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RESEARCH ARTICLE

Campylobacter colonization of the chicken induces a proinflammatory response in mucosal tissues

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Abstract

Campylobacter jejuni is a major cause of human inflammatory enteritis, but colonizes the gastrointestinal tract of poultry to a high level in a commensal manner. *In vitro*, *C. jejuni* induces the production of cytokines from both human and avian-model epithelial cell and macrophage infections. This suggests that, *in vivo*, *Campylobacter* could induce proinflammatory signals in both hosts. We investigated whether a proinflammatory cytokine response can be measured in both day-of-hatch and 2-week-old Light Sussex chickens during infection with *C. jejuni*. A significant induction of proinflammatory chemokine transcript was observed in birds of both ages, compared with levels in mock-infected controls. This correlated with an influx of heterophils but was not associated with any pathology. These results suggest that in poultry there may be a controlled inflammatory process during colonization.

Introduction

Campylobacter is the main bacterial cause of human enteritis in the developed world and a major source of infection is through the consumption of contaminated poultry meat. It is regarded as a commensal of the avian host, colonizing the avian gastrointestinal tract to a high level ($9\log_{10}$ CFU g^{-1} contents) without overt disease.

The ability of *Campylobacter* to colonize in chickens, and other poultry, has been well documented, with primary sites of colonization being the caeca, colon and cloaca (Beery *et al.*, 1988). However, due to the absence of pathological changes, little is known about its interactions with the host in terms of the immune response. Experimental infection of the chicken with *Campylobacter* strains induces secretory and serum IgG antibodies specific for the bacteria (Myszewski & Stern, 1990). Some evidence also exists that *Campylobacter* can cause disease in day-of-hatch chicks (Newell, 2001) but the lack of a consistent reported pathology in naturally colonized birds has led to the view that these bacteria are commensal in the chicken.

The attachment and invasion of human epithelial cells by *Campylobacter* is often described as a major component of the infection process and has been studied extensively using human cell culture models (Everest *et al.*, 1992; Konkel *et al.*, 1992; Al-Salloom *et al.*, 2003). While the invasion of human

epithelia is well described, the attachment to and invasion of chicken epithelia by *C. jejuni* strains is more debatable. There is no direct evidence that *Campylobacter* adheres or attaches to the avian gut (Beery *et al.*, 1988). However, there is some evidence that *Campylobacter* can traverse the intestinal epithelium and they have been recovered from the spleen and liver of young chicks (Young *et al.*, 1999). This invasion, whether transient or sporadic, would require a close interaction between the host and the bacteria. The clear antibody response to *Campylobacter* by poultry (Myszewski & Stern, 1990) indicates that some bacterial/host cell interaction must take place during the colonization process for the adaptive response to have been initiated.

The development of this immunoglobulin response requires the stimulation of innate signals that drive the cell-to-cell interactions required for antigen presentation. The same inflammatory signals that drive adaptive responses have been correlated with intestinal disease in humans (Enocksson *et al.*, 2004) and these are associated with induction of pathology. *Campylobacter* can stimulate the induction of cytokines from *in vitro* cultured avian macrophage and epithelial cell models (Smith *et al.*, 2005), similar to that seen during *Campylobacter* stimulation of human macrophage and epithelial cells (Hickey *et al.*, 1999, 2000; Al-Salloom *et al.*, 2003; Jones *et al.*, 2003). The responses to *Campylobacter in vitro* are similar to those seen with the pathogenesis of *Salmonella* during *in vivo* infection of poultry (Withanage *et al.*, 2004), which correlate with intestinal pathology in the chicken. We were, therefore, interested in determining whether *Campylobacter* colonization of the chicken induced a cytokine response in day-of-hatch and 2-week-old chickens not previously exposed to these bacteria.

Materials and methods

Bacterial strains

Campylobacter jejuni G1 has been used previously for *in vitro* cytokine studies and can persistently colonize the intestinal tract of birds to a high level (Linton *et al.*, 2000; Jones *et al.*, 2004; Smith *et al.*, 2005). The bacteria were cultured on sheep blood agar (Oxoid, UK) under microaerophilic conditions (10% CO_2, 5% O_2 and 85% N_2). For inoculation of birds, bacteria were grown for 48 h in 10 mL of Muller–Hinton broth (Oxoid, UK).

Poultry infections

Specific pathogen-free (SPF) Light Sussex chickens were produced at the Institute for Animal Health. Birds were set up as two groups hatched from the same parent flock; the first was set up 2 weeks before the second to obtain groups of two ages, which could be infected with the same culture of *Campylobacter*. Both groups were given 0.1 mL of *Campylobacter*-free gut flora prepared as described previously (Jones *et al.*, 2004); birds in group 1 were dosed at 14 days of age and those in group 2 were dosed on day of hatch. Birds were maintained under high biosecurity and were monitored for *Campylobacter*, before infection, by cloacal swab tests and found to be free of these bacteria. Uninfected control birds remained free of *Campylobacter* throughout the study. All experimental animal procedures were approved by the local ethical review committee.

Colonization trials

Both groups of birds were infected with $8 \log_{10}$ CFU in a 0.1 mL phosphate-buffered saline (PBS) from the same culture of *C. jejuni* G1 either on day of hatch or at 2 weeks of age. At the same time, in control birds, a mock infection of PBS was induced. At 6 and 12 h and 1, 2 and 7 days postinfection, five birds from each infected group, and three control birds, were taken for postmortem analysis.

Birds were killed and tissue samples were immediately taken for RNA extraction by immersion in RNAlater (Ambion), histology by storage in 10% formaldehyde and bacterial counts.

Bacterial counts

To determine levels of colonization, caecal contents were removed postmortem and diluted in ice-cold PBS to 1 g contents mL^{-1}. Samples were serially diluted, 10-fold, in ice-cold PBS and plated directly onto *Campylobacter*-selective blood-free agar (CCDA; C739; Oxoid) and CCDA-selective supplement (SR155; Oxoid). Samples of contents were also used to inoculate 3 mL of Exeter enrichment broth (Jones *et al.*, 2004), which was incubated for 3 days, and swab-plated onto CCDA-selective plates to determine the presence of bacteria in low numbers.

Real-time quantitative reverse transcriptase (RT)-PCR

RNA expression was determined using real-time quantitative RT-PCR, using the ABI PRISM 7700 as described previously (Kaiser *et al.*, 2000). Primers and probes for 28S rRNA gene, IL-1β, IL-6, CXCLi2 (previously referred to as IL-8), CXCLi1 (previously referred to as K60), IL-13, TGF-β4 and IL-10 have been described previously (Kaiser *et al.*, 2000; Avery *et al.*, 2004; Rothwell *et al.*, 2004). RT-PCR was performed using the RT qRT-PCR^{tm} Mastermix (Eurogentec, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (Perkin Elmer Applied Biosytems, Boston) with the following cycle profile: one cycle of 50 °C for 2 min, 60 °C for 30 min and 95 °C for 5 min and 40 cycles

of 94 °C for 20 s, 59 °C for 1 min. Each RT-PCR experiment contained three no-template controls, test samples and a standard $\log_{10}$ dilution series. Each experiment was performed in triplicate with replicates performed on different days. Regression analysis of the mean values of six replicate RT-PCRs for the $\log_{10}$-diluted RNA was used to generate standard curves.

Data were calculated as fold changes compared with the mean of the mock-infected bird samples and averaged. All the data shown are the mean from five independent infected samples and represent the mean of the fold changes between birds with SD. Statistical analysis was carried out using ANOVA to analyse variance between experiments and controls.

Histology

An assessment of heterophil infiltration was carried out by histology on tissue sections from the caeca and ileum from the same infected and uninfected birds used for cytokine analysis. Samples were analysed at 1 and 4 days postinfection, as it was at these times that we observed maximal cytokine differences between groups. Formalin-fixed samples were stained with haematoxylin and eosin (H&E) using standard protocols. Heterophils were enumerated in a blind assessment using five random fields of view for each tissue section.

Results

Campylobacter counts

Initially, at 6 h postinfection, *Campylobacter* were present in caeca at levels between 4 and 5 $\log_{10}$ CFU g^{-1} and increased rapidly within the first 24 h postinfection to 9 $\log_{10}$ CFU g^{-1} (Fig. 1). A greater variation between counts was observed in day-of-hatch birds than those of 2-week-old birds at 6 h postinfection, probably due to the lack of gut flora present in the gastrointestinal tract of these birds (Fig. 1a). No significant difference was observed between birds inoculated at day of hatch and at 2 weeks of age for caecal counts over the 7 days of infection. No *Campylobacter* were isolated from mock-infected age-matched control birds either by direct count or enrichment culture.

Cytokine induction in day-of-hatch birds

RNA was isolated from the ileum and caecal walls of the day-of-hatch infected birds at various times postinfection. Fold changes in cytokine mRNA expression between infected and uninfected birds were determined for CXCLi1, CXCLi2, IL-1β and IL-6 (Fig. 2). We also measured the levels of IL-10, IL-13 and TGF-β4 in the caecal wall, ileal wall and spleens from the same birds but no expression was detected (data not shown). Significant differences, compared with uninfected controls, were observed for CXCLi1 and CXCLi2 at time-points from 12 h and 1 day postinfection, respectively, in caecal samples. However, a more sporadic response was observed from ileal samples with an early CXCLi2 response at 6 h ($P < 0.05$) and CXCLi1 at 4 days postinfection ($P < 0.08$) (Fig. 2a and b). Levels of IL-1β and IL-6 in the caecal and ileal walls were either absent or not significantly above those of uninfected birds in the early stages of infection. No significant response was observed for IL-6 or IL-1β until day 4 or 7, respectively (Fig. 2d, g and h). The most consistently observed responses in day-of-hatch infected birds were for the chemokines CXCLi1 and CXCLi2 in caecal tissues (Fig. 2e and f).

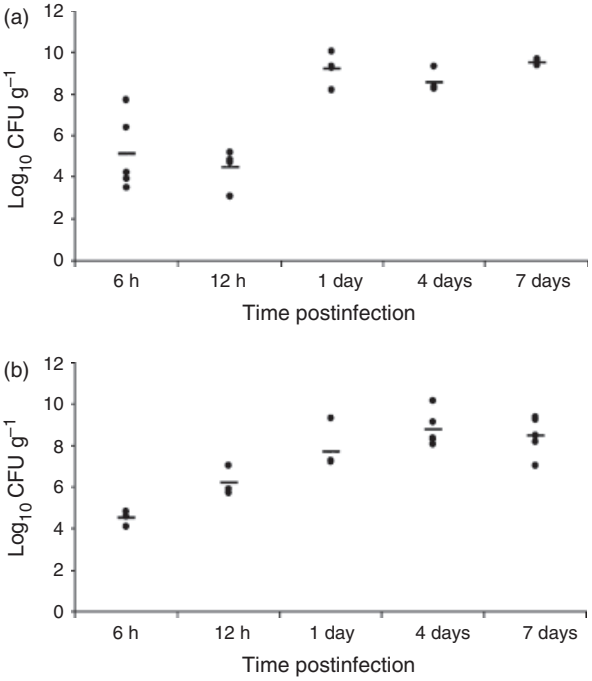

Fig. 1. Colonization of poultry by *Campylobacter*. Birds were infected with 8 $\log_{10}$ CFU of *Campylobacter jejuni* G1 at either (a) day of hatch or (b) 2 week of age. Counts are shown as log CFU g^{-1} of caecal contents.

Cytokine induction in 2-week-old birds

RNA was isolated from the ileal and caecal walls of the birds infected at 2 weeks of age at various times postinfection and the fold difference between infected and uninfected birds was determined for CXCLi1, CXCLi2, IL-1β and IL-6 (Fig. 3). As with the day-of-hatch birds, no mRNA expression could be measured for IL-10, IL-13 or TGF-β4 from intestinal wall, caecal wall or spleen samples (data not shown). In contrast to the day-of-hatch birds, CXCLi1 was clearly expressed in both ileal and caecal walls (Fig. 3a and b). CXCLi1 was expressed at significant levels from 1 day postinfection in infected ileal tissue (Fig. 3a) while in caecal

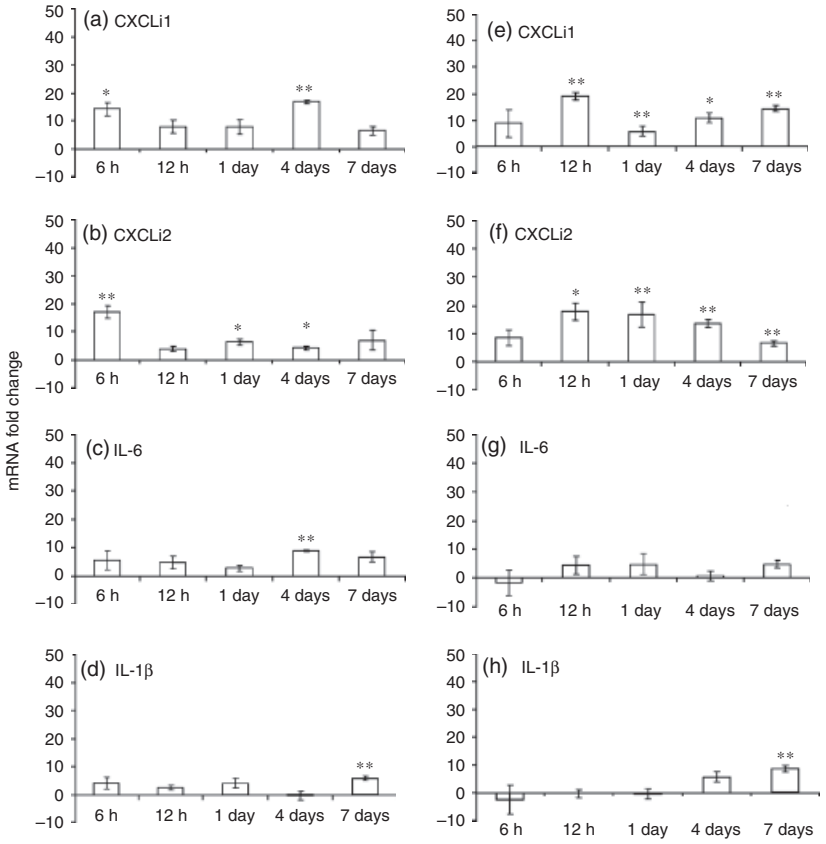

Fig. 2. Quantification of cytokine and chemokine transcripts from day-of-hatch birds. Cytokine transcript levels were determined from both infected and uninfected birds at 6 and 12 h and 1, 4 and 7 days postinfection. The data are shown as fold difference in mRNA expression levels in infected birds compared with levels in uninfected control birds at the same time-points. Samples were taken from the ileal (a–d) and caecal walls (e–h). Significant differences compared with uninfected birds are indicated by $^{**}P < 0.05$ and $^{*}P < 0.1$.

tissue early expression from 6 h to 1 day postinfection was observed for CXCLi1 (Fig. 3e) and from 12 h postinfection for CXCLi2 (Fig. 3f). The fold change differences for IL-1β and IL-6 appeared to be clearer than for day-of-hatch birds, with expression at both 6 and 12 h postinfection in the ileal samples for IL-6 and later at 7 days postinfection in the caecal samples.

The largest fold difference observed was for CXCLi1, which peaked at 40-fold induction at 1 day postinfection but was reduced by day 7 (Fig. 3a). This level of response was not observed for CXCLi1 in caecal tissue but significant changes were observed at earlier time-points (Fig. 3e). Significant expression was observed at 12 h postinfection for CXCLi2, which again appeared reduced by day 7 (Fig. 3f). In contrast to the younger birds, differences in expression of IL-1β and IL-6 could be observed at all but one time-point, although the differences were not statistically significant ($P > 0.05$). As with the data from young birds, changes in expression levels of IL-1β mRNA were the lowest.

Heterophil migration counts

CXCLi1 and CXCLi2 have been implicated in the chemoattraction of phagocytes (Kogut, 2002). We determined whether there was a difference in heterophil influx by histology of sections taken at 1 and 4 days postinfection for both the day-of-hatch and the 2-week-old birds. We chose these time-points as this was when we observed the most significant mRNA expression levels of the CXCLi chemokines in tissues (Figs 2 and 3) and they correlate to the times postinfection at which symptoms can occur in man. The data clearly show that there were significant differences between the 2-week-old infected and uninfected birds at 1 and 4 days postinfection in the caecal sections and 4 days postinfection in the ileal sections (Fig. 4). We observed a considerable variation between birds. This could be due to bird-to-bird differences as we used an out-bred flock. However, we observed no significant differences in heterophil numbers for the samples from day-of-hatch birds at either time-point (Fig. 4).

Discussion

Campylobacter is well adapted to life within the avian intestinal tract and the majority of bacteria are found in the caeca and can colonize the caeca of birds to very high cell numbers (Beery *et al.*, 1988). It does not cause any obvious pathology in the field and has been described as a

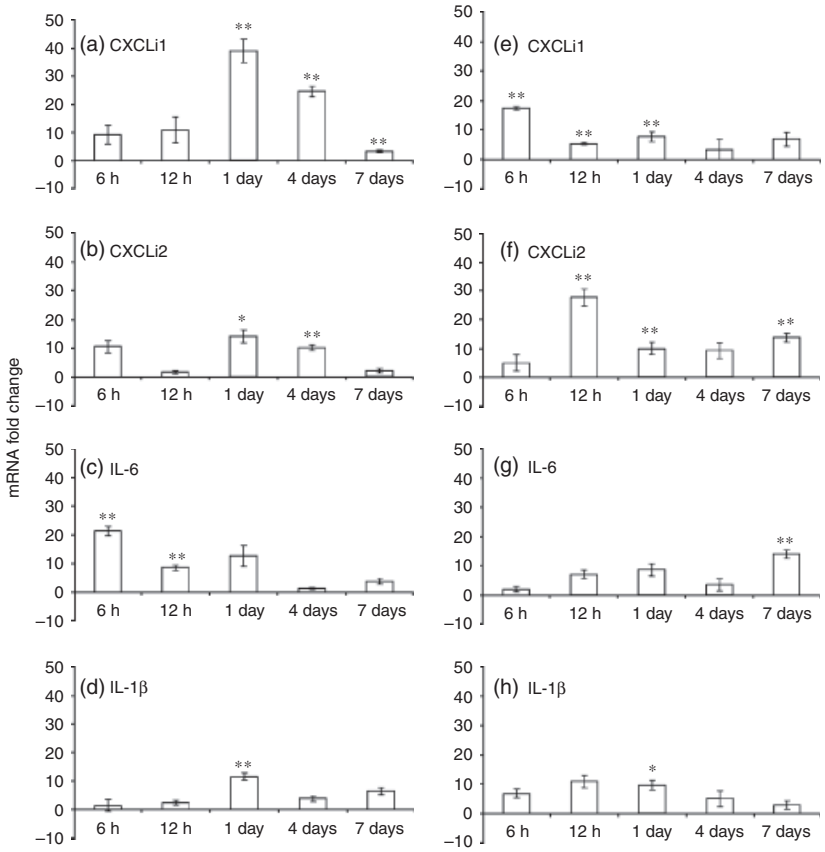

Fig. 3. Quantification of cytokine and chemokine transcripts from 2-week-old birds. Cytokine transcript levels were determined from both infected and uninfected birds at 6 and 12 h and 1, 4 and 7 days postinfection. The data are shown as fold difference in mRNA expression levels in infected birds compared with levels in uninfected control birds at the same time-points. Samples were taken from the ileal (a–d) and caecal walls (e–h). Significant differences compared with uninfected birds are indicated by $^{**}P < 0.05$ and $^{*}P < 0.1$.

commensal of poultry. *Campylobacter* is an important zoonosis, causing gastroenteritis in humans that has been associated with the induction of proinflammatory signals (Enocksson *et al.*, 2004). Recently, the repertoire of cytokines in the chicken has been described (Kaiser *et al.*, 2005), allowing their expression during infection to be analysed. While the production of proinflammatory markers correlates with disease in humans, recent evidence has shown that *Campylobacter* can also induce proinflammatory cytokines in *ex vivo* avian cells (Smith *et al.*, 2005). Thus, *Campylobacter* have the potential to induce inflammatory responses in poultry (Smith *et al.*, 2005). *Campylobacter*-specific antibodies develop after colonization, indicating that there is a bacteria/host cell interaction *in vivo*. Such a close cell interaction may be predicted to cause an inflammatory response and this should be measurable.

There are a number of chicken models in use in the *Campylobacter* research field. Day-of-hatch chicks have been used in both colonization and disease models (Ruiz-Palacios *et al.*, 1981; Sanyal *et al.*, 1984; Lowenthal *et al.*, 1994). However, at this age, the immune system is immature. We compared day-of-hatch with 2-week-old birds, where the intestinal immune system will be more developed and that represents the age of infection that is most common in the field (Lowenthal *et al.*, 1994; Stern *et al.*, 2001; Bar-Shira *et al.*, 2003; Bar-Shira & Friedman, 2006). The older birds will develop a gut flora, standardized in this study to reduce bird-to-bird variation, and this is involved in the development of the immune system.

Birds were infected with *C. jejuni* G1. In both groups of birds, *C. jejuni* G1 rapidly established itself (Fig. 1). Bacteria were distributed rapidly through the gastrointestinal tract and gave an initial level of about $4 \log_{10} \mathrm{CFU\,g^{-1}}$ of caecal contents over the first 6 h of infection, replicating to the expected level of $9 \log_{10} \mathrm{CFU\,g^{-1}}$ over the first 24 h (Fig. 1). Postmortem samples were taken at various times postinfection and analysed for the presence of the proinflammatory cytokines IL-1β and IL-6 and the inflammatory chemokines CXCLi1 and CXCLi2. There was a sharp contrast in the variability of the data within groups between the day-of-hatch and the 2-week-old birds. The data for the day-of-hatch birds were more variable, showing minimal induction of both IL-1β and IL-6. This was in contrast to 2-week-old birds, which showed a low but significant increase in IL-1β and IL-6. This could be due to an incomplete population of immune cells within the mucosal tissue in the day-of-hatch birds, although the cytokines can also be expressed by epithelial cells. The chemokine CXCLi1 showed reduced

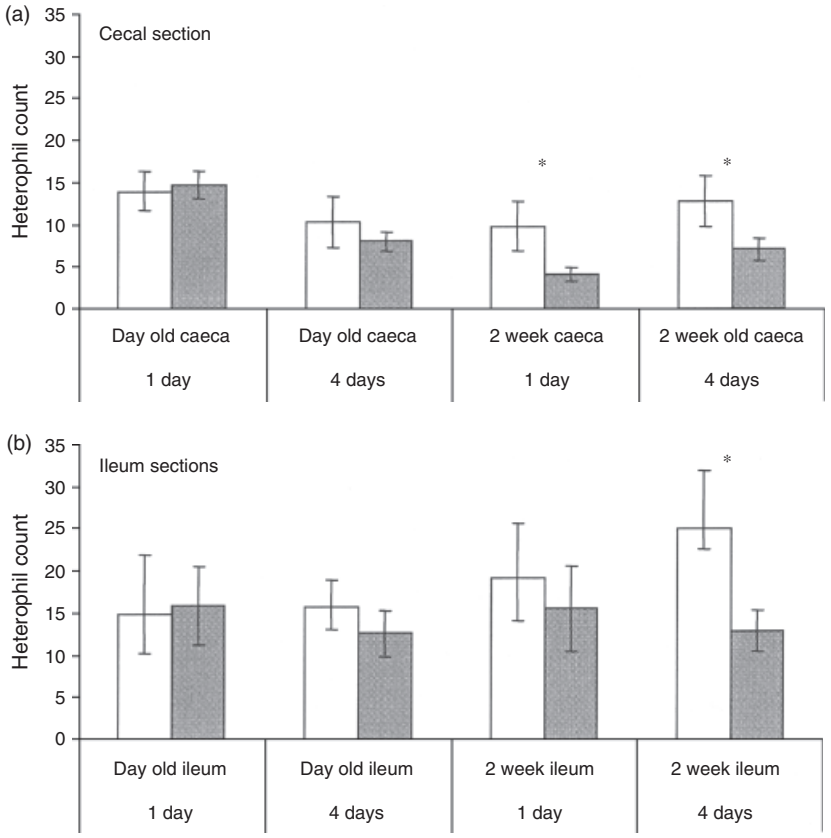

Fig. 4. Counts of heterophil infiltration. Heterophil counts were performed in a blind count assessment on formalin-fixed H&E-stained samples. Five fields of view were counted for each bird. Counts are from the caecal (a) and the ileal (b) walls. Open bars represent counts from infected birds; grey bars represent counts from uninfected control birds. Significant differences; *P-value < 0.05.

expression in the ileum of day-of-hatch birds at 1 and 4 days postinfection compared with levels in 2-week-old birds (Figs 2a, e and 3a, e). However, there was a significant production of CXCLi2 in the caeca, which matched the levels observed in 2-week-old birds.

CXCLi1 and CXCLi2 are homologues of human CXCL8 and have been implicated in the attraction of heterophils to the site of infection in poultry (Kogut, 2002; Kaiser *et al.*, 2005). We, therefore, examined H&E sections of the caecal and ileal wall for changes in host cell populations that may be indicative of cell migration due to chemokine induction in the tissues (Fig. 4). We observed significant differences between heterophils in the caecal and ileal tissues of 2-week-old birds but not in day-of-hatch birds. The lack of observable heterophilia in day-of-hatch birds could be due to the immaturity of this cell type in young birds, leading to poor migration in response to enterocyte signalling (Bar-Shira & Friedman, 2006). No obvious lesions were seen in the caecal tissue postmortem, suggesting that the heterophilia did not cause excessive damage to the tissues. While previous studies have hypothesized that macrophages could contribute to disease in humans (Jones *et al.*, 2003), our study clearly shows induction of a heterophilia without pathology. This is not unprecedented as Foster *et al.* (2003) clearly observed that *Salmonella* strains can initiate polymorphonuclear infiltration without pathology.

The induction of proinflammatory chemokines in birds of both ages was most clear at 12 h and 1 day postinfection (Figs 2 and 3), after which it appeared to peak and then decline at later time-points. This downregulation of cytokine production was not due to a reduction in bacterial load in the gastrointestinal lumen, as the level of colonization was constant over these time-points. Other signals may well be involved in the modulation of these responses and be critical in the outcome of the infection. Not all cytokines are proinflammatory and several of them play a role in controlling inflammatory processes. We measured IL-10, IL-13 and TGF-β4 transcript levels in the ileum and caecum and spleen. We observed no induction of these cytokines in either the infected or the mock-infected birds. This may suggest that the avian host is not modulating the response to *Campylobacter* by the direct action of these cytokines.

If campylobacteriosis in humans is purely driven by inflammatory responses, then we might expect to find pathological changes in the chicken. We did not observe any pathology. However, the chicken genome sequence has highlighted many innate immune differences between poultry and humans (Kaiser *et al.*, 2005) that may be crucial

in driving pathological changes. *Campylobacter* does induce a proinflammatory response in the chicken during colonization; however, this response clearly does not lead to disease.

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