

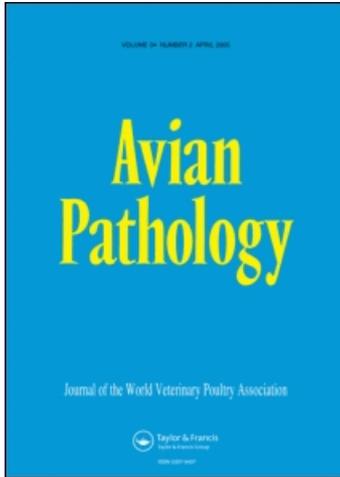
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Immunopathology and cytokine responses in commercial broiler chickens with gangrenous dermatitis

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Immunopathology and cytokine responses in commercial broiler chickens with gangrenous dermatitis

Guangxing Li^{1,2}, Hyun S. Lillehoj^{1*}, Kyung Woo Lee¹, Sung Hyen Lee¹, Myeong Seon Park¹, Seung I. Jang¹, Gary R. Bauchan³, Cyril G. Gay⁴, G. Donald Ritter⁵, Daniel A. Bautista⁶ and Gregory R. Siragusa⁷

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Gangrenous dermatitis (GD) is an emerging disease of increasing economic importance in poultry resulting from infection by *Clostridium septicum* and *Clostridium perfringens* type A. Lack of a reproducible disease model has been a major obstacle in understanding the immunopathology of GD. To gain better understanding of host–pathogen interactions in GD infection, we evaluated various immune parameters in two groups of birds from a recent commercial outbreak of GD, the first showing typical disease signs and pathological lesions (GD-like birds) and the second lacking clinical signs (GD-free birds). Our results revealed that GD-like birds showed: reduced T-cell and B-cell mitogen-stimulated lymphoproliferation; higher levels of serum nitric oxide and α -1-acid glycoprotein; greater numbers of K55⁺, K1⁺, CD8⁺, and MHC class II⁺ intradermal lymphocytes, and increased K55⁺, K1⁺, CD8⁺, TCR1⁺, TCR2⁺, Bu1⁺, and MHC class II⁺ intestinal intraepithelial lymphocytes; and increased levels of mRNAs encoding proinflammatory cytokines and chemokines in skin compared with GD-free chickens. These results provide the first evidence of altered systemic and local (skin and intestine) immune responses in GD pathogenesis in chickens.

Introduction

Gangrenous dermatitis (GD) is a disease of poultry that is caused by the Gram-positive, spore-forming, anaerobic bacilli *Clostridium septicum* and *Clostridium perfringens* type A (Wages & Opengart, 2003; Tellez *et al.*, 2009). In chickens as well as in turkeys, GD infection causes significant economic losses in meat production (Bains & MacKenzie, 1975; Fowler & Hussaini, 1975). Since the first reported case in 1930, GD infections have been reported worldwide (Wages & Opengart, 2003). While the incidence of GD outbreaks has decreased with the application of in-feed antibiotics, recent voluntary or legally mandated withdrawal of antibiotic growth promoters, and other anti-microbial drugs, threatens the re-emergence of GD as a major poultry disease (Neumann & Rehberger, 2009; Tellez *et al.*, 2009). According to the US Animal Health Association's Committee on Transmissible Diseases of Poultry and other Avian Species, GD has consistently ranked as a top disease concern for the poultry industry (Mataragas *et al.*, 2008). Furthermore, human food-borne illnesses due to the consumption of poultry products contaminated with *C. perfringens* pose a risk to public health (Holtby *et al.*, 2008).

Clostridium spp. are widely distributed in the environment and are commonly recovered from diseased

chickens (Songer, 1996; Bryant & Stevens, 1997). Following *C. septicum* and/or *C. perfringens* infection, a rapid and fatal toxemia usually results (Tveten, 2001). Gangrenous lesions are often observed and characterized by excessively red patches of skin devoid of feathers with underlying serosanguinous fluid accompanied by emphysema. The musculature is usually crepitant with gas present between muscle groups (Gomis *et al.*, 2002). In spite of these severe anatomic effects, the only immunopathologic outcome of GD that has been reported was atrophy of the bursa of Fabricius with an associated depletion of lymphocytes (Willoughby *et al.*, 1996).

Recently, an outbreak of GD in commercial broiler chickens with typical signs and pathological lesions in various tissues including the skin and intestine was reported (Li *et al.*, 2010). In the present study, we investigated systemic and local (skin and intestine) immune responses at the molecular and cellular levels in broilers with GD-like signs and their healthy cohorts from the same production facility. Our results demonstrated that GD-like birds with characteristic clinical signs of *Clostridium* infections showed altered immune functions compared with GD-free chickens, thereby

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enhancing our understanding of the *Clostridium*-chicken interactions that will facilitate the future development of effective prevention strategies against GD.

Materials and Methods

Background on chickens and GD outbreak. The chickens used in the present study were obtained from a poultry farmhouse where a GD outbreak occurred in March 2009 as described (Li *et al.*, 2010). The broiler chickens involved in field GD outbreak received normal seasonal vaccinations including Marek's disease, Newcastle disease, infectious bronchitis, and infectious laryngotracheitis. No therapeutic medication was used, and the feeding programme consisted of a corn, soybean and meat product diet with a shuttle drug programme of salinomycin (60 g/ton), bacitracin methylene disalicylate (50 g/ton), roxarsone (3-nitro: 45.4 g/ton) in the starter feed (0 to 18 days of age) followed by narasin/nicarbazin (Maxiban: 81 g/ton), bacitracin methylene disalicylate (50 g/ton), 3-nitro (45.4 g/ton) in the grower feed (19 to 34 days of age). Clinical signs included varying degrees of growth depression, loss of appetite, leg weakness, reluctance to move, diarrhoea, and incoordination. Total mortality in the affected house during the disease outbreak period of 5 days was 2.0%.

Tissue samples. Ten GD-like birds with obvious GD lesions and five clinically healthy birds, aged 34 days, from a commercial farm were selected for serum and tissue collection. Both *C. septicum* and *C. perfringens* have been isolated from the affected lesions of birds with GD lesions; that is, skin and intestine (Li *et al.*, 2010). Serum was collected by cardiac puncture and frozen until tested. After euthanasia, spleens were prepared aseptically in Hank's balanced salt solution (HBSS) (Sigma, St Louis, Missouri, USA). Skin, muscle, and intestine (including the duodenum, jejunum and ileum) were collected aseptically for mRNA analysis. Skin and intestinal jejunum near the Meckel's diverticulum were collected and snap-frozen in liquid nitrogen for immunochemistry analysis.

Mitogen-induced splenocyte proliferation. A single-cell suspension of the spleen was prepared by gently pressing through a screen sieve into Petri dishes containing HBSS. Single-cell suspensions were obtained by centrifugation through Histopaque-1077 (Sigma), and the cells were washed three times with HBSS and resuspended in RPMI medium containing 10% foetal bovine serum, 1.0 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 0.1 mM non-essential amino acid, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, pH 7.3, 2.0 mM glutamine, and 5 µg/ml 5-fluorocytosine (all from Sigma). Splenocytes were seeded at 5×10^5 /well in 96-well microtitre plates, stimulated with concanavalin A (Con A) (Sigma) or *Escherichia coli* lipopolysaccharide (LPS) (Sigma) at 0.625, 1.25, or 2.5 µg/ml in a humidified incubator at 41 °C with 5% CO₂ for 24 or 48 h. Splenocytes cultured with medium alone were used as negative controls. Following incubation, cell proliferation was measured using the Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Gaithersburg, Maryland, USA).

Serum nitric oxide levels. Serum nitric oxide (NO) levels were measured as described (Lillehoj & Li, 2004). Briefly, serum samples were centrifuged at $1000 \times g$ for 30 min at 4 °C, 100 µl was mixed with an equal volume of freshly prepared Griess reagent (Sigma) containing 1% (w/v) sulphanilamine in 5% phosphoric acid and 0.1% (w/v) *N*-naphthylethylenediamine, the mixture was incubated for 10 min at room temperature, and the optical density at 540 nm (OD₅₄₀) was measured using a microtitre plate reader (Bio-Rad, Richmond, California, USA). Nitrite concentrations were calculated from a standard curve using NaNO₂ (Green *et al.*, 1982).

Serum α -1-acid glycoprotein levels. Chicken α -1-acid glycoprotein (α -1-AGP) in serum was measured by enzyme-linked immunosorbent assay (Life Diagnostics, West Chester, Pennsylvania, USA) according to the manufacturer's instructions. Briefly, serum samples were diluted appropriately and incubated in 96-well microtitre plates for 45 min at room temperature, the plates were washed three times, and horseradish peroxidase-conjugated anti-chicken α -1-AGP antibody was added for 45 min at room temperature. The plates were washed, tetramethylene

blue substrate was added for 20 min at room temperature, and the OD₄₅₀ was measured.

Immunohistochemistry analysis. Frozen skin and jejunum samples were embedded in OCT medium (Sakura Finetek USA, Torrance, California, USA) and 7 µm cryostat sections were prepared (Ultrapro 500; Vibratome, St Louis, Missouri, USA), air-dried, and fixed in acetone for 10 min (Withanage *et al.*, 2003). The slides were blocked with diluted normal horse serum (1:30) (Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 30 min at room temperature and incubated for 2 h at room temperature with mouse monoclonal antibodies against total chicken lymphocytes (K55), macrophages (K1), T cells (CD4, CD8, TCR1, and TCR2), B cells (Bu1) or major histocompatibility complex (MHC) class I and II surface antigens (Table 1) followed by incubation for 2 h at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, Carlsbad, California, USA). The slides were counterstained with 0.01 mg/ml propidium iodide (Sigma) and observed with a fluorescent microscope (Carl Zeiss, Thornwood, New York, USA).

Quantitative reverse transcriptase-polymerase chain reaction. Total RNA was extracted from the intestine (including duodenum, jejunum, and ileum) and skin and the underlying muscle using TRIzol (Invitrogen) as described elsewhere (Hong *et al.*, 2006b; Park *et al.*, 2008). Five micrograms of total RNA were treated with 1.0 U DNase I and 1.0 µl of 10x reaction buffer (Sigma), incubated for 15 min at room temperature, then 1.0 µl stop solution was added, and the mixture was heated at 70 °C for 10 min. RNA was reverse transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, California, USA) according to the manufacturer's recommendations. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) oligonucleotide primers for chicken cytokines, chemokines, and GAPDH are presented in Table 2. Amplification and detection were carried out using equivalent amounts of total RNA from the intestine and skin/muscle using the M × 3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described elsewhere (Hong *et al.*, 2006b; Park *et al.*, 2008). Standard curves were generated using log₁₀-diluted standard RNA and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program (Muller *et al.*, 2002). Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle values for the amplification products was calculated by pooling values from all samples in that experiment.

Statistical analysis. All values were expressed as the mean ± standard deviation. Mean values were compared among the different groups by the Duncan's Multiple Range test following analysis of variance using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Differences between means were considered significant at $P < 0.05$. The levels of cytokine transcripts were plotted using GraphPad Prism version 4.03 for Windows (GraphPad, San Diego, California, USA).

Table 1. Monoclonal antibodies used in immunohistochemistry analysis.

Monoclonal antibody	Antigen specificity	Reference
CTLA 4	CD4	Lillehoj <i>et al.</i> (1988a)
CTLA 8	CD8	Lillehoj <i>et al.</i> (1988a)
K1	Macrophage/thrombocyte	Kaspers <i>et al.</i> (1993)
TCR1	$\gamma\delta$ T-cell receptor	Chen <i>et al.</i> (1986)
TCR2	$\alpha\beta$ T-cell receptor	Chen <i>et al.</i> (1986)
MHC I	MHC class I molecule	Pink <i>et al.</i> (1985)
MHC class II	MHC class II molecule	Lillehoj <i>et al.</i> (1988b)
Bu1	Bu 1 antigen	Rothwell <i>et al.</i> (1996)
K55	Pan lymphocyte	Cheung <i>et al.</i> (1991)

Table 2. Oligonucleotide primers used for quantitative real-time RT-PCR of chicken cytokines/chemokines.

RNA target	Primer sequence		PCR product (base pairs)	GenBank accession number
	Forward primer	Reverse primer		
GAPDH	5'-GGTGGTGCTAAGCGTGTAT-3'	5'-ACCTCTGTCATCTCTCCACA-3'	264	K01458
IFN- α	5'-GACATCCTTCAGCATCTTCA-3'	5'-AGGCGCTGTAATCGTTGTCT-3'	238	AB021154
IL-1 β	5'-TGGGCATCAAGGGCTACA-3'	5'-TCGGGTGGTTGGTGATG-3'	244	Y15006
IL-6	5'-CAAGGTGACGGAGGAGGAC-3'	5'-TGGCGAGGAGGGATTCT-3'	254	AJ309540
IL-17	5'-CTCCGATCCCTTATTCTCCTC-3'	5'-AAGCGTTGTGGTCCCTCAT-3'	292	AJ493595
TNFSF-15	5'-CCTGAGTATCCAGCAACGCA-3'	5'-ATCCACCAGCTTGATGTCCTAAC-3'	292	AB194710
LITAF	5'-TGTGTATGTGCAGCAACCCGTAGT-3'	5'-GGCATTGCAATTTGGACAGAAGT-3'	229	AY765397
IFN- γ	5'-AGCTGACGGTGGACCTATTATT-3'	5'-GGCTTTGCGCTGGATTTC-3'	259	Y07922
IL-2	5'-TCTGGGACCACTGTATGCTCT-3'	5'-ACACCAGTGGGAAACAGTATCA-3'	256	AF000631
IL-10	5'-CGGGAGCTGAGGGTGAA-3'	5'-GTGAAGAAGCGGTGACAGC-3'	272	AJ621614
IL-12p40	5'-AGACTCCAATGGGCAAATGA-3'	5'-CTCTTCGGCAAATGGACAGT-3'	274	NM_213571
IL-4	5'-ACCCAGGGCATCCAGAAG-3'	5'-CAGTGCCGGCAAGAAGTT-3'	258	AJ621735
IL-13	5'-CCAGGGCATCCAGAAGC-3'	5'-CAGTGCCGGCAAGAAGTT-3'	256	AJ621735
IL-8	5'-GGCTTGCTAGGGGAAATGA-3'	5'-AGCTGACTCTGACTAGGAAACTGT-3'	200	AJ009800

Results

Mitogen-induced splenocyte proliferation. Splenocytes from the clinically healthy and GD-like birds were used to assess general lymphocyte function following *in vitro* stimulation with Con A (T-cell mitogen) or LPS (B-cell mitogen). Decreased lymphoproliferation in response to Con A and LPS were observed in the GD-like birds compared with GD-free chickens at wide ranges of mitogen doses (Table 3).

NO production. Serum NO concentrations are commonly measured as an indicator of inflammation using an *in vitro* assay (Stuehr & Nathan, 1989; Pertile *et al.*, 1996). As shown in Figure 1, serum NO levels in GD-like chickens were greater than those of the healthy controls.

Acute phase protein in serum. GD-like chickens exhibited higher levels of serum α -1-AGP compared with GD-free chickens (Figure 2).

Immunohistochemistry analysis. While no infiltrating cells that stained for the K55 pan lymphocyte marker were seen in the subcutaneous layer of the skin from GD-free

chickens (Figure 3a), abundant K55⁺ cells were evident in GD-like birds (Figure 3b,c). Infiltrating cells expressing the K1 (macrophage) and CD8 cell surface antigens also were evident in GD-like chickens (Figure 3d,e). Cells expressing MHC class II antigens were observed in the dermis and epidermis, and large cell aggregates of MHC class II-expressing cells were occasionally seen in the dermis of GD-like chickens (Figure 3g,h). By contrast, few MHC class II⁺ cells were found beneath the epidermis (Figure 3f). No cell staining in the skin of GD-like or GD-free chickens was seen using monoclonal antibodies against CD4, TCR1, TCR2, or Bu1 (data not shown).

In the intestine, K55⁺ cells were distributed in the mucosal and submucosal layers of GD-like chickens (Figure 4a), whereas an obvious decrease was observed in the clinically healthy birds (data not shown). K1⁺ cells were relatively few in the submucosal layer (Figure 4b), while a higher number of CD8⁺ cells were detected mainly in the mucosal layer (Figure 4c) of GD-like chickens. Neither cell types were seen in GD-free birds (data not shown). TCR1⁺ cells in the mucosal layer (Figure 4d) and TCR2⁺ cells in the lamina propria (Figure 4e) of chickens with GD-like signs were also

Table 3. Splenocyte proliferative response of GD-like and clinically healthy chickens.

Treatment ^a	Birds ^b	Con A group ^c		LPS group ^c	
		24 h	48 h	24 h	48 h
Medium	H	0.869 ± 0.147	1.080 ± 0.299	0.938 ± 0.204	0.984 ± 0.272
	S	0.739 ± 0.283*	0.770 ± 0.173**	0.682 ± 0.131**	0.724 ± 0.173*
2.5 μ g/ml	H	0.902 ± 0.214	1.109 ± 0.283	1.053 ± 0.179	1.089 ± 0.198
	S	0.757 ± 0.192*	0.771 ± 0.114**	0.677 ± 0.174**	0.677 ± 0.228**
1.25 μ g/ml	H	1.093 ± 0.154	1.097 ± 0.297	1.057 ± 0.173	1.081 ± 0.194
	S	0.713 ± 0.133**	0.728 ± 0.134**	0.722 ± 0.163**	0.733 ± 0.256**
0.625 μ g/ml	H	1.030 ± 0.233	1.002 ± 0.296	0.992 ± 0.185	1.089 ± 0.195
	S	0.683 ± 0.148**	0.798 ± 0.166**	0.700 ± 0.136**	0.669 ± 0.216**

^aSplenocytes from GD-like and clinically healthy birds at 5×10^6 /ml were stimulated with medium control or Con A or LPS at the indicated concentrations for 24 h or 48 h.

^bH, clinically healthy; S, GD-like.

^cEach value represents the mean \pm standard deviation of triplicate samples from five healthy birds and 10 GD-like chickens.

* $P < 0.05$.

** $P < 0.01$ decreased proliferative response in GD-like chickens compared with healthy chickens.

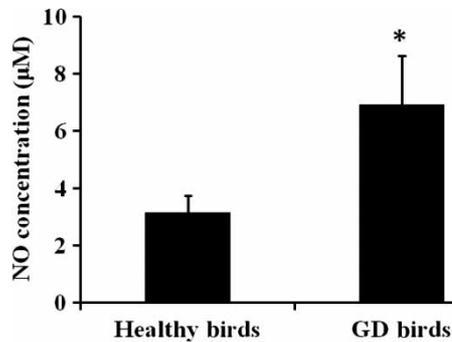


Figure 1. NO concentrations in the serum of GD-like and clinically healthy chickens. Each bar represents the mean \pm standard deviation of triplicate samples from 10 GD-like birds and five clinically healthy birds. *Significantly increased NO levels in GD-like birds compared with healthy birds ($P < 0.01$).

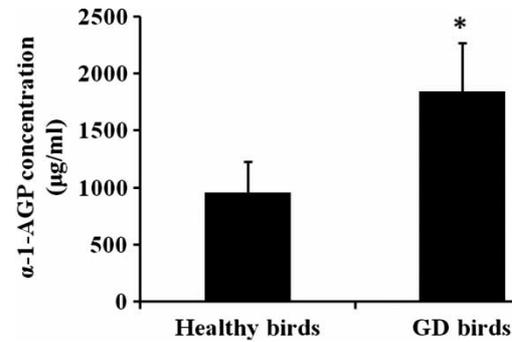


Figure 2. Chicken α -1-AGP concentrations in the serum from GD-like and clinically healthy chickens. Each bar represents the mean \pm standard deviation of triplicate samples from 10 GD-like birds and five clinically healthy birds. *Significantly increased α -1-AGP levels in the GD-like birds compared with the healthy birds ($P < 0.01$).

apparent. A significantly decreased number of Bu1⁺ cells was found in the mucosal and submucosal layers (Figure 4f,g) of the intestine in the GD-like chickens compared with the healthy birds. In the case of cells expressing MHC class II, GD-free birds showed staining in the submucosa and lamina propria (Figure 4h,i) while GD-like chickens demonstrated staining in the mucosal and submucosal layers (Figure 4j,k). Furthermore, there appeared to be more MHC class II⁺ cell aggregates in the submucosal layer of GD-like birds (Figure 4l).

Gene expression of immune-related genes in the skin and intestine. The skin/muscle and intestinal levels of mRNAs

encoding proinflammatory (interferon (IFN)- α , interleukin (IL)-1 β , IL-6, IL-17, TNFSF-15, LITAF), Th-1 type (IFN- γ , IL-2, IL-12 p40), Th-2 type (IL-4, IL-10, IL-13) cytokines and the chemokine IL-8 from GD-like and clinically healthy chickens were examined by quantitative RT-PCR. In general, the levels of mRNAs encoding immune-related molecules other than proinflammatory cytokines were decreased. As shown in Figure 5, the levels of nine transcripts (IFN- γ , IL-1 β , IL-2, IL-4, IL-10, IL-12 p40, IL-13, IL-17) were suppressed in the skin of GD-like compared with GD-free birds. In contrast, four transcripts (IFN- α , TNFSF-15, LITAF, IL-8) were

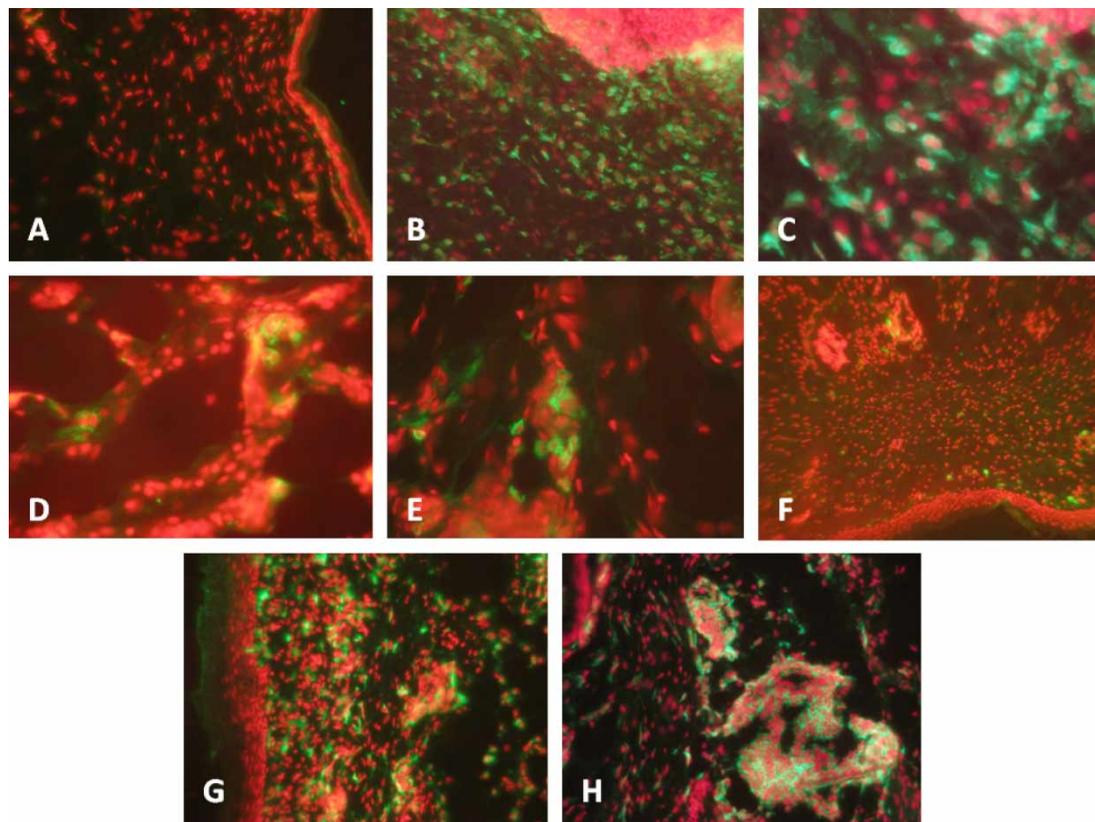


Figure 3. Immunohistochemical analysis of leukocyte subpopulations in the skin of GD-like and clinically healthy chickens. 3a: No K55⁺ cells were detected in healthy chickens. 10x. 3b: GD showing thickened epidermis with large infiltration of K55⁺ cells in the subcutaneous layer of GD-like chickens. 10x. 3c: The higher magnification of (3b). 40x. 3d: K1⁺ cells in GD-like chickens. 40x. 3e: CD8⁺ T cells in the dermis of GD-like chickens. 40x. 3f: MHC class II⁺ cells beneath the epidermis of healthy chickens. 10x. 3g: MHC class II⁺ cells in the dermis and epidermis of GD-like chickens. 10x. 3h: MHC class II⁺ cell aggregate in the dermis of GD-like chickens. 10x.

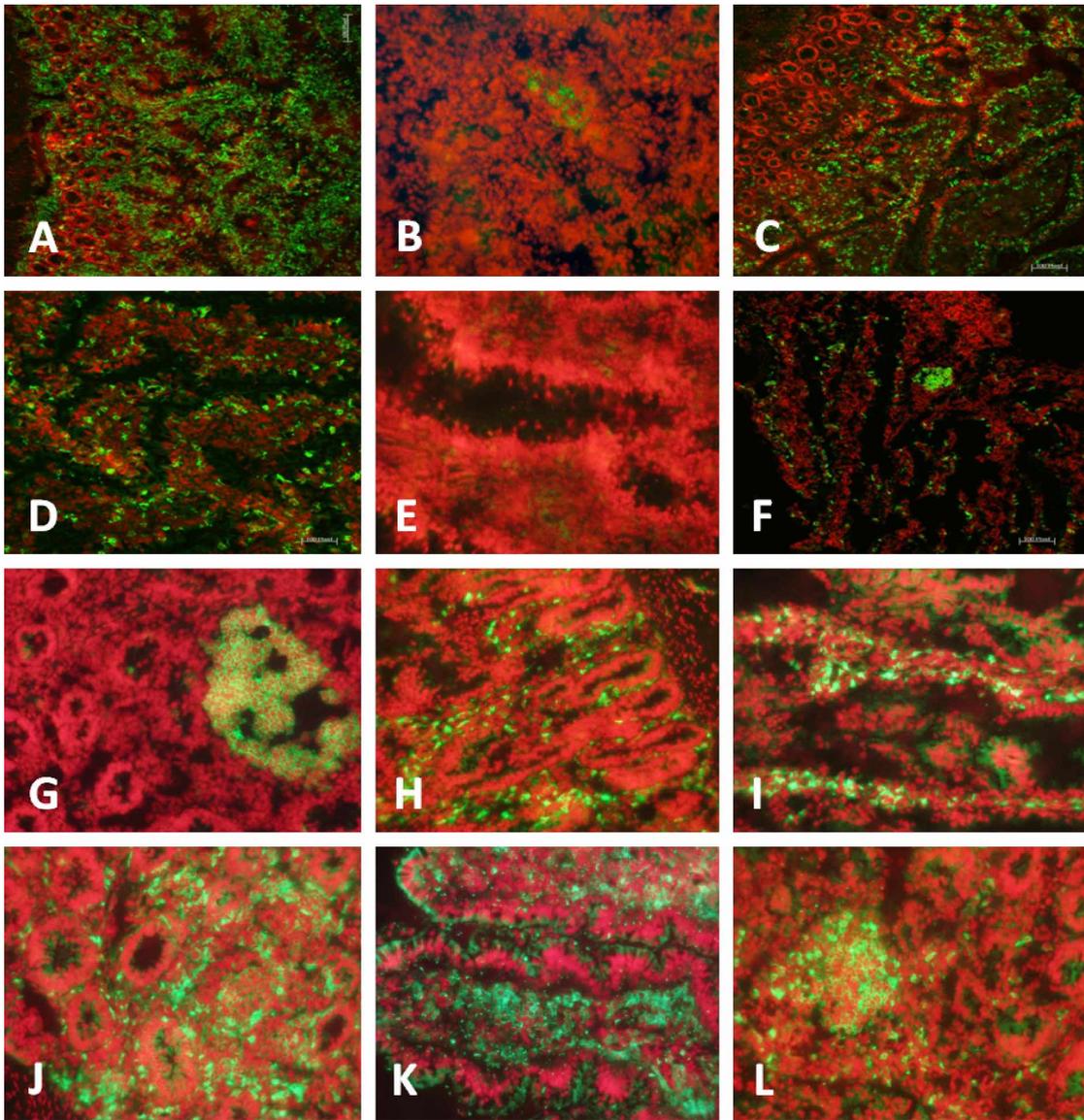


Figure 4. Immunohistochemical analysis of leukocyte subpopulations in the intestine of GD-like and clinically healthy chickens. 4a: $K55^+$ cells in GD-like chickens. 10x. 4b: $K1^+$ cells in the submucosal layer of GD-like chickens. 10x. 4c: $CD8^+$ cells in the mucosal layer of GD-like chickens. 10x. 4d: $TCR1^+$ cells in the mucosal layer of GD-like chickens. 10x. 4e: $TCR2^+$ cells in the lamina propria of GD-like chickens. 10x. 4f: Bul^+ cells in the mucosal and submucosal layers of GD-like chickens. 10x. 4g: A single large lymphoid follicle with Bul^+ cells in the submucosal layer of GD-like chickens. 10x. 4h: MHC class II^+ cells in the submucosal layer of healthy chickens. 10x. 4i: MHC class II^+ cells in the lamina propria of healthy chickens. 10x. 4j: MHC class II^+ cells in the submucosal layers of GD-like chickens. 10x. 4k: MHC class II^+ cells in the mucosal layer of GD-like chickens. 10x. 4l: MHC class II^+ cell aggregate in the submucosal layer of GD-like chickens. 10x.

up-regulated under the same conditions. In the intestine, 12 transcripts (IFN- α , IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 p40, IL-13, IL-17, LITAF) were decreased in GD-like chickens compared with healthy birds (Figure 6).

Discussion

There has been increasing interest in clostridial infections, especially GD in poultry (Williams, 2005; Ritter, 2006), which is now being recognized as an emerging disease of poultry with increasing economic importance. However, due to difficulties in understanding complex aetiology, and a lack of a chicken model, there has been limited progress in our understanding of host-pathogen interactions in GD (Songer, 1996; Wages & Opengart, 2003). In

the present study, we investigated a commercial flock of broiler chickens with typical signs and pathological lesions of GD to compare systemic and local (skin and intestine) immune responses with those of clinically healthy birds from the same flock. In summary, our results demonstrated that, compared with GD-free chickens, GD-like birds showed: reduced T-cell and B-cell mitogen-stimulated spleen lymphoproliferation; higher levels of serum NO and α -1-AGP; greater numbers of $K55^+$, $K1^+$, $CD8^+$, and MHC class II^+ intradermal lymphocytes and increased $K55^+$, $K1^+$, $CD8^+$, $TCR1^+$, $TCR2^+$, Bul^+ , and MHC class II^+ intestinal intraepithelial lymphocytes; and increased skin levels of mRNAs encoding proinflammatory cytokines and chemokines.

In general, GD outbreaks are facilitated by prior infection with immunosuppressive pathogens, such as chicken anaemia virus, infectious bursal disease virus, and

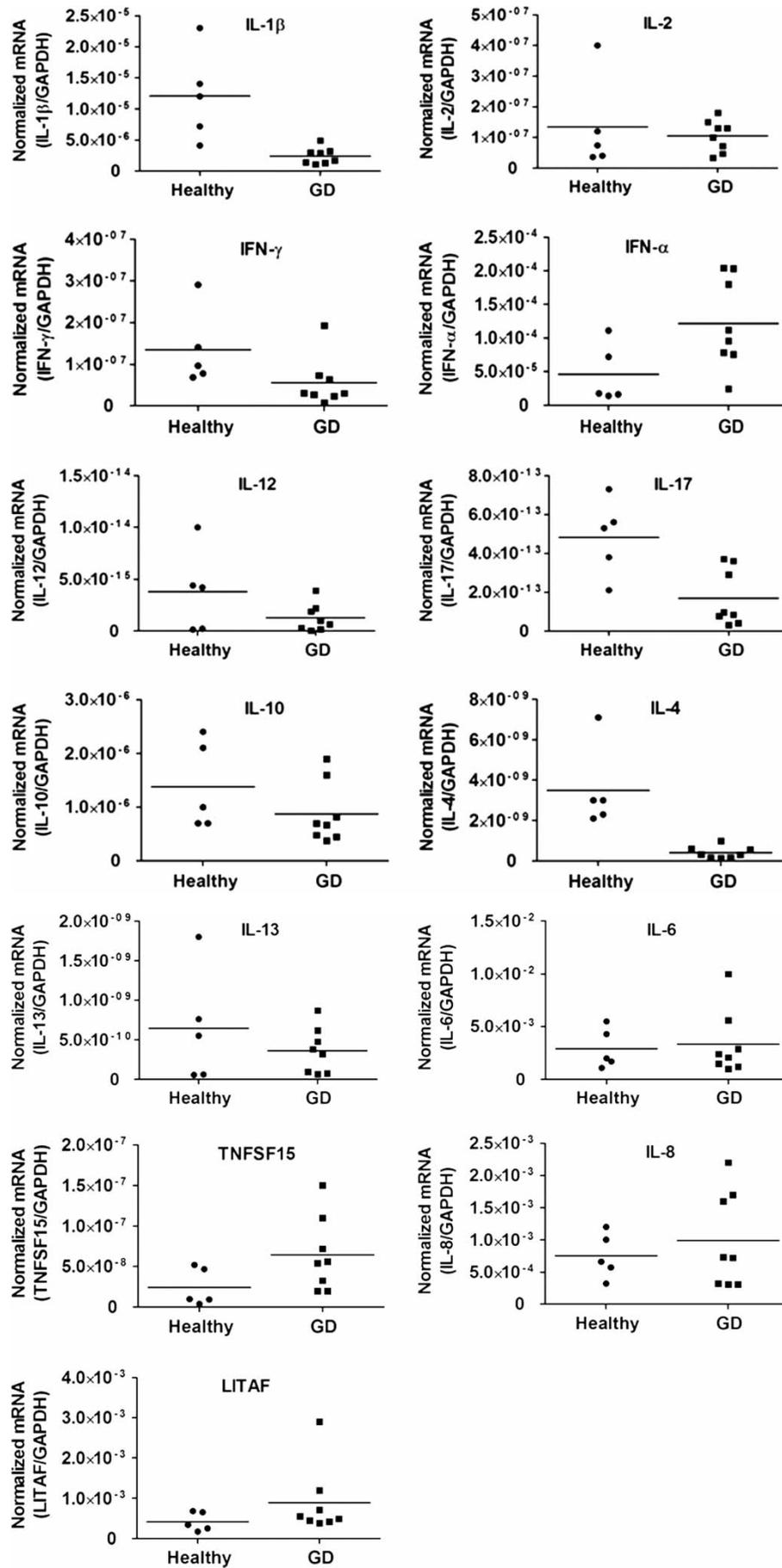


Figure 5. Levels of transcripts encoding cytokine, chemokine, and other immune-related molecules in the skin/muscle from clinically healthy and GD-like chickens. Each dot plot represented the mean of triplicate samples from five healthy birds and eight GD-like chickens. Mean levels indicated with horizontal lines.

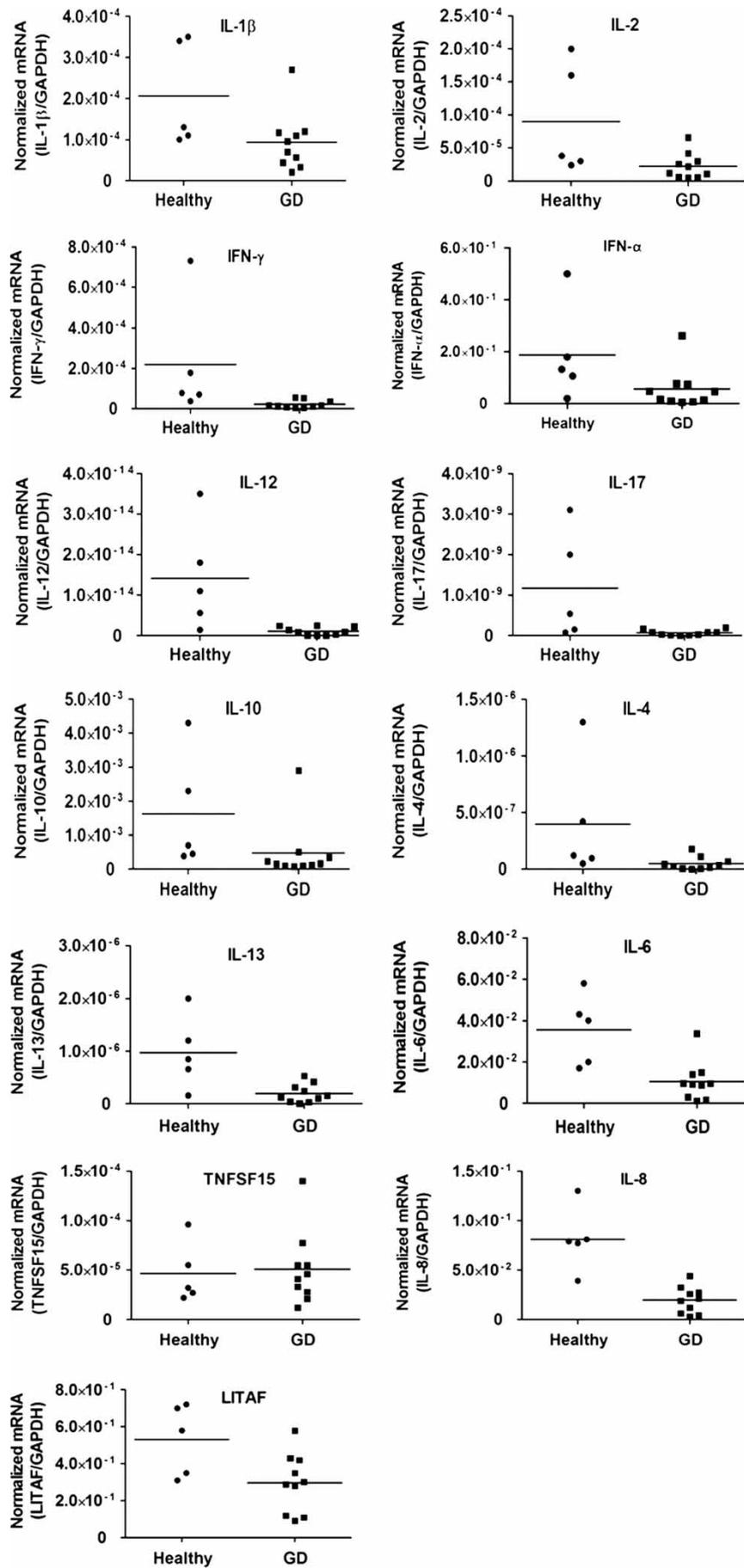


Figure 6. Levels of transcripts encoding cytokine, chemokine, and other immune-related molecules in the intestine from clinically healthy and GD-like chickens. Each dot plot represented the mean of triplicate samples from five healthy birds and 10 GD-like chickens. Mean levels indicated with horizontal lines.

reovirus. These viral infections are associated with atrophy of the bursa of Fabricius and concomitant suppression of host immune function, which promote *Clostridium* infections (Willoughby *et al.*, 1996; Williams, 2005). Our previous report indicated that sera from GD-like chickens, as well as clinically healthy chickens, contained high antibody titres against chicken anaemia virus and infectious bursal disease virus (Li *et al.*, 2010). Furthermore, immunohistological examination demonstrated reduced lymphocyte numbers in the bursa of Fabricius that was associated with bursal injury and necrosis (Li *et al.*, 2010). In addition to viral and bacterial implications, it is understood that management factors such as high flock stocking density, low diet quality, and increased moisture contents in the litter may be contributing to GD outbreaks (Ritter, 2006; Li *et al.*, 2010).

The overall immune status of GD-like birds appears to be abnormal compared with GD-free birds. Therefore, we measured splenocyte proliferation as an indicator of host immune function in GD-like and healthy chickens. Both T-cell and B-cell mitogenic responses were significantly depressed in chickens with disease signs, suggesting that suppressed immune status of the host may have contributed to enhanced pathogenicity and immunopathology of the clostridial infection. Another indication of abnormal host immune response was the production of high levels of NO, an important mediator of immunity. Primary monocytes/macrophages produce large amounts of NO when activated through pathogen-associated molecular patterns, particularly CpG oligodeoxynucleotides (Babu *et al.*, 2006; He *et al.*, 2009). The primary function of NO is to kill bacteria, fungi, protozoa and tumour cells via the production of the potent oxidant peroxynitrite following radical-radical reaction with superoxide (Rubbo *et al.*, 1994; Lillehoj & Li, 2004). Additionally, NO plays an active role in inflammatory responses (Karsten *et al.*, 2005) and, when its production is uncontrolled, causes tissue damage (Allen, 1997; MacMicking *et al.*, 1997; Karsten *et al.*, 2005). The fact that GD-like chickens contained higher NO serum levels compared with disease-free chickens suggests a similar induction of the free radical in the skin and intestine that may, in part, be responsible for the increased inflammation seen immunohistochemically. Additionally, higher serum levels of α -1-AGP, a predominant avian acute phase protein (Juul-Madsen *et al.*, 2008), in GD-like chickens versus GD-free birds further supports the increased systemic inflammatory response during this disease.

Clostridium organisms normally grow optimally within the devitalized, anaerobic milieu. However, acute invasion and destruction of healthy, living tissue ensues following production of bacterial toxins (Bryant & Stevens, 1997). Alpha-toxin, the principal virulence factor of *C. septicum*, is a lethal, necrotizing, membrane pore-forming protein that binds to glycosylphosphatidylinositol-anchored proteins (Hang'ombe *et al.*, 2004). *C. perfringens* type A also produces alpha-toxin, together with NetB toxin, β -2-toxin, enterotoxin, and enzymes that contribute to its virulence (Titball *et al.*, 1993; Bueschel *et al.*, 2003; Jost *et al.*, 2005; McDevitt *et al.*, 2006; Keyburn *et al.*, 2008; Cooper & Songer, 2009; Martin & Smyth, 2009). In human clostridial myonecrosis, cytotoxic effects of clostridial exotoxins were seen in the interfascial planes and within capillary vasculature near the demarcation between healthy and necrotic tissues (Bryant *et al.*, 1993). These afflicted areas

exhibited prominent leukocyte/lymphocyte infiltration. In the present study, increased infiltration of lymphocytes (K55⁺), T cells (CD8⁺), macrophages (K1⁺), and antigen-presenting cells (MHC class II⁺) in the skin, and higher levels of these cells in combination with B cells (Bu1⁺) in the intestine were observed in GD-like chickens compared with the clinically healthy birds. Furthermore, both *C. perfringens* and *C. septicum* were isolated from affected intestinal tissues of GD-like chickens (Li *et al.*, 2010).

Recruitment and activation of leukocytes to sites of microbial infections is mediated by chemokines and cytokines. We observed that IL-8, a CXC chemokine that is responsible for leukocyte recruitment, was one of four transcripts to be up-regulated in the skin of GD-like chickens compared with disease-free chickens. Transcripts encoding IFN- α , TNFSF-15, and LITAF also were increased in skin, while none of the transcripts were increased in the intestine. Chicken IFN- α plays an important role in defence against viral infection, including viruses that are known to predispose to *Clostridium* infection and development of GD (Ritter, 2006). TNFSF-15 plays crucial roles in inflammation, apoptosis, and cell proliferation (Park *et al.*, 2007), and LITAF is a transcription factor that up-regulates TNF- α gene expression (Hong *et al.*, 2006a). By contrast, the transcript levels for the majority of cytokines examined were decreased in GD-like chickens compared with GD-free chickens. We speculate that reduced expression of these cytokines in GD-like birds may reflect general immunosuppression associated with the disease. At this stage, the underlying causes of immunosuppression with respect to GD are not clearly understood, but can be addressed with the development of a genetically susceptible GD chicken model that is under study in our laboratory.

In summary, the data presented in this paper demonstrate that: splenocyte proliferation in response to Con A or LPS was diminished in GD-like chickens compared with their healthy cohorts; GD-like chickens had greater levels of serum NO and α -1-AGP compared with healthy birds; higher levels of T cells, B cells, macrophages, and activated cells were present in the skin and intestine of GD-like chickens compared with disease-free chickens; and the levels of transcripts encoding IL-8, IFN- α , TNFSF-15, and LITAF were increased in skin, while those for the majority of other cytokines were decreased in skin and the intestine, of GD-like chickens compared with GD-free chickens. These results provide insights into the host-pathogen interaction in GD infection that will facilitate the future development of effective control strategy against *Clostridium* infections in chickens.

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