 0.05$) and a tendency for iNOS on day 14 ($p < 0.1$).

4. Discussion

In chicken and other vertebrates, the juvenile period is of major importance for (mucosal) immune development. During this period, the intestinal mucosa is exposed to a wide variety of dietary antigens, commensal- and pathogenic microorganisms. Nowadays, it is assumed that the early maturation phase of the intestinal immune system affects the functionality of the whole immune system later in life (Noble, 2009). The present study addresses aspects of the development of the chicken intestinal immune system in the absence of deliberate immunization, especially with respect to the appearance of sIgA. This immunoglobulin isotype has important functions in mammalian species (Favre et al., 2005; Corthesy, 2007; Cerutti and Rescigno, 2008). In the current study,

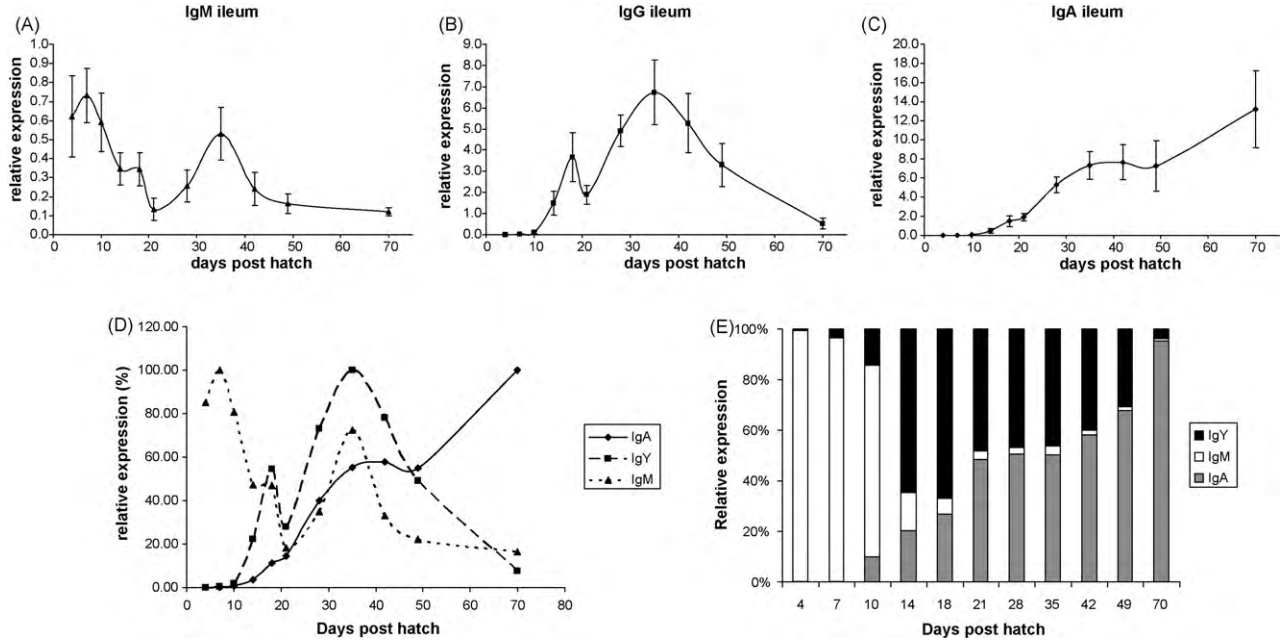

Fig. 4. Average relative mRNA expression of the three immunoglobulin isotypes in ileum ($n = 10$/time point). Standard errors are represented per time point in figure A–C. (A) Relative mRNA expression levels of IgM. (B) Relative mRNA expression levels of IgY. (C) Relative expression levels of IgA. (D) Relative expression levels are expressed as percentage of the maximum expression of that isotype. (E) Representation of the relative mRNA levels as percentage of the total immunoglobulin expression levels to visualize the contribution of each isotype per time point.

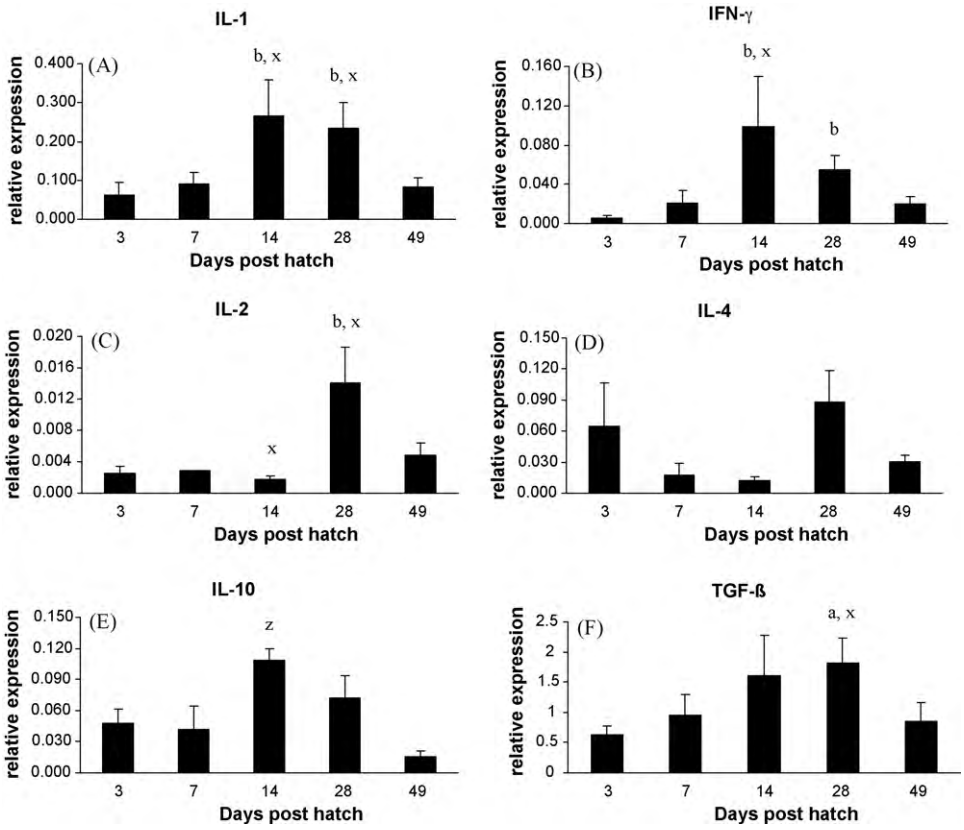

Fig. 5. qRT-PCR results: relative cytokine mRNA expression levels in jejunum of juvenile chickens ($n = 5$ for each time point; A, IL-1; B, IFN-γ; C, IL-2; D, IL-4; E, IL-10, F, TGF-β). Expression values are standardized using 28S as an internal standard. Significant differences in expression levels were tested against day 3 or 49. Within each experimental period, relative expression levels that differ from day 3 are indicated with the superscripts *a* and *b* above the bars ($a = p < 0.05$ and $b = p < 0.01$). Relative expression levels that differ significantly from day 49 are indicated with superscripts *x* and *z* above the bars ($x = p < 0.1$ and $z = p < 0.01$).

immunohistochemistry and qRT-PCR analyses show that endogenous IgA in the chicken intestine is almost undetectable before 14 days of age. However, the mRNA expression levels of IgA increase rapidly from 21 days on and reach maximum levels at 70 days of age (Fig. 2). Between 2 and 6 weeks of age, mRNA expression of various cytokines is enhanced compared to days 3–4 post hatch (Figs. 5 and 6). Maximum sIgA expression is accompanied with a return of cytokine mRNA expression to basal levels after week 7. In mammals, sIgA promotes immune exclusion by entrapping dietary antigens and microorganisms in the mucus, and prevents translocation of (pathogenic) microorganisms across the mucosal barrier (Macpherson, 2006). In addition, if microbes succeed in reaching and penetrating the surface epithelium, sIgA can also act as a clearance system from the basolateral surface back to the lumen (Cerutti and Rescigno, 2008; Macpherson et al., 2008). In pIgR knock-out mice, which lack IgA in the intestinal lumen, the mucosa is no longer protected from penetration by the intestinal microbiota (Corthesy, 2007). Consequently, these $pIgR^{-/-}$ mutants had enhanced microbiota specific serum IgG levels, indicating induction of systemic immune responses, which are the result of increased translocation of intestinal bacteria (Johansen et al., 1999; Sait et al., 2007). The neutralizing activities of sIgA result in downmodulation or blocking of bacteria mediated inflammation (Cerutti and Rescigno, 2008). Our observation that pro-inflammatory cytokines and iNOS expression are upregulated in the absence of IgA is in accordance with the proposed functions of sIgA in mammals. This cytokine upregulation may also be responsible for the enhanced IgM and IgY expression at 5 weeks of age. Our observations, and given the role of (the non-inflammatory isotype) IgA in mammals (Cerutti and Rescigno, 2008; Corthesy, 2007) suggest that also chicken sIgA contributes to maintenance of mucosal homeostasis.

The observation of age dependent sIgA expression in the present study is comparable with earlier studies in chicken (Jeurissen et al., 1989; Yamamoto et al., 1977). Also in mammals, IgA is poorly expressed during the first weeks after birth (Huling et al., 1999; Pérez-Cano et al., 2005). Correlation between IgA protein production and mRNA expression was corroborated by the positive staining of IgA producing cells and IgA mRNA levels. In most mammals, the lack of endogenous neonatal IgA production is compensated by the intake of colostrum and milk, which contain moderate or high concentrations of sIgA. Whether similar is true for chickens is unknown. During the absence of intestinal IgA, innate immunity and maternal antibodies may provide protection in chickens. Low amounts of IgA are found in the yolk of freshly-laid eggs, but significant amounts of IgA (0.3–0.4 mg; Hamal et al., 2006) are actively transported from the egg albumen to the yolk 1 day prior to hatch (Kaspers et al., 1996), and subsequently to the intestine through enclosure of the yolk sac around hatch. Due to physiological and biological processes maternal IgA is disappeared at the age of 2 weeks. Therefore, due to the low IgA levels between 2 and 4 weeks, penetration of bacteria across the mucosa may be enhanced. Translocated bacteria may then induce enhanced mRNA expression levels of IL-1 and IFN-γ, IL-12p40 and iNOS. It remains to be established whether this "IgA gap" plays a functional role in immune development. In jejunum as well as ileum, the ratio between IL-4/IFN-γ and IL-10/IFN-γ expression levels decreased strongly (5 and 7.5 times, respectively) between days 3 and 14 (results not shown). This is similar as in mammalian species, where the gut microbiota has a role in the establishment of an equilibrium between Th1 and Th2 responses (Hrncir et al., 2008).

In mammals, mucosal IgA is produced in both a T cell dependent and independent manner (He et al., 2007). Commensal bacteria

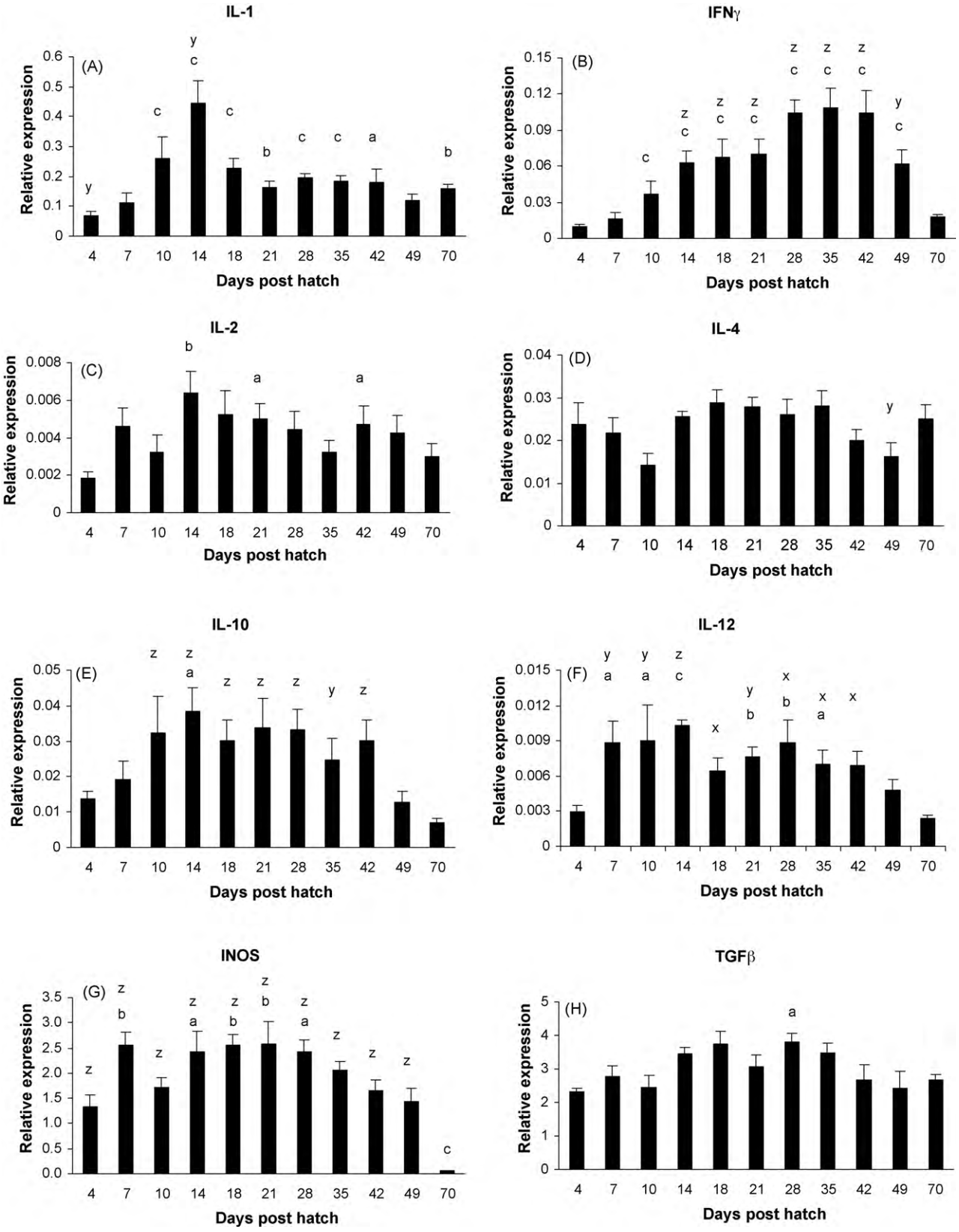

Fig. 6. qRT-PCR results: relative mRNA expression levels of different immune related genes in ileum of juvenile chickens ($n = 10$ for each time point; A, IL-1; B, IFN-γ; C, IL-2; D, IL-4; E, IL-10, F, IL-12p40; G, INOS; H, TGF-β). Expression values are standardized using 28S as an internal standard. Significant differences in expression levels were tested against day 3 or 70. Within each experimental period, relative expression levels that differ from day 3 are indicated with the superscripts *a*, *b* and *c* above the bars ($a = p < 0.05$, $b = p < 0.01$ and $c = p < 0.001$). Relative expression levels that differ significantly from day 49 are indicated with superscripts *x*, *y* and *z* above the bars ($x = p < 0.05$, $y = p < 0.01$ and $z = p < 0.001$).

contribute to IgA class switching through mechanisms that have not been fully elucidated yet (Fagarasan and Honjo, 2004). In general, TGF-β, together with IL-4 and IL-10, enable mucosal B cells to skew their isotype towards IgA (Corthesy, 2007; Fagarasan and Honjo, 2004; He et al., 2007). Little is known about the mechanisms behind class switching to IgA in aves. In the present study, we found that enhanced levels of IL-10 mRNA precede the increase in IgA mRNA levels, but a significant increase in TGF-β expression is less clear. However, the observed correlations between IgA and IFN-γ, TGF-β and IL-10 in weeks 3–5 p.h., the period in which a strong increase of IgY and IgA expression was observed, suggests a role for these cytokines in class switching to IgY and IgA in chicken. The correlation between IgA and IFN-γ expression could indicate that class switching in chicken is T cell dependent. In human, IFN-γ is involved in class switch to IgA1, whereas TGF-β and IL-10 together with APRIL induce the production of IgA2 in a T cell-independent manner (Cerutti et al., 2005; He et al., 2007). In chickens, so far no evidence exists for the presence of a homologue for APRIL and its receptor BCMA (Reddy et al., 2008). In addition, different IgA subclasses are not identified in chicken so far. Therefore, future studies are required to elucidate whether chicken IgA is produced in a T cell dependent or independent way or both.

Expression of pIgR followed the same kinetics as IgA and may even preceded the production of IgA (Fig. 2). Chicken pIgR mRNA expression levels are relatively low immediately after hatch, but increase after day 14 p.h. (Fig. 2). This is comparable to mammals, where intestinal pIgR expression is also low immediately after birth (Huling et al., 1999). In mammalian cell lines, pIgR expression was induced by the pro-inflammatory cytokines IFN-γ, TNF-α and IL-1β (Kaetzel, 2005), and the Th2 promoting cytokine IL-4 has a stimulatory effect (Ackermann et al., 1999). For the production of these cytokines influx of antigen presenting cells and T cells and subsequent activation of these immune cells is required. Activation of immune cells is triggered by the commensal microbiota, most likely via TLR signalling (Hempen et al., 2002). In chickens, a strong influx of CD3+ cells was observed on day 4 p.h., which continued to increase after day 4, but with a smaller magnitude (Bar-Shira et al., 2003). In addition, a significant increase of IFN-γ expression on day 4, which continued to increase in the second week p.h. was reported. In accordance with Bar-Shira et al. (2003), we also found a significant increase of IL-1β and IFN-γ expression in the second week p.h. (Figs. 5 and 6). These cytokines are probably responsible for induction of pIgR expression, because regulatory regions within and flanking the human pIgR gene contain several transcription factor-binding sites, including a NF-κB/Rel site, which mediates transcriptional responses to pro-inflammatory cytokines (Kaetzel, 2005; Schjerven et al., 2001). Indeed, like in mammalian species, a site with strong sequence homology to the human and murine NF-κB/Rel binding site is present within 1 kb distance of the startcodon of the chicken pIgR gene (results not shown). Therefore, as in mammals, expression of the chicken pIgR gene is likely to be induced by pro-inflammatory cytokines, which are produced in response to bacterial colonization of the intestinal tract. As in rabbits and rats, high pIgR expression was measured in the liver of chickens. Northern blot experiments also showed that expression levels of pIgR were higher in liver than in jejunum (Wieland et al., 2004). Rabbits and rodents obtain most of their proximal intestinal sIgA from hepatobiliary transfer of circulating pIgA (Lamm, 1998). Humans in contrast, have no hepatobiliary transport and obtain 95% of intestinal sIgA from production in the submucosa. The high concentrations of SC and sIgA in chicken bile (Rose et al., 1981) suggests that the hepatobiliary route of sIgA delivery may also be of importance for avian species.

Taken together, this study provides indirect evidence that, like in mammals, expression of immune related genes in the intestine of juvenile chickens is highly dynamic. Chicken sIgA probably plays a role in the maintenance of mucosal homeostasis. Future studies should be directed on the modulation of either cytokine and/or antibody isotype responses in juvenile chickens to establish functional relationships, especially during early periods of life.

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