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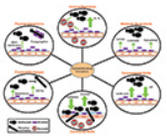

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RESEARCH PAPER

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# Invasive behavior of *Campylobacter jejuni* in immunosuppressed chicken

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**ABSTRACT**

*Campylobacter jejuni* is a predominant cause of gastroenteritis in humans but rather harmless in chickens. The basis of this difference is unknown. We investigated the effect of the chicken immune defense on the behavior of *C. jejuni* using glucocorticoid (GC)-treated and mock-treated 17-day old Ross 308 chicken bearing in mind that GCs have immunosuppressive effects and dampen the innate immune response. The effect of GC administration on the behavior of *C. jejuni* was compared with that on infection with *Salmonella* Enteritidis to address possible microbe-associated differences. Our results revealed that GC treatment fastened the intestinal colonization of *C. jejuni* ($p < 0.001$) and enhanced its dissemination to the liver ($p = 0.007$). The effect of GC on intestinal colonization of *S.* Enteritidis was less pronounced ($p = 0.033$) but GC did speed up the spread of this pathogen to the liver ($p < 0.001$). Cytokine transcript analysis showed an up to 30-fold reduction in baseline levels of *IL-8* mRNA in the cecal (but not spleen) tissue at Day 1 after GC treatment ($p < 0.005$). Challenge with *C. jejuni* strongly increased intestinal *IL-8, IL-6,* and *iNOS* transcript levels in the non-GC treated animals but not in the GC-treated birds ($P < 0.005$). *In vitro* assays with chicken macrophages showed that GC dampened the TLR agonist- and *C. jejuni* induced-inflammatory gene transcription and production of nitric oxide ($P < 0.005$). Together, the results support the hypothesis that *C. jejuni* has the intrinsic ability to invade chicken tissue and that an effective innate immune response may limit its invasive behavior.

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## Introduction

The bacterial food-borne pathogen *Campylobacter jejuni* (*C. jejuni*) is estimated to cause about 100 million cases of diarrheal illness each year.[1,2] One major source of *C. jejuni* is contaminated chicken meat.[2] *C. jejuni* frequently colonizes the ceca of chicken at high concentrations ($10^9$ CFU/g cecal content) usually without or with very mild clinical manifestations.[3-7] The molecular basis of the different effects of *C. jejuni* in humans and chickens is still unknown. Factors that may contribute include a differential expression of bacterial virulence traits in the different hosts, a variable composition of the local microbiota, intrinsic differences in the intestinal mucosal architecture, and/or differences in immune defense between the species. Detailed analysis of the interaction of *C. jejuni* with chicken cecal tissue has yielded variable results ranging from a lack of *C. jejuni* penetration of the intestinal mucus layer to *C. jejuni* invasion of chicken tissue with signs of a local inflammatory response.[6-10]

One important step in the mucosal defense against bacterial pathogens is the early recognition of microbial products by the innate immune system. One class of host receptors that signals the presence of bacteria is the Toll-like receptor (TLR) family. Activation of members of this receptor family by bacterial ligands initiates downstream signaling events that result in nuclear translocation of transcription factors (such as NF-$\kappa$B) that regulate the expression of pro- or anti-inflammatory genes. Comparative analysis of the human and chicken TLR repertoire revealed several differences between the mammalian and avian species (for a review see refs. 11,12). Chicken-specific TLR characteristics include a broader subset of TLR1/2 receptors,[13] the absence of a TLR4/MyD88-independent signaling pathway,[14] the presence of a protease-activated TLR15,[16] and the presence of TLR21 as a functional ortholog of human TLR9.[16,17] *C. jejuni* can activate both human and chicken TLRs,[18] but the contribution of TLRs to the chicken defense against *C. jejuni* remains to be determined.

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One major class of regulators of mammalian biological systems including the immune system, is the adrenal-derived glucocorticoids. These steroid hormones bind to the cytosolic glucocorticoid (GC) receptor. The formed complex translocates to the nucleus and binds to response elements in the promoter regions of GC-responsive genes.[19,20] GCs also influence transcription factors that induce potent anti-inflammatory activity.[19,21,22] GCs are always detectable in serum, but their concentration strongly varies with the environmental stress encountered by the host. The hypothalamus-pituitary-adrenal axis responsible for the stress-related production of GC in mammals is also functional in chicken.[23,24] On poultry farms, environmental stress influences serum corticosterone levels and affects the susceptibility of chicken to infectious diseases,[25,26] possibly through its dampening effect on the immune system.

In the present study, we applied GC-induced immunosuppression to investigate the contribution of the chicken immune defense to the apparent commensal behavior of *C. jejuni* in chicken. GC-treated and control chickens were challenged with *C. jejuni* or (as control) with *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis). Bacterial colonization, systemic dissemination, and tissue expression of pro-inflammatory genes were followed in time. In addition, the effect of GCs on *C. jejuni*-induced chicken innate immune (TLR) activation was assessed in cultured chicken cells. Our results demonstrate that bacterial challenge of GC treated chicken results in a poor inflammatory response and a more rapid intestinal colonization and dissemination of *C. jejuni.*

## Results

### *Effect of glucocorticoids on the colonization and dissemination of* C. jejuni *in chicken*

To investigate the reason for the behavior of *C. jejuni* in chicken, we first investigated the effect of GC administration on the intestinal colonization and dissemination of *C. jejuni*. Hereto, different groups of SPF chicken were injected with either the glucocorticoid Depo-Medrol (groups 1 and 3) or PBS (groups 2 and 4). After 24 h, groups 3 and 4 were challenged orally with $10^5$ CFU of *C. jejuni* strain 81116. Groups 1 and 2 served as non-challenged controls. The following days, at least 5 chicken of each group were sacrificed to determine the number of *C. jejuni* in the ceca and liver, and to isolate tissue RNA for gene expression analysis.

Enumeration of *C. jejuni* in the collected cecal contents demonstrated significantly higher loads of *Campylobacter* in the ceca of the GC-treated group (group 3) compared to those of the PBS-injected group (group 4) ($\beta$ = 0.56, 95%CI 0.26–0.86, p < 0.001) (Fig. 1A, left panel). In the GC-treated animals, the number of *C. jejuni* had geometric mean values of $3.0 \times 10^9$ CFU/g content at Day 2 to $4.5 \times 10^9$ CFU/g content at Day 4 after challenge. In the control animals, *C. jejuni* values reached mean levels of $1.0 \times 10^3$ and $1.5 \times 10^6$ CFU/g at Days 2 and 3 respectively, rising to $7.1 \times 10^7$CFU at Day 4. Bacterial culture of liver tissue demonstrated significantly higher numbers of *C. jejuni* in the liver of the GC-treated group compared to the control group ($\beta$ = 0.46, 95%CI 0.13–0.79, p = 0.007). At Day 2 and Day 3 after challenge, the number of *C. jejuni* in the liver reached mean values of $2.2 \times 10^3$ CFU/g and $4.1 \times 10^3$ CFU/g, respectively. At these time points, *C. jejuni* was still virtually absent in the liver of the control animals (1.1 CFU/g tissue at Day 2 and 16.7 CFU/g tissue at Day 3) (Fig. 1A, right panel).

Experiments with chicken challenged with *S.* Enteritidis strain CVI-1 instead of *C. jejuni* revealed a slightly faster intestinal colonization ($\beta$ = 0.10, 95%CI 0.01–0.19, p = 0.033) (Fig. 1B, left panel) and much faster bacterial spread to the liver ($\beta$ = 0.75, 95%CI 0.47–1.02, p < 0.001) (Fig. 1B, right panel) for the GC-treated group (group 5) compared to the control group (group 6).

### *Inflammatory gene expression in chicken challenged with* C. jejuni

To learn more about the mechanism(s) contributing to the more rapid colonization and dissemination of *C. jejuni* after GC administration, we first determined the effect of the *C. jejuni* challenge on the expression of inflammatory genes for the non-GC treated animals. RT-qPCR analysis on mRNA derived from cecal tissue at Day 1 after challenge with *C. jejuni* (group 4) showed a 50–100 fold up-regulation of *IL-6, IL-8*, and inducible nitric oxide synthase (*iNOS*) transcripts when compared to transcript levels in the non-challenged control group (group 2) (Fig. 2A). At Day 4 after bacterial challenge, the difference in cecal *IL-6, IL*-8, *IL-1β* and *iNOS* transcripts was less pronounced (Fig. 2A). In spleen tissue, the challenge with *C. jejuni* caused an increase in *IL-6* and *IL-8* transcripts at Day 1 and an additional increase in *IL-1β* mRNA at Day 4. The bacterial challenge did not change *iNOS* and *IFNβ* transcript levels (Fig. 2B).

To determine the microbe specificity of the host response, we performed similar transcript analysis after challenge with *S.* Enteritidis. This pathogen induced an increase in cecal *IL-8* and *IL-6* transcript levels at Day-1 and Day-4. Transcript levels of iNOS did not differ between the infected and non-infected groups of animals (Fig. 2C). This result clearly differed from the cecal response upon challenge with *C. jejuni* (cf. Figs. 2C versus 2A). Like *C. jejuni*, infection with *S.* Enteritidis

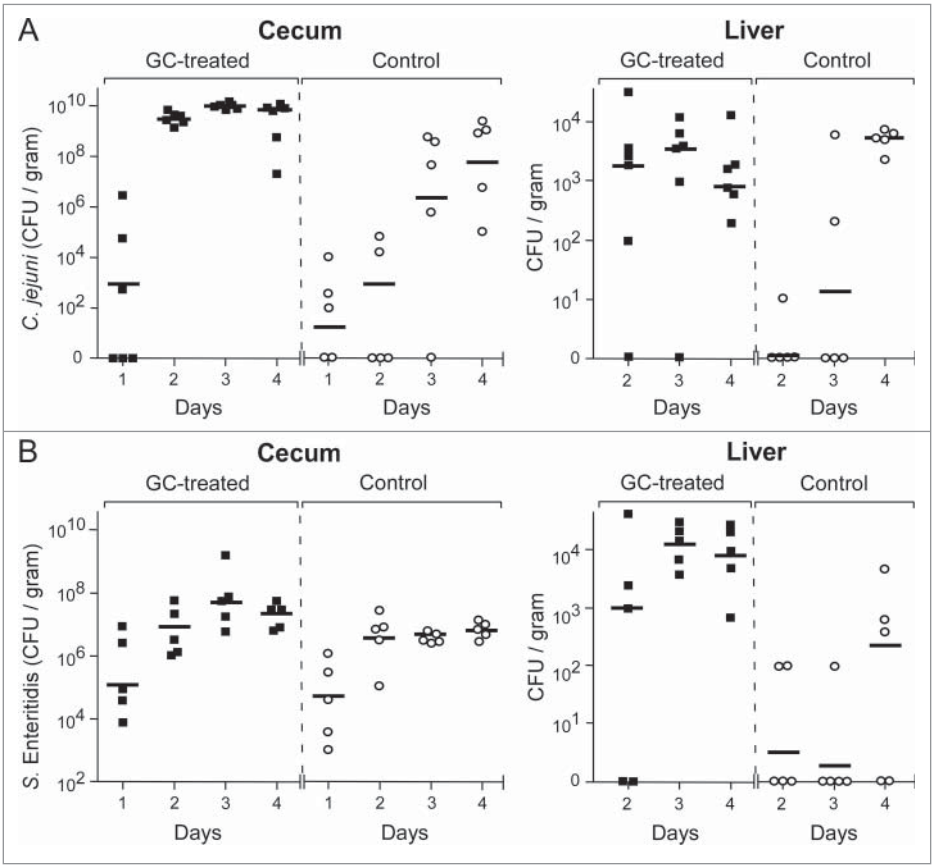


**Figure 1.** *C. jejuni* and *S.* Enteritidis colonization kinetics in GC-treated and control chickens. Chicken were injected with GC (closed blocks) or PBS (open circles) and 24 h later challenged orally with $10^5$ CFU of *C. jejuni* or *S.* Enteritidis. At Day 1–4 post-challenge, *C. jejuni* (panel A) and *S.* Enteritidis (panel B) colonization of the ceca and the liver was estimated by CFU counting. Data are plotted as CFU per gram of cecal content or liver tissue for each chicken and expressed as the geometric mean (horizontal bars) of CFU per group of chicken. Statistical analysis of differences between treated and non-treated chickens were calculated with a gamma generalized linear model and gave the following values: *Campylobacter* in the ceca: $\beta = 0.56$, 95%CI 0.26–0.86, $p < 0.001$; *Campylobacter* in the liver: $\beta = 0.46$, 95%CI 0.13–0.79, $p = 0.007$; *Salmonella* in the ceca: $\beta = 0.10$, 95%CI 0.01–0.19, $p = 0.033$; *Salmonella* in the liver: $\beta = 0.75$, 95%CI 0.47–1.02, $p < 0.001$.

variably increased *IL-8*, *IL-6* and *IL-1β* transcript levels in spleen tissue (Fig. 2D). Neither *C. jejuni* nor *S.* Enteritidis significantly increased *iNOS* transcript levels in the spleen (Figs 2B and D). Overall, our results indicate that colonization of chicken with *C. jejuni* induces a robust host response and that *C. jejuni* and *S.* Enteritidis elicit bacteria- and tissue-specific cytokine responses.

### *Glucocorticoid-induced downregulation of pro-inflammatory gene expression*

Transcript analysis on the same panel of inflammatory genes at Day 1 after administration of GC (i.e. without additional bacterial challenge)(group 1) revealed a 30-fold reduction of cecal baseline *IL-8* mRNA levels compared to those in the PBS-injected control group (Fig. 3). At Day 4, only a minor difference in the cytokine mRNA levels between both groups was measured (Fig. 3). RT-qPCR assays on spleen tissue revealed no significant differences in baseline transcripts between the GC-treated and control group during the period of analysis (Fig. 3). The transient downregulation of baseline cytokine transcript levels in the ceca may reflect the suppression of the mucosal immune response elicited by the commensal bacterial flora. Notably, comparison of cytokine transcript levels between PBS-injected and non-injected chickens revealed no differences (data not shown), indicating that the acute stress that may be associated with the handling of the animals did not cause changes in the expression of the tested genes.

### *Effect of glucocorticoids on* C. jejuni *induced inflammatory gene transcripts*

Next, we determined cytokine transcript levels for the complex combination of GC treatment plus bacterial challenge. Comparison of transcript levels in tissues from the GC-treated, *C. jejuni* challenged birds (group

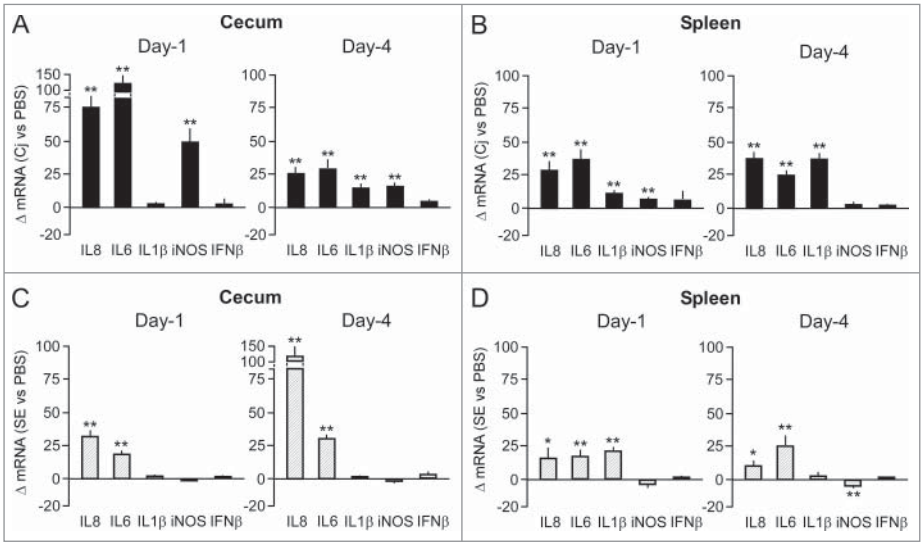


**Figure 2.** Effect of *C. jejuni* and *S.* Enteritidis colonization on cytokine mRNA levels. Transcript levels for the indicated cytokines were determined in cecal mucosa and spleen tissue isolated from individual birds at Day 1 and 4 after challenge with *C. jejuni* (panels A and B) or *S.* Enteritidis (panels C and D). RT-qPCR results were expressed as fold difference between the average mRNA levels in the indicated tissues of challenged chicken compared to (PBS-injected) control birds. Significant differences in ΔmRNA values were analyzed using log transformed data as described in Materials and Methods. Significant differences are indicated: $^{**}P < 0.005$; $^{*}P < 0.05$.

3) with those in the PBS-injected, non-challenged animals (group 2) demonstrated significantly reduced cecal mRNA levels for *IL-6, IL-8*, and *IL-1β* at Day-1 post challenge. In spleen tissue, the effect was limited to a minor increase in *IL-6* mRNA in the GC-treated,

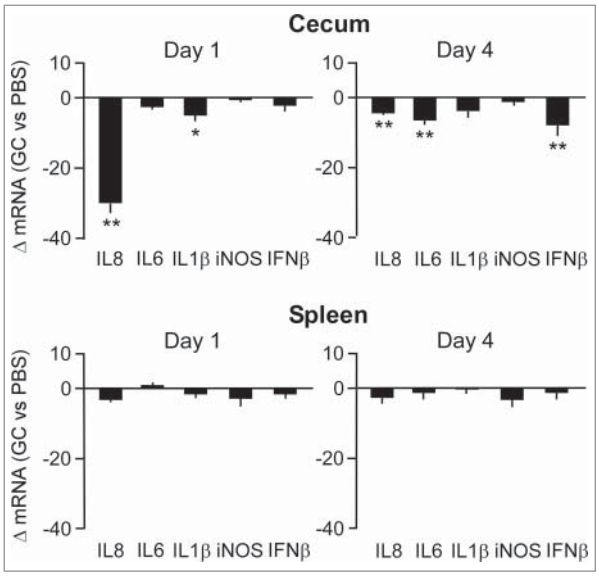


**Figure 3.** Effect of GC treatment on inflammatory gene expression in cecal mucosa and spleen tissue. Transcript levels of the indicated genes at Day 1 and 4 after injection of chicken with GC or PBS were determined by real-time RT-qPCR. Results are expressed as the mean ± SEM fold difference in tissue mRNA levels in the GC-treated vs. control animals. Significant differences in ΔmRNA values were analyzed using log transformed data as described in Materials and Methods. Significant differences are indicated: $^{**}P < 0.005$; $^{*}P < 0.05$.

*C. jejuni* challenged group (Fig. 4A). At Day 4 post-challenge, the reduced cytokine transcript levels in cecal tissue of the GC-treated and challenged animals had returned to baseline levels. At this point in time, a moderate increase in multiple pro-inflammatory gene transcripts was measured in spleen tissue (Fig. 4A).

To more specifically determine the strong dampening effect of the GC treatment on the *C. jejuni*-induced tissue response, we compared gene transcript levels in the GC-treated, *C. jejuni* challenged animals (group 3) with those in the non-GC treated, *C. jejuni*-challenged chickens (group 4). This demonstrated that GC treatment reduced the *C. jejuni*-induced *IL-8* and *IL-6* response by >150-fold at Day 1 post-challenge (Fig. 4B). On Day 4, the strong immunosuppressive effect had virtually disappeared. In spleen tissue, GC treatment reduced several of the measured *C. jejuni*-induced cytokine transcripts by a factor 5 to 20 on both Day 1 and Day 4 (Fig. 4B).

To learn whether *C. jejuni* was still able to induce a cytokine response in the GC-treated birds, we compared the gene transcript levels in the cecal tissue of the GC-treated, *C. jejuni* challenged animals (group 3) with those of the GC-treated, non-challenged chickens (group 1). This showed only a small increase in the cecal *IL-8* and *iNOS* mRNA levels after *C. jejuni* challenge (Fig. 4C). This indicates that GC treatment largely prevents the robust *C. jejuni*-induced inflammatory response that is observed after *C. jejuni* infection of non GC-treated chickens (Fig. 2A). This effect may contribute to the observed more rapid colonization of the ceca and liver in these animals (Fig. 1).

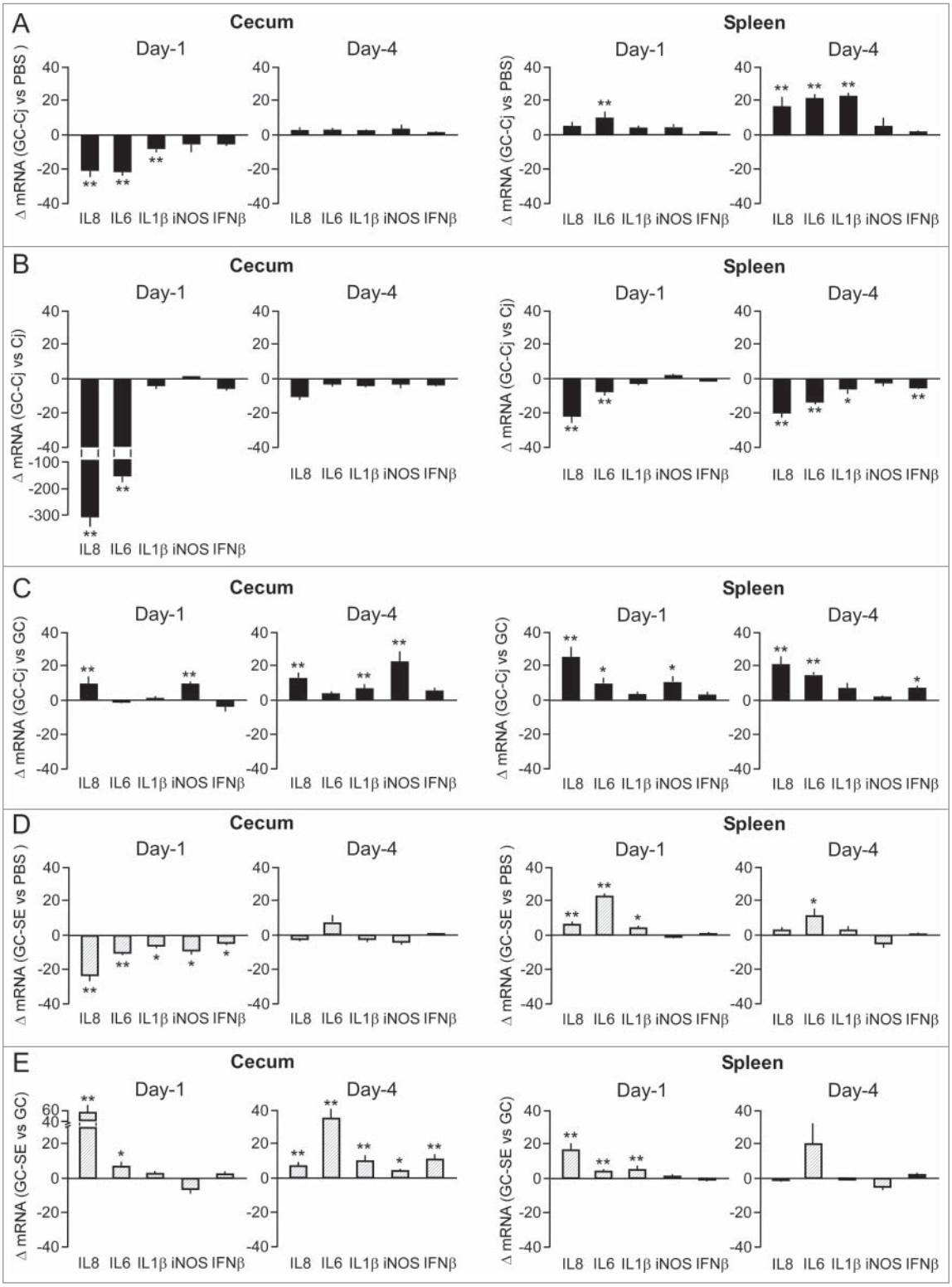


**Figure 4.** Effect of the combination of GC treatment plus challenge with *C. jejuni* or *S.* Enteritidis on inflammatory gene expression. Chicken were injected with GC or PBS and after 24 h challenged with *C. jejuni* (panels A and C) or *S.* Enteritidis (panels D and E). Real-time RT-qPCR was performed on mRNA isolated from cecal mucosa and spleen tissue collected at Days 1 and 4 post-challenge. Results are expressed as the mean ± SEM fold difference in mRNA levels in GC-treated, treated and challenged chicken versus PBS-injected and non-challenged animals (panels A and D), PBS-injected and *C. jejuni* challenged chicken (panel B), or GC-treated and non-challenged chicken (panels C and E). Significant differences in ΔmRNA values were analyzed using log transformed data as described in Materials and Methods. Significant differences are indicated: $^{**}P < 0.005$; $^{*}P < 0.05$.

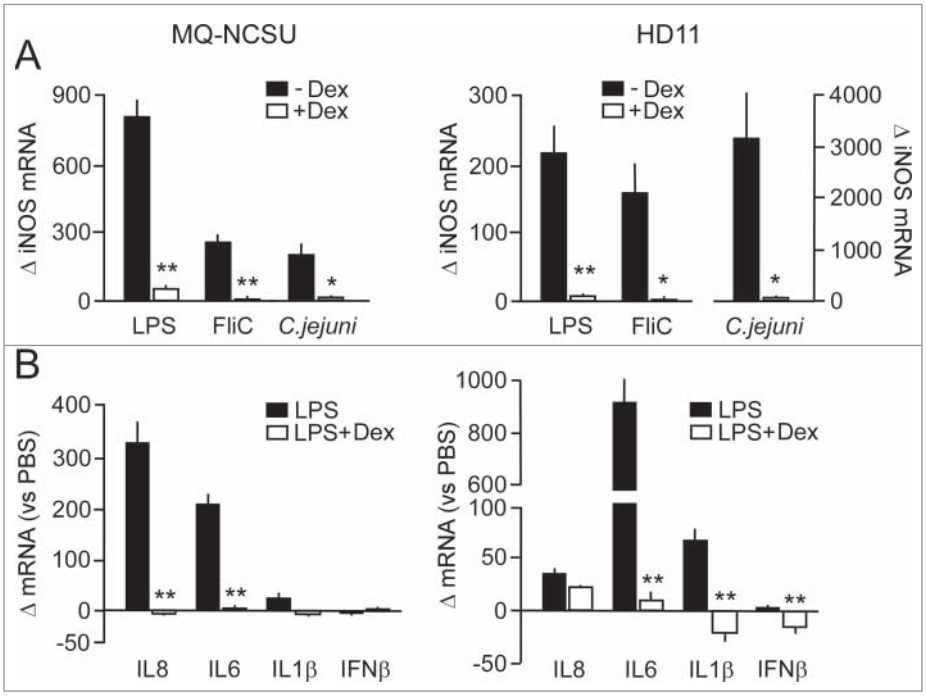


**Figure 5.** Effect of GC-treatment gene TLR- and *C. jejuni*-induced gene transcription in chicken macrophages. (A) MQ-NCSU and HD11 cells pre-incubated (17 h) with or without dexamethasone were stimulated with LPS (100 ng/ml) or flagellin (FliC, 1 $\mu$g/ml) for 16 h or with live *C. jejuni* ($2 \times 10^5$) for 8 h. Then, real-time RT-qPCR was performed on mRNA isolated from the cells to measure differences in *iNOS* mRNA levels between the Dex-treated and control cells. (B) Fold difference in the indicated inflammatory gene transcript levels in the GC-treated and non-GC treated cells after stimulation with LPS (100 ng/ml, 16 h). All results are expressed as the mean ± SEM fold difference in the transcript levels between the Dex-treated ands control cells (n = 3). For each response significant differences between Dex-treated and control cells are indicated: $^{**}P < 0.001$; $^{*}P < 0.05$.

### *Effect of glucocorticoids on S. enteritidis induced inflammatory gene transcripts*

Comparative analysis of gene transcripts in tissues derived from GC-treated chicken challenged with *S.* Enteritidis (group 5) with those from non-infected control chickens (group 2) indicated that the GC treatment inhibited the *Salmonella*-induced increase in intestinal *IL-8* and *IL-6* response at both Day-1 and Day-4 (compare Fig. 4D vs 2C). In spleen tissue the changes in *IL-8, IL-6* and *IL-1β* transcript levels were also less pronounced than in the non-GC treated, *S.* Enteritidis challenged animals (cf. Fig. 4D vs 2D). When the transcript data of the GC-treated, non-challenged animals were used as a reference, moderate increases in mainly *IL-8* (Day-1) and *IL-6* transcript levels (Day-4) were measured for both intestinal and spleen tissue samples (Fig. 4E). Overall, these data point to the presence of a GC treatment-induced attenuation of the host response toward *S.* Enteritidis, as was seen for *C. jejuni*.

### *GC modulation of the TLR response*

In search for a potential explanation of the apparent attenuated inflammatory response in the GC-treated chickens, we investigated the expression of inflammatory genes and the production of nitric oxide (NO) in 2 different chicken macrophages cell lines (MQ-NCSU and HD11). Hereto, cells were exposed to the glucocorticoid dexamethasone ($10^{-6}$ M) or solvent solution for 17 h prior to the addition of defined bacterial TLR agonists. RT-qPCR analysis on mRNA isolated from the non GC-treated macrophages demonstrated a strong induction (150–200 fold) of *iNOS* transcript after exposure of the cells to LPS, flagellin, and live *C. jejuni* strain 81116 (Fig. 5A). GC pretreatment of the cells fully abrogated this response in both cell lines. A similar strong reduction was measured for the *IL-8* and *IL-6* response in MQ-NCSU cells and for the *IL-6, IL-1β* and *IFNβ* transcripts in HD11 cells (Fig. 5B).

To ensure that the observed changes in inflammatory gene transcription translated to the protein level, we measured the production of cellular nitric oxide (NO) under the different conditions. This revealed that both bacterial LPS and flagellin (FliC) induced a dose-dependent increase in NO production in the chicken macrophages (Fig. 6A and B, filled bars), in line with the observed induction of *iNOS* mRNA (Fig. 5A). Again, GC pretreatment ($10^{-6}$ M, 17 h) of the cells strongly reduced these responses (Figs. 6A and B, open bars). The

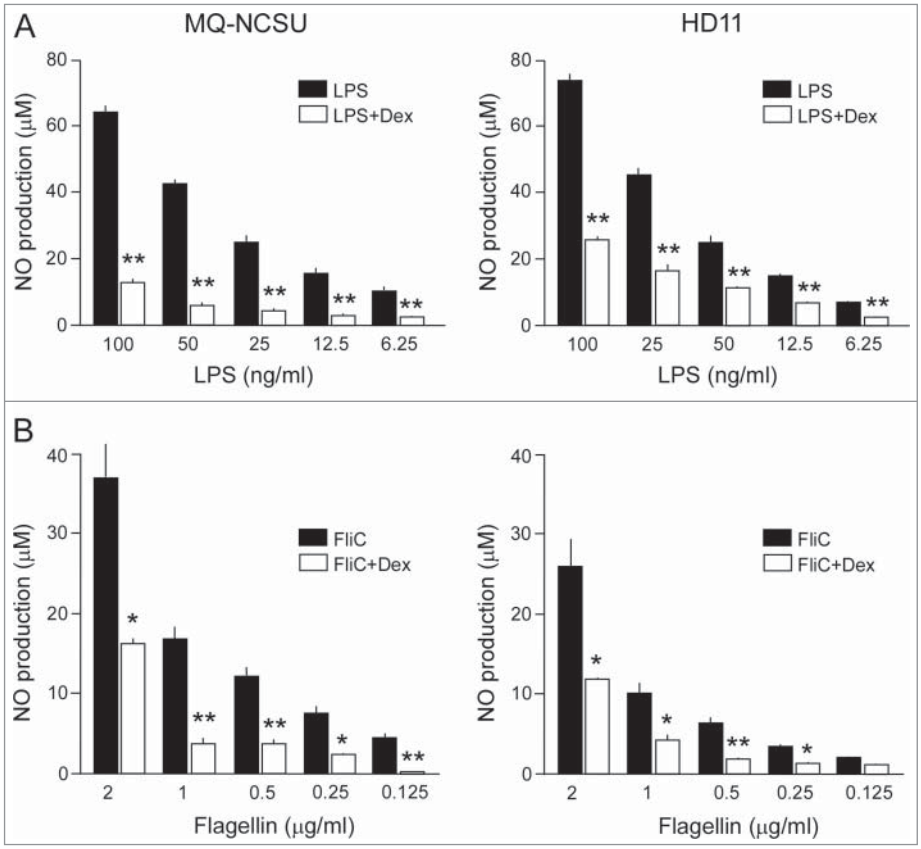


**Figure 6.** Effect of GC treatment on LPS or flagellin-induced production of nitric oxide in chicken macrophages. NCSU and HD11 chicken macrophages were incubated with dexamethasone (Dex, $10^{-6}$ M) for 17 h and then stimulated with the indicated concentrations of LPS (A) or flagellin (FliC) (B). After 24 h of stimulation, NO production was measured using the Griess assay. Results are the mean ± SEM of 6 (A) and 4 (B) experiments. Significant differences in ligand-induced NO production between Dex-treated and control cells are indicated: $^{**}P < 0.005$; $^{*}P < 0.05$.

inhibitory effect required preincubation of the cells with GC. The effect was not observed when GC and the TLR agonists were added simultaneously (data not shown). Together, these results indicate that GC strongly inhibits both the TLR agonist- and bacteria-induced stimulation of the inflammatory response in chicken macrophages. This is in line with the observed attenuated *in vivo* response and may contribute to the observed invasiveness of *C. jejuni* in GC-treated chickens.

## Discussion

In the present study, we investigated the role of the host immune defense in the behavior of *C. jejuni* in chickens. We provide evidence that GC treatment results in more rapid intestinal colonization and dissemination of *C. jejuni* to the liver in conjunction with a reduced pro-inflammatory gene expression at the infection niche. Stimulation of macrophages with different TLR ligands demonstrated strong inhibition of the chicken TLR response for GC-treated cells confirming that GC weakens at least one component of the innate host defense. Our results support the concept that *C. jejuni* has the intrinsic property to spread in chicken but that the natural host defense may limit *C. jejuni* invasion and dissemination to distant organs.

The rationale for our study was the still unexplained host specificity of *C. jejuni* infection. *C. jejuni* is the leading cause of bacterial enterocolitis worldwide but relatively rarely induces clinical manifestations in the chicken.[8,10] Potential reasons for this difference are numerous and may range from a different expression of bacterial virulence traits or mucosal receptors to the existence of a different microbiota in the human and chicken host. Alternatively, it can be imagined that chickens have a more effective host defense against *C. jejuni*, although this appears to vary between chicken breeds.[7,27] We investigated the influence of the chicken immune response on the colonization and dissemination of *C. jejuni* in chicken by administering GC to the animals prior to *C. jejuni* challenge. GC has immunosuppressive effects, also in the chicken.[28,29] Our results indicate that administration of GC to chicken indeed results in a downregulation of baseline transcript levels of distinct

inflammatory genes in the cecal tissue (Fig. 3). This result likely reflects the GC-induced dampening of the innate immune response.

Challenge of Ross 308 chicken with *C. jejuni* strain 81116 resulted in a more rapid colonization of the ceca and bacterial spread to the liver in the GC-treated animals (Fig. 1). The faster colonization of *C. jejuni* after GC treatment was unexpected. This effect was less pronounced for *S.* Enteritidis and has not been observed with *S.* Typhimurium.[30] One possible explanation for the more rapid colonization with *C. jejuni* may be that GC treatment induces *C. jejuni* favorable alterations in the intestinal microenvironment by influencing e.g. the composition of the microbiota, the production or composition of the mucus, or the status of the local antimicrobial defense.[6,31] Alternatively, the lack of a potent innate immune response may contribute to the rapid bacterial expansion after GC treatment.

The rapid dissemination of *C. jejuni* to the liver in the GC-treated birds indicates that the bacterium has the intrinsic ability to spread to distant organs. Dissemination of *C. jejuni* to the blood and liver of chickens occurs infrequently and seems to vary between bacterial strains and chicken lines.[5,32,33] Multiple mechanisms may contribute to the dissemination in the GC-treated animals. One hypothesis is that the more rapid cecal colonization ($3.10^9$ CFU at Day-2 after challenge) simply results in a more rapid bacterial dissemination. Another is that GC treatment causes a general increased translocation of bacteria across the intestinal barrier. We considered these explanations as less likely as GC treatment has been reported to support rather than weaken intestinal barrier function.[34,35] We favored the scenario that the GC-induced dampening of the local innate immune response facilitates the bacterial spread to distant organs.

Analysis of the expression of inflammatory genes in the cecal tissue after challenge with *C. jejuni* revealed a strong increase *IL-6, IL-8* and *iNOS* transcripts. This pro-inflammatory signature in the non-GC treated animals indicates that *C. jejuni* is sensed by the chicken immune system and elicits a potent local immune response as previously noted.[7-10,36-38] The measured gene expression levels induced by *C. jejuni* were relatively high but this may obviously vary between chicken lines and depend on the composition of the microbiota which likely determines the baseline cytokine transcript levels. GC treatment reduced the baseline transcript levels in the cecal tissue and these levels barely increased after challenge with *C. jejuni* (Fig. 4B). This lack of response is likely caused by the immunosuppressive effect of GC on the mucosal cells but perhaps also by a reduced influx of inflammatory cells into the mucosal tissues. Both instances result in a weakened host defense and thus may contribute to the more invasive behavior of *C. jejuni*.

To ascertain that GC administration can limit the innate immune response in chicken cells, we tested the effect of GC on chicken macrophages *in vitro*. Avian cells respond to *C. jejuni* with increased transcript levels of cytokines and chemokines and the production of nitric oxide.[18,39-41] This response may involve the activation of different types of pathogen recognition receptors. One major molecular mechanism via which GC may limit the mucosal host defense is the inhibition of the TLR response.[42,43] TLRs are key sensors of environmental danger signals including microbial products and are major drivers of the innate immune response. The chicken TLR repertoire is well established and has been shown to respond to *C. jejuni*.[18] Our finding that GC administration severely dampens chicken macrophage gene transcription and nitric oxide production in response to the TLR agonists LPS (TLR4) and flagellin (TLR5) as well as after exposure to *C. jejuni* (Figs. 5 and 6) shows that the TLR pathway in chickens is responsive to GC. These results support the scenario that GC administration contributes to the invasive behavior of *C. jejuni* at least partially by suppression of the local innate immune response. To some extent, this situation may resemble observations in mice that show that animals with innate immune deficiencies become prone to *C. jejuni* infection.[44-46]

Overall, our results for the first time demonstrate that GC treatment of chickens dampens the intestinal immune defense and causes a more rapid colonization and dissemination of *C. jejuni* in chicken. The data imply that *C. jejuni* has the intrinsic ability to invade chicken tissue and that the innate defense is important to limit this invasive behavior.

## Materials and methods

### *Bacterial culture*

*C. jejuni* strain 81116 (NTCT 11828)[47,48] was grown on Blood agar base II medium (Oxoid) containing 5% horse blood lysed with 0.5% saponin at 42°C under microaerophilic (5% $O_2$, 10% $CO_2$, 85% $N_2$) conditions. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis, nalidixic acid resistant) strain CVI-1 was grown on Luria-Bertani (LB) plates (Biotrading) at 37°C. Bacteria from cloacal swabs and serial dilutions of tissue homogenates were grown on *Campylobacter* selective blood-free agar plates with CCDA-selective (Oxoid SR0155E) supplement (*C. jejuni*) or on Brilliant Green Agar supplemented with 100 $\mu$g/ml of nalidixic acid (*S.* Enteritidis) to suppress growth of resident flora. For use in challenge

experiments, *C. jejuni* and S. Enteritidis were grown in Heart Infusion broth and Brain Heart Infusion broth (Biotrading) with nalidixic acid (Sigma-Aldrich) respectively, collected by centrifugation (4,000 × *g*, 10 min), and resuspended in PBS. Bacterial suspensions ($10^5$ CFU in 0.25 ml) were administered orally to the chicken using a 1 ml syringe that was carefully placed deeply into the mouth (7 cm).

### Cell culture

The chicken macrophage HD11[49] and MQ-NCSU[50] cell lines were propagated in 25 $cm^2$ tissue culture flasks (Corning) containing 5 ml of Dulbecco's modified Eagle medium (Life Technologies) supplemented with 5% fetal bovine serum (FBS) at 37°C and 10% $CO_2$. For use in cell stimulation assays, cells were seeded onto 12- or 24-well plates ($10^5$ cells/well) in 1 ml of DMEM plus 5% FBS per well.

### Chicken experiments

Fertilized SPF (*Campylobacter* and *Salmonella* free) chicken eggs (Ross 308) were kept at 38°C and 65–75% relative humidity in a forced air chicken egg incubator. After hatch, the chickens were divided into 6 groups (1–6) of 27 birds and housed in pens with *ad libitum* access to water and feed. Bacterial cultures of cloaca swabs taken at Day 1 after hatch confirmed the *Campylobacter* and *Salmonella* negative status of the animals. At Day 17 after hatch groups 1, 3, and 5 received the synthetic glucocorticoid methylprednisolone (Depo-Medrol®, intramuscular, 10 mg/kg body weight; Pfizer), while groups 2, 4, and 6 were injected with phosphate buffered saline (PBS). At 24 h after administration, groups 3 and 4 were challenged orally with $10^5$ CFU of *C. jejuni* strain 81116, while group 5 and 6 were challenged with the equivalent number of *S.* Enteritidis. Groups 1 and 2 served as non-challenged controls.

At Days 1, 2, 3, and 4 after bacterial challenge, at least 5 birds from each group were sacrificed by cervical dislocation by professional staff to be able to determine the number of *C. jejuni* or *S.* Enteritidis in the cecal contents and liver, and to collect tissues for transcript analysis (see below). For bacterial enumeration, serial dilutions of 1 g of cecal content or 1 g of liver tissue homogenized in 3 ml of peptone water, were grown as described above. The number of colonies (CFU) was counted after 24–48 h of incubation. Collected tissues were snap-frozen in liquid nitrogen and stored at −80°C until further analysis. All chicken were cared for in accordance with accepted procedures of the Dutch law of animal welfare and all animal experiments were approved by the Ethics Committee of the Central Veterinary Institute of Wageningen University, Lelystad, the Netherlands.

A gamma generalized linear model with a log link function (as the CFU data were continuous, positive, right-skewed and with both constant variance and normally distributed residuals on the log scale) was used to indicate statistically significant differences between treated and non-treated chicks. This method allows assessing the overall effect of GC treatment on *Campylobacter* and *Salmonella* CFUs in the chicks' ceca and livers over the entire course of bacterial colonization, while accounting for the day of sampling.

### In vitro *treatment of cultured chicken cells*

To measure the effects of GC on cultured chicken cells, dexamethasone (Sigma) or the equivalent amount of solvent (ethanol, final concentration <0.5%) was added at 12 h after seeding of the cells onto the 12- or 24-well plates (low cell density). Seventeen hours later, cells were stimulated with the indicated amounts of purified LPS, flagellin, or bacteria. After the indicated incubation periods (37°C, 10% $CO_2$), cell culture supernatants were collected for measurement of nitric oxide (NO). The cells were treated with RNA-Bee™ (Bio-connect) to extract RNA for transcript analysis (see below). Purified LPS and *Salmonella* flagellin (FliC) were isolated as previously described.[14,51]

### RNA isolation from tissue and cells

RNA was isolated from the collected tissues by placing approximately 50 mg of tissue in a LYSING matrix tube (MP Biomedical GmbH) containing 1 ml of RNA-BEE™ on ice. Cells were disrupted in MagNA lyser instrument (Roche) (6,500 × g, 50 sec, 20°C). Total RNA was extracted from the lysate using the RNA-Bee™ isolation kit according to the instruction of the manufacturer. Contaminating DNA was removed by treatment of the RNA samples with DNase (1 U/$\mu$g of RNA, Fermentas). The quantity and purity of the isolated RNA was verified using a NanoDrop ND-1000 spectrophotometer. For isolation of RNA from cultured cells, $10^5$ cells were collected in 250 $\mu$l of RNA-Bee™ and directly subjected to the RNA-Bee extraction method described above. Purified RNA was stored in 25 $\mu$l of RNase-free water and stored at -80°C until further analysis.

### Quantitative real-time RT-PCR analysis

Quantitative real-time PCR on isolated RNA was routinely performed using the Reverse Transcriptase RT-qPCR Master Mix kit (Eurogentec). The reaction was performed in a Roche LightCycler®480 using 50 ng of

**Table 1.** Primers and probes used in this study.

| | | |
|---|---|---|
| chGAPDH | Forward | GCCGTCCTCTCTGGCAAAG |
| | Reverse | TGTAAACCATGTAGTTCAGATCGATGA |
| | probe | (FAM)-AGTGGTGGCCATCAATGATCC-(TAMRA) |
| chIL-8 (CXCL8) | Forward | GCCCTCCTCCGGTTTCAG |
| | Reverse | CGCAGCTCATTCCCCATCT |
| | probe | (FAM)-TGCTCTGTCGCAAGGTAGGACGCTG(TAMRA) |
| chIL-1ß | Forward | GCTCTACATGTCGTGTGTGATGAG |
| | Reverse | TGTCGATGTCCCGCATGA |
| | probe | (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA) |
| chIFN$\beta$ | Forward | ACAACTTCCTACAGCACAACAACTA |
| | Reverse | GCCTGGAGGCGGGACATG |
| | probe | (FAM)-TCCCAGGTACAAGCACTG-(TAMRA) |
| chIL-6 | Forward | 5′- GCT CGC CGG CTT CGA |
| | Reverse | 5′-GGT AGG TCT GAA AGG CGA ACA G |
| | Probe | 5′-AGG AGA AAT ACC TGA CGA AGC TCT CCA-(TAMRA) |
| chiNOS | Forward | GGCAGCAGCGTCTCTATGACTTG |
| | Reverse | GACTTTAGGCTGCCCAGGTTG |
| chG6PDH | Forward | CGGGAACCAAATGCACTTCGT |
| | Reverse | CGCTGCCGTAGAGGTATGGGA |

DNase I-treated RNA samples as template and the primer sets and probes listed in Table 1. Probes for *IL-8 (CXCL8-CXCLi2), IL-6, IL-1β* and *IFNβ* were labeled with the fluorescent reporter dye 5-carboxy-fluorescein (FAM) at the 5′-end and with the quencher N, N, N, N'- tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′-end (Eurogentec). Inducible isoform of nitric oxide synthase (iNOS) transcripts were measured using the one-step RT-qPCR Master Mix Plus SYBR Green1 kit (Eurogentec). The following reaction conditions were used: Reverse Transcription step at 48°C for 30 min followed by incubation for 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. Each sample was run in duplicate. Non-reverse transcriptase-treated samples, a template free sample, and a nuclease free water sample served as controls. Transcript levels were normalized to those for the chicken internal control genes glyceraldehyde-3-phosphate dehydrogenase (*chGAPDH*) or glucose-6-phosphate dehydrogenase (*chG6PDH*, HD11 cells only). For each gene, results were expressed as fold change in mRNA level compared to the PBS-injected, GC-injected or *C. jejuni* challenged control group according to Schmittgen & Livak[52] using the formula: (1) $\Delta$ $C_t$ target gene - $\Delta$ $C_t$ GAPDH (or G6PDH) for each sample, (2) $\Delta$ $C_t$ target gene treated - $\Delta$ $C_t$ target gene control. The fold change for each gene transcript was determined using: Fold change $= 2^{-\Delta(\Delta \text{ Ct gene treated } -\Delta \text{ C gene controll})}$. To calculate the relative expression levels for each of the bird groups, the transcript levels in samples of individual birds were compared with the mean value of the group of control birds, yielding a mean $\pm$ SEM value for the treated groups. The SEM values thus represent the variation in fold difference between individual chicken. The expression of the internal control genes was not influenced by the GC treatment.

Statistical analysis on all RT-qPCR results was performed on log transformed data the GraphPad Prism 6 multiple *t* test corrected for multiple comparison using the Holm-Šidák method, with $\alpha = 5.000\%$

### *Nitric oxide assay*

For measurement of nitric oxide (NO) production, the Griess assay was employed. In brief, cell supernatants collected after 17 h of incubation with the bacterial TLR agonist (or controls) were incubated (10 min, 20°C, in dark) with an equal volume (50 $\mu$l) of Griess reagent 1 (1% sulfanilamide, Sigma-Aldrich). Then, an equal volume of Griess reagent 2 (0.1% N-naphthyl ethylene diamine dihydrochloride (Sigma-Aldrich) in 2.5% phosphoric acid) was added. After an additional 5 min of incubation in the dark, absorbance was measured at 550 nm in a spectrophotometer. The amount of produced NO was calculated from a calibration curve established by serial dilution (1–100 $\mu$M) of sodium nitrite ($NaNO_2$) in tissue culture medium. Data were analyzed using the GraphPad Prism 6 multiple *t* test. Values are expressed as the mean +/− SEM of at least 3 independent experiments.

## Abbreviations

| | |
|---|---|
| *C. jejuni* | *Campylobacter jejuni* |
| chGAPDH | chicken glyceraldehyde-3-phosphate dehydrogenase |
| chG6PDH | chicken glucose-6-phosphate dehydrogenase |
| CFU | Colony forming units |
| Dex | Dexamethasone |
| DMEM | Dulbecco's modified Eagle medium |
| FBS | Fetal bovine serum |
| GC | Glucocorticoid |
| IL | Interleukin |
| iNOS | Inducible isoform of nitric oxide synthase |
| LPS | Lipopolysaccharide |
| NO | Nitric oxide |
| PBS | Phosphate buffered saline |
| RT-qPCR | Reverse transcription polymerase chain reaction |
| *S.* Enteritidis | *Salmonella enterica* serovar Enteritidis |
| SPF | Specific pathogen free |
| TLR | Toll-like receptor. |

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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