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Original Article

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Cellular and molecular insights on the regulation of innate immune responses to experimental aspergillosis in chicken and turkey poults

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Abstract

Across the world, many commercial poultry flocks and captive birds are threatened by infection with *Aspergillus fumigatus*. Susceptibility to aspergillosis varies among birds; among galliform birds specifically, morbidity and mortality rates seem to be greater in turkeys than in chickens. Little is known regarding the features of avian immune responses after inhalation of *Aspergillus* conidia, and to date, scarce information on inflammatory responses during aspergillosis exists. Thus, in the present study, we aimed to improve our understanding of the interactions between *A. fumigatus* and economically relevant galliform birds in terms of local innate immune responses. Intra-tracheal aerosolization of *A. fumigatus* conidia in turkey and chicken poults led to more severe clinical signs and lung lesions in turkeys, but leukocyte recovery from lung lavages was higher in chickens at 1dpi only. Interestingly, only chicken CD8+ T lymphocyte proportions increased after infection. Furthermore, the lungs of infected chickens showed an early upregulation of pro-inflammatory cytokines, including IL-1β, IFN-γ and IL-6, whereas in turkeys, most of these cytokines showed a downregulation or a delayed upregulation. These results confirmed the importance of an early pro-inflammatory response to ensure the development of an appropriate anti-fungal immunity to avoid *Aspergillus* dissemination in the respiratory tract. In conclusion, we show for the first time that differences in local innate immune responses between chickens and turkeys during aspergillosis may determine the outcome of the disease.

Lay Summary

Aspergillus fumigatus infection may cause mortality in poultry, depending on species sensitivity. This study confirms the earlier activation of chickens' pro-inflammatory effectors to control *Aspergillus* dissemination, whereas turkeys' immune response enables the exacerbation of lung lesions.

Key words: aspergillosis, chicken, turkey, immune response, cytokines.

Introduction

Avian respiratory diseases are responsible for substantial economic losses to the poultry industry worldwide[1,2] and to heavy drawbacks to bird conservation programmes.[3,4] This is particularly true for aspergillosis caused by the ubiquitous fungal species *Aspergillus fumigatus*; susceptible individuals are infected by this opportunistic and major airborne pathogen through inhaling dormant conidia.[2]

Acute and chronic forms of aspergillosis in birds have been known for centuries, and previously described clinical expressions of this mycotic disease include anorexia, aphony, ruffled feathers, gasping, lethargy, lateral recumbency, and often death

24 to 48 h after onset of clinical signs.[5] Different levels of susceptibility to the infection among avian species may be clinically expressed as digestive, neurological, or dermatological symptoms.[6] Lesions can be localized or disseminated but granulomatous lesions in lungs and air sacs are recurrently seen.[7] Unfortunately, *ante mortem* diagnosis remains difficult.[8]

As confirmed *in vitro* and in mouse models, *Aspergillus fumigatus* infection and germination can be detected and limited by the host's innate immune response.[9–13] However, if the immune system fails to clear *Aspergillus* conidia, they swell, germinate, and grow into large hyphal structures that invade surrounding airways and initiate a disseminated infection; by spreading via the bloodstream, the fungus may finally reach the central nervous system.[2,14,15] In birds, susceptibility to *Aspergillus* infection has been associated with bad management practices, poor hygiene, or extreme avian genetic selection aimed at maximum productivity.[16] However, information remains scarce regarding predisposing immunological factors that may enable aspergillosis pathophysiology.

In mammals, host immune response to invasive aspergillosis are mostly mediated by neutrophils and alveolar macrophages, which may inhibit conidia swelling and germination to some extent.[17,18] Importantly, the early activation of innate immune responses results in more effective phagocytosis and killing of the fungus.[12,19] This phenomenon is linked to a switch in cytokine signalling toward a pro-inflammatory profile, leading to the activation of phagocytes, the regulation of epithelial host defence and the setting up of an adequate adaptive immune response through the activation of specific T-cell subsets.

The early detection of fungal elements by pattern recognition receptors (PRRs) and the release of pro-inflammatory cytokines are crucial for controlling *Aspergillus* dissemination.[20] The activation of mammalian Toll-like receptors (TLR) 2, 3, and 4, the regulation of inflammatory chemokines[21] and the production of interleukins (ILs) 1-β and 17A upon infection are examples for the importance of Th1-associated immune responses against pulmonary aspergillosis.[22–26] Other cytokines have also been highlighted as key factors during host response to *Aspergillus* infection. This is exemplified by the fact that the absence of anti-inflammatory or Th2-associated cytokines such IL-10 and IL-4, which may counteract locally beneficial pro-inflammatory responses, increases resistance to infection.[27–29]

The aim of the present study is to compare early innate immune response against acute aspergillosis in chickens (*Gallus gallus domesticus*) and turkeys (*Meleagris gallopavo*). The choice of these galliform species enables a side-by-side comparison of their different levels of susceptibility to *Aspergillus* infection, which has already been observed in field conditions but never proved under controlled conditions.[5,30] We believe that our results will shed light on why the development and implementation of new strategies targeting early diagnosis and prophylaxis must take into account species-specific immunological differences in order to ensure the control of avian aspergillosis.

Methods

Fungal strain and inoculum preparation

Aspergillus fumigatus reference strain CBS 144.89 was used in all experiments. This clinical strain, also known as CEA10, was isolated from an invasive aspergillosis patient at a Paris hospital in 1989. The strain was first characterized at the Pasteur Institute in Paris and is available at the Fungal Genetics Stock Center (FGSC) (accession number A1163). All reagents were purchased from Sigma-Aldrich (France) unless otherwise stated. Mycological cultures were performed on Sabouraud dextrose agar (SDA) supplemented with chloramphenicol (50 mg/l) and incubated at 37°C. To prepare the inoculum, the cryopreserved strain was thawed and grown on SDA plates for two days at 37°C. After 2 to 3 days of subculture in 75 cm^2 SDA-filled flasks, conidia were harvested by flooding the flasks with sterile phosphate-buffered saline (PBS) containing 0.01% (v/v) Tween 20 (PBST). The conidia were purified using 70 μm nylon cell strainers (ClearLine® Dominique Dutcher, France), concentrated by centrifugation (at 3500*g* for 10 min) and diluted in PBST to a concentration of 2×10^8 conidia/ml.

Animal experiments

The design and execution of the present study was in strict accordance with good animal practices as defined by the French and European code of practice for the care and use of animals for scientific purposes. All animal experiments were approved by the local ethics committee for animal experimentation (Anses/ENVA/UPEC, approval no. 10/03/15-11) and by the French Ministry of National Education, Higher Education, and Research (Project no. 1164).

All experiments were performed using Lohmann brown laying hens and Hybrid grade maker female turkeys, provided by local commercial breeders (Lohmann France and Grimaud Frères Sélection, respectively). Animals were purchased at 1 day old and housed under specific pathogen-free (SPF) conditions at the animal care facility of the Biomedical Research Centre at the Veterinary College of Alfort (CRBM-EnvA, France). They were fed *ad libitum* with water and appropriate commercial food until the day of euthanasia. Two independent experiments per avian species were performed.

The experimental model of acute aspergillosis followed the protocol described by Melloul et al.[30] with slight modifications. Briefly, after an acclimation period of 1 week, a group of 20 seven-day-old chickens or turkeys were anaesthetized by isoflurane inhalation (Vetflurane® Virbac, France), randomly inoculated with 2×10^7 conidia intra-tracheally using a 19-gauge Microsprayer® aerosolizer (Penn Century, Wyndmoor, PA, USA) and identified with numbered leg rings. Birds were weighed once daily and monitored twice a day to survey the presence of clinical signs and mortality. A group of 10 chickens or turkeys inoculated with PBST alone was used as a negative control. At 1, 2, or 3 days postinoculation (dpi), five *Aspergillus*-infected birds

Table 1. List of primary and secondary antibodies.

Panel	Antibody target	Isotype	Species specificity	Clone	Fluorochrome	Secondary antibody
I	CD45 (leukocytes)	Mouse IgM	Chicken	LT40	Alexa Fluor 488	Conjugated
	CD41/CD61 (thrombocytes)	Mouse IgG1	Chicken	11C3	PerCP	Goat anti mouse IgG1
II	CD45 (leukocytes)	Mouse IgM	Chicken	LT40	Alexa Fluor 488	Conjugated
	Monocyte-Macrophage	Mouse IgG1	Chicken	KUL01	PerCP	Goat anti mouse IgG1
III	CD4 (CD4 + lymphocytes)	Mouse IgG2b	Chicken	2–35	FITC	Conjugated
	CD8a (CD8+ lymphocytes)	Mouse IgG1	Chicken	11–39	RPE	Conjugated

and three control birds were sequentially selected according to their leg ring number and euthanatized with 1 ml/kg of pentobarbital (Dolethal® Vétoquinol, France) injected into the occipital sinus.

Sample recovery

Samples were only recovered from euthanized birds. Whole blood was collected using 5 ml heparinized tubes (Vacutainer® BD, France) and placed on ice for further leukocyte purification and analysis. Pulmonary fluid contents were recovered through respiratory lavage according to the method described by Nganpiep and Maina.[31] In total, 10–12 ml of cell suspension was recovered from 15 ml of PBS instilled into the lungs and air sacs. Respiratory lavages were conserved on ice and used for leukocyte purification and analysis.

After visual examination of both the lungs and the air sacs, the presence of lesions[7] was scored for each lung as 0 (without gross lesions), 1 (lesions covering <50% of the organ but not the air sacs) or 2 (covering ≥50% of the organ, including the air sacs or not). Next, the right lung was fixed in 10% neutral buffered formalin for histopathological examination to identify lesions associated with acute aspergillosis. The left lung was divided into two pieces. One part was snap-frozen in liquid nitrogen and stored at −80°C for further RNA isolation, and the other was conserved in cold PBST for *Aspergillus* culture.

Mycological culture

To compare *A. fumigatus* lung colonization between chickens and turkeys, lungs from euthanized birds were sampled at 1, 2, and 3 dpi. Two grams of lung were collected and homogenized in 6 ml of sterile PBST, and 100 μl was plated on SDA and incubated at 37°C for 24 and 48 h. Growth and counts of *A. fumigatus* colonies (cfu) were assessed.

Histopathological examination

Samples were fixed in 10% buffered formalin for 48 h, dehydrated in successive ethanol baths (from 70 to 100%). and impregnated in paraffin in a LOGOS One automated device (Milestone, Italy). Next, samples were embedded in paraffin, and blocks were cut in 4-μm-thick sections. Slides were stained with haematoxylin-eosin-saffron (HES) using a Leica CV 5030 automated device (Leica, Germany), or with periodic acid-Schiff (PAS) according to standard histological procedures in order to improve the visualization of hyphae in tissue lesions. The images were captured in a Zeiss Axio Imager Z2 microscope equipped with an AxioCam MRm camera (Carl Zeiss SAS, France).

Leukocyte purification and flow cytometry

Chicken and turkey blood leukocytes were obtained through gradient separation using Histopaque 1077. Briefly, 2 ml of whole blood was gently layered on 3 ml of Histopaque solution and centrifuged at 800 rcf for 30 min without brakes at room temperature. The white cell ring zone, which contains leukocytes, was recovered in a 50-ml Falcon® tube and washed twice with 30 ml of PBS. Leukocytes from lung lavages were purified by filtration through 70 μm nylon cell strainers, followed by centrifugation at 400 rcf for 5 min at room temperature and two washing steps with 5 ml of PBS. Cell concentration was calculated with a Malassez haemocytometer and purified blood, and lung lavage cells were placed in 3 ml tubes containing one million cells. Next, the cells were suspended in 0.5 ml of cold flow cytometry (FACS) buffer containing PBS 1X, 0.5% BSA, and 0.2 M EDTA (Sigma-Aldrich, France).

For leukocyte immunophenotyping, cells were incubated with three different combinations of monoclonal antibodies (mAbs). These antibody panels were previously titrated for their optimal concentrations and included mouse anti-chicken CD45 (Southern Biotech, Birmingham, AL, USA), mouse anti-chicken CD41/CD61 (Bio-Rad, UK), mouse anti-chicken monocyte/macrophage (targeting chicken MRCL1) (Southern Biotech, USA), mouse anti-chicken CD4 (Bio-Rad, UK) and mouse anti-chicken CD8 alpha (Bio-Rad, UK). Detailed information on antibody combinations for the two avian species and, where applicable, fluorescent secondary antibodies is given in Table 1.

Flow cytometry analysis of antibody-stained leukocytes was performed on a FACSCanto II (BD Biosciences, San Jose, CA, USA) flow cytometer equipped with three lasers (405, 488 and

Table 2. List of primer pairs targeting gene coding for avian cytokines.

Gene	Target species	NCBI Target gene(s)	Forward primer	Reverse primer	Target length
Avian GAPDH	Chicken Turkey	NM_204305.1 NM_001303179.1	CCACATGGCATCCAAGGAGT	CTCCAACAAAGGGTCCTGCT	74 bp
Chicken IL-2		AF017645.1	TTGGCTGTATTTCGGTAGCA	TCCTGGGTCTCAGTTGGTGT	160 bp
Turkey IL-2		AJ007463.1	GAGCATCGCTATCACCAGAA	GCAGAGTTTGCTGACTGCAC	141 bp
Avian IL-6	Chicken Turkey	AJ309540.1 XM_003207130.3	AGGGCCGTTCGCTATTTGAA	ACGGAACAACACTGCCATCT	112 bp
Avian IL-10	Chicken Turkey	EF554720.1 NM_001303189.1	GCTGCGCTTCTACACAGATG	TCCCGTTCTCATCCATCTTC	203 bp
Avian IFN-γ	Chicken Turkey	X99774.1 XM_003202048.3	CTGAAGAACTGGACAGAGAG	CACCAGCTTCTGTAAGATGC	264 bp
Avian TLR3	Chicken Turkey	NM_001011691.3 XM_019614820.1	AACACCCCGCCTAAATATCA	CCACCCTTCAAAATGGATGA	153 bp
Avian IL-1β	Chicken Turkey	NM_001004414.2 XM_010723207.2	AGGCTCAACATTGCGCTGTA	CTTGTAGCCCTTGATGCCCA	93 bp
Avian CCL5	Chicken Turkey	NM_001045832 XM_003211628.3	TATTTCTACACCAGCAGCAAATG	GCAGACACCTCAGGTCC	74 bp 152 bp
Chicken CxCLi2		NM_205498.1	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC	139 bp

633 nm). The acquisition criteria included the recording of 10,000 total events per sample. Subsequently, singlet cells were gated and specific cell subpopulations were analyzed within the CD45 + population. The sequential steps of the flow cytometry analysis are depicted in Fig. 3A. Data are presented as the percentage of a given population. Raw data analysis was performed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Finland).

RNA isolation and RT-qPCR analysis

One hundred mg of frozen lung samples were thawed and homogenized (Tissue Master Homogenizer® Omni International, Kennesaw, GA, USA) in a 15 ml conical tube containing 1 ml of TRIzol™ Plus (Thermo Fisher Scientific, The Netherlands). Total RNA was isolated according to the TRIzol method and followed by a DNAse treatment step (RNase-Free DNase set, Qiagen Benelux, Venlo, The Netherlands) using the manufacturer's instructions. Purified RNA was eluted in 50 μL RNAse-free water and the RNA was quantified by absorbance measurement at 280 nm (Nanodrop 2000, ThermoFisher Scientific, The Netherlands). Complementary DNA (cDNA) was generated from 2.5 μg of RNA using a Superscript IV VILO Master Mix kit (ThermoFischer Scientific, The Netherlands).

Real-time qRT-PCR was performed using using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, The Netherlands) in a Roche LightCycler 96 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 10 ng of cDNA was used to detect the expression of gene coding for selected cytokines during *Aspergillus* infection (Table 2).

The selection of a pair of primers was performed using NCBI software, in order to confirm their ability to amplify the same sequence from both the chicken and the turkey target gene. Otherwise, specific primer pairs for each species were used. Next, the *GAPDH* gene was validated as a reference gene following stability analysis of its expression in control and infected birds.[32,33] Mean threshold cycle values (Ct) were determined based on triplicates and the results presented as 2^(ΔΔCt) values, calculated by subtracting the experimental ΔΔCt value from the control ΔΔCt value.[34]

Statistical analysis

Statistical differences were assessed for the leukocyte percentages and relative cytokine expression using a two-way analysis of variance (ANOVA) test and Sidak's multiple comparisons test (GraphPad Prism v8). Differences were considered statistically significant when P-values $\leq .05$.

Results

Clinical severity of experimental *aspergillosis* is species-dependent

Following *Aspergillus* inoculation, both chickens and turkeys showed a decrease in body weight compared to the control groups from 1 dpi onward, with statistically significant differences being observed in chicken at 3 dpi (Fig. 1A). Mild clinical signs (e.g. sneezing and nasal discharge) appeared at 2 dpi in two out of 15 chickens, while one chicken was found gasping. At 3 dpi, the severity of clinical signs was progressive but heterogeneous, and all chicken were mostly sneezing. The clinical

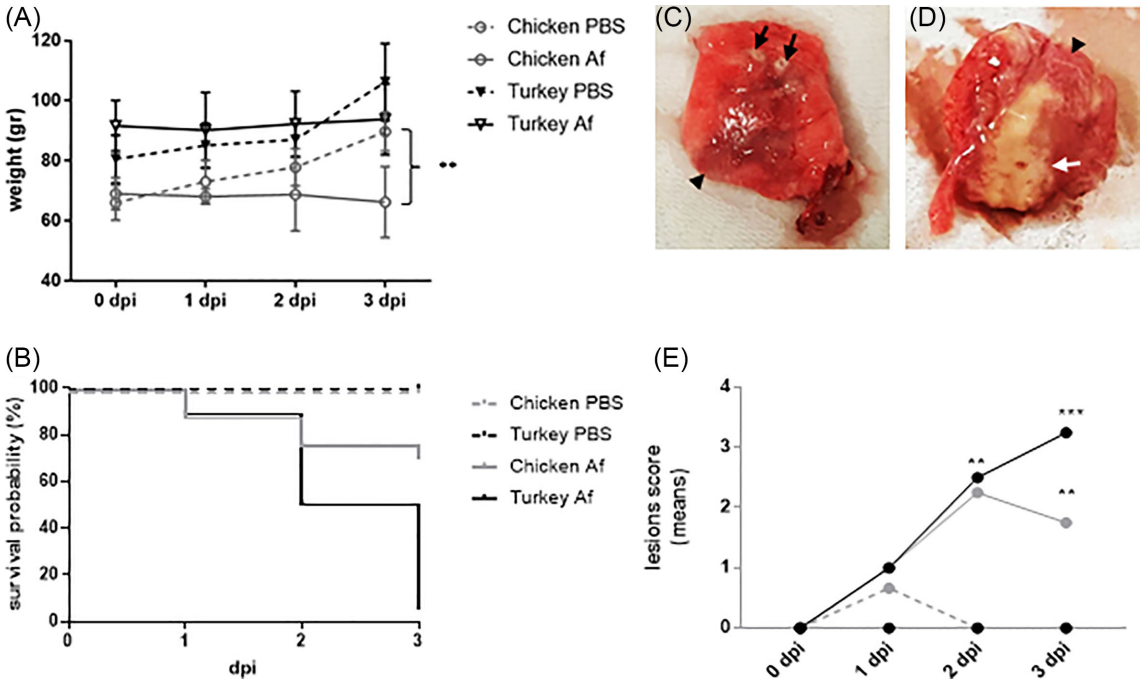

Figure 1. Weight progression, survival curves, and lung lesions of chicken and turkeys during experimental infection with *Aspergillus fumigatus* CBS144.89 strain. Seven-days old chicken and turkey were inoculated by Microsprayer® aerosolization with 2×10^7 conidia of *Aspergillus fumigatus* strain CBS144.89, suspended in 100 μl of PBS-tween 20 at 0.01%. Daily mean weights were compared between infected and control groups (A). All infected birds lost weight from 1 dpi onwards with a significant difference between the chicken groups at 3 dpi. (B) Kaplan-Meier survival curves of the four experimental groups 80% (12/17) of infected chicken survived until the end of the experiment while only one turkey survived until 3 dpi. Photographs of infected turkey lungs showing gross lesions at 1 dpi (C) and 3 dpi (D). Lesions included congestion, caseous nodules (black arrow), granulomas (white arrow) and focal or diffuse congestive lesions (arrowheads). Gross lesions were scored for each lung as no visible lesions (0), less than a half (1) or a half or more of the lung (2). After the addition of the lesion scores for both lungs per animal, mean lesion scores were calculated and confirmed the observation of more severe lung lesions in infected turkeys than in infected chicken (E). *: $P < .05$; **: $P < .005$ and ***: $P < .0005$ by Dunn's multiple comparisons test.

signs exhibited by the infected turkeys appeared earlier than in infected chickens, and the severity of the clinical signs in turkeys progressed in all individuals over time. Two birds out of 15 showed mild clinical signs as soon as 1 dpi. At 2 dpi, six birds out of 10 manifested ruffled feathers and intermittent gasping, and all turkeys showed either lateral recumbency or severe gasping at 3 dpi. Mortality was observed from 1 dpi (two birds per infected group out of 20), and at 3 dpi all turkeys and one chicken were severely diseased or dying (Fig. 1B).

During necropsy, the presence of congestive and granulomatous tissue lesions in the lungs of all infected birds was observed, independent of the species. Lesion extensions increased over time in both avian groups, although turkey lungs presented larger lesions from 1 dpi. Moreover, in all infected turkeys, more than half of the lung surface showed lesions compatible with an *Aspergillus* infection at 3 dpi (Fig. 1E). Posterior thoracic air sacs appeared thickened and were associated with extensive lung lesions, while no exudate or caseous plaques were observed.

Aspergillus-associated lung tissue colonization and damage were confirmed by histopathological analysis and fungal culture from tissue homogenates in all infected birds. Histological analysis of lung sections showed pulmonary lesions with oedema surrounding the air capillaries and the presence of leukocyte infiltrates predominantly characterized by polymorphonuclear cells (heterophils) in the peribronchial region (Fig. 2). The presence of *Aspergillus* hyphae was confirmed by PAS staining in both chicken and turkey samples, although this was less abundant in chickens. HES staining revealed leukocyte infiltration foci (macrophages, heterophils, lymphocytes), signs of bronchial wall damage, and peribronchial necrosis, which were more extensive in turkeys than in chickens. In turkeys, this tissue damage profile appeared as early as day 1 dpi.

Aspergillus development was only observed in fungal cultures from infected animals. Chicken lung cultures presented variable fungal colonization, and some cultures revealed a high fungal burden (>100 cfu), whereas others had no more than 10 cfu, no matter the day after infection. On the other hand, all turkey lung cultures displayed higher cfu values than 100 from 1 dpi onward.

Aspergillosis leads to different leukocyte recruitment profiles in the lungs of chicken and turkey poults

Purification and flow cytometry immunophenotyping of leukocytes from blood samples enabled proper identification of targeted leukocyte subpopulations in both chickens and turkeys. We used parameters such as morphology (size and granularity)

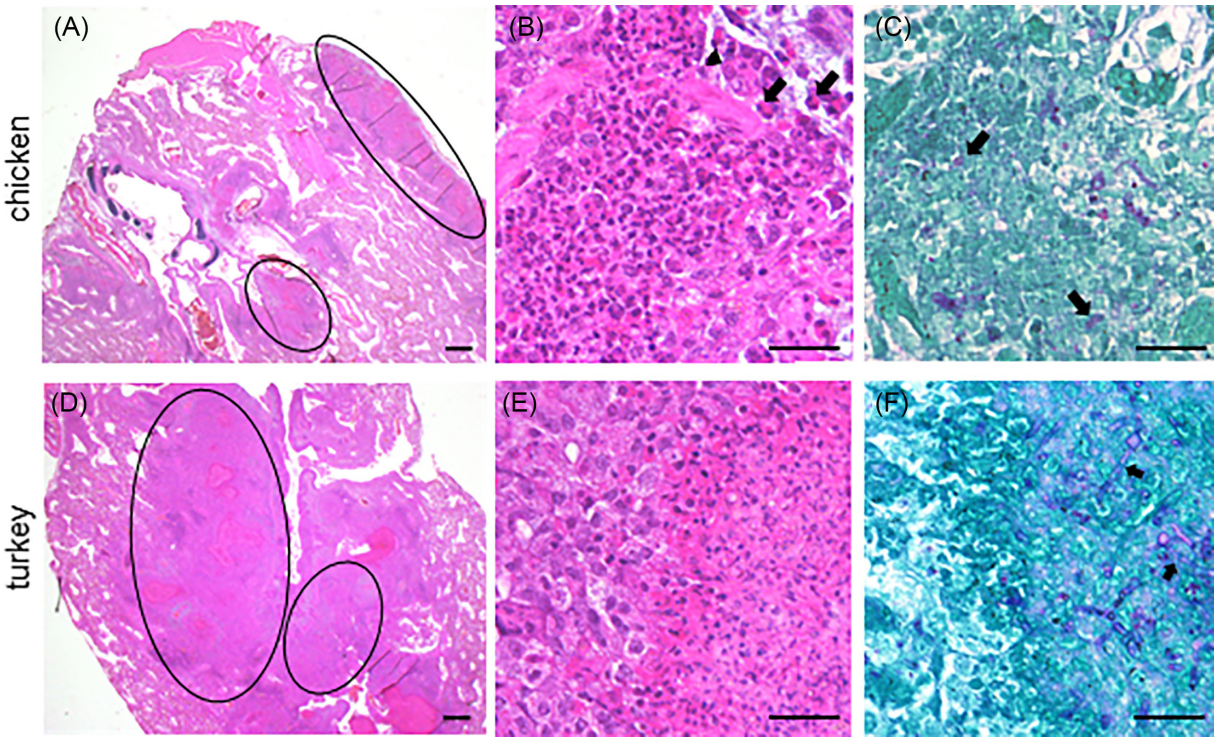

Figure 2. Representative histopathology sections of chicken and turkeys lungs 3 days after infection with *Aspergillus fumigatus* CBS144.89 strain. Seven-days old chicken and turkey were inoculated by Microsprayer® aerosolization with 2×10^7 conidia of *Aspergillus fumigatus* strain CBS144.89, suspended in 100 μl of PBS-tween 20 at 0.01%. Birds were euthanized 3 days after infection and hematoxiline-eosine-saffron staining and PAS staining were performed. Chicken lung sections showed reduced histological damage, with fewer and smaller infiltrates (A), located mostly at the periphery of the lung parenchyma or in the peribronchial regions (circles). Lesions consisted of moderate leucocyte infiltrations comprising predominantly heterophils (B, arrows) and giant cells (B, arrowhead), but rarely fungal elements (C, arrows). In contrast, turkey lung sections revealed large infiltrates and patterns of bronchial damage and peribronchial necrosis (D). Abundant necrotic material surrounded by infiltrates predominantly consisting of mononuclear cells (E). Lesions contained many septate hyphae (F, arrows). Scale bars, 0.5 mm (A, D) and 25 um (B-C, E-F).

and specific leukocyte immunostaining of CD45+ cells to reveal heterophils, thrombocytes, monocytes/macrophages, and CD4+ and CD8+ T cell subsets. Cytometry data from blood samples did not reveal differences between infected and control groups. On the other hand, cytometry analysis of respiratory lavages confirmed the presence of the same leukocyte subpopulations and allowed an in-depth comparison of cell recruitment profiles in the lungs of *Aspergillus*-infected chickens and turkeys (Fig. 3A, B).

Both avian species exhibited modest cellular loads in control respiratory lavages that needed up to two minutes to reach 10 000 events per sample. Interestingly, leukocyte recovery from turkeys' control lung lavages was low compared to chickens (1–4% in noninfected turkeys vs 15–40% in noninfected chickens). At 1 dpi, a significant increase in the total number of leukocytes ($P = .0038$) was observed in chickens compared to the control group. By contrast, infected turkeys did not exhibit significant differences in leukocyte count compared to the control groups, although an overall increase was observed in both infected and PBS control turkeys at 2 dpi and 3 dpi (Fig. 3C).

In lavages from chickens and turkeys, heterophils were the dominant leukocyte subpopulation in infected and noninfected birds. However, in both avian species the number of heterophils, thrombocytes, macrophages, and CD4+ lymphocytes in lung lavages were comparable at the time points analyzed. Only chicken CD8+ T lymphocytes showed a slight variation in cell proportions relative to the control group at 1 dpi ($P = .058$).

Chickens develop an earlier pro-inflammatory cytokine response in the lungs than turkeys

We next assessed whether the expression of cytokine genes could explain the divergent infection patterns observed for chickens and turkeys. Real-time RT-PCR analyses of lung samples revealed an early expression of gene coding for pro-inflammatory cytokines IL-1β, CXCLi2, and interferon γ (IFN-γ) in chickens (Fig. 4A, B). This upregulation started at 1 dpi and reached values of more than 50-fold, 38-fold, and 4-fold, respectively, compared to the control group at 3 dpi. Whereas CXCLi2 and IFN-γ upregulation was essentially homogeneous, IL-1β expression showed a remarkable degree of individual variability at 1 dpi. On the other hand, IL-1β and IFN-γ gene expressions were downregulated at 1 dpi and 2 dpi in turkeys compared to the control groups, while a moderate increase in IL-1β expression was only

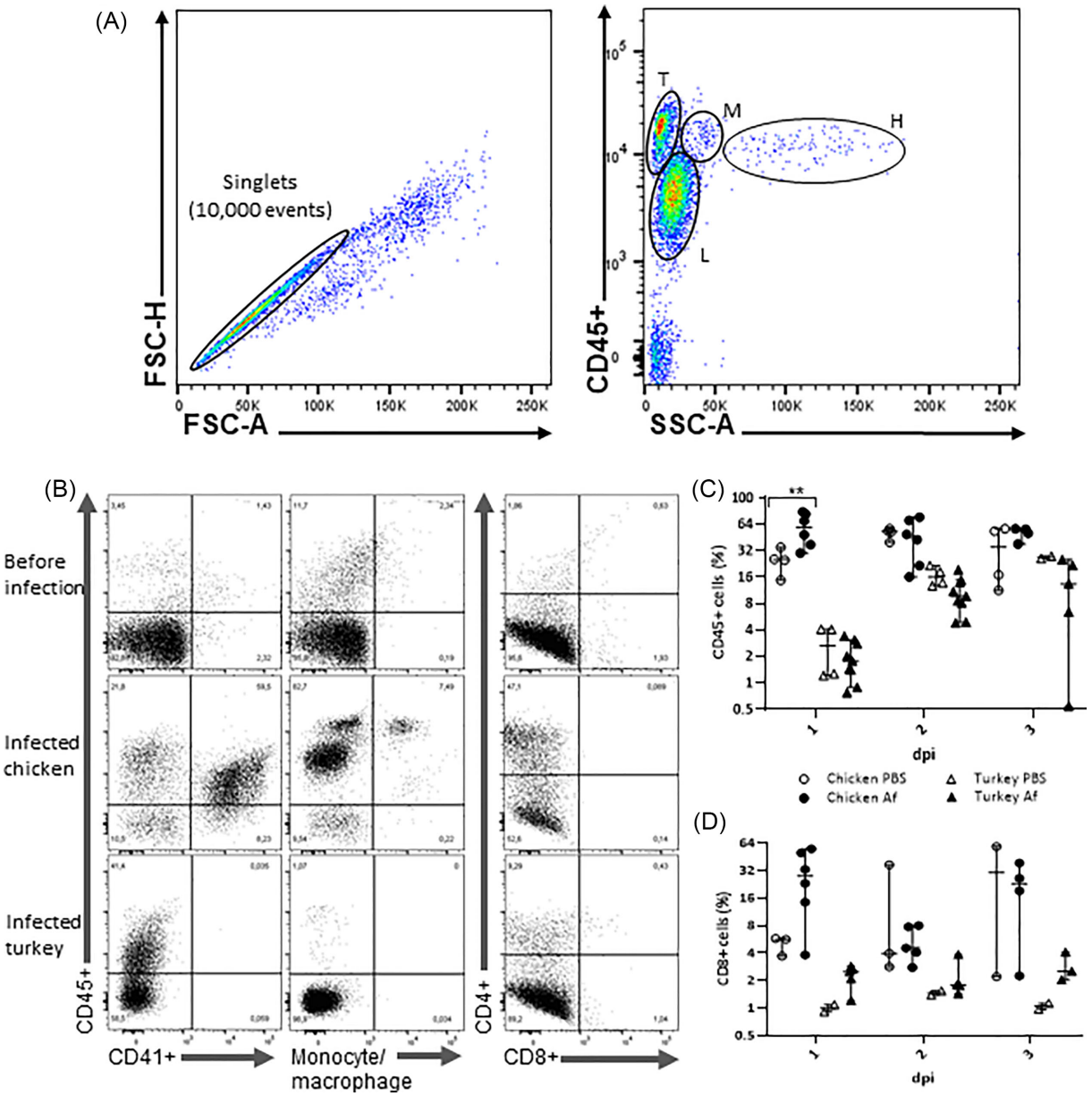

Figure 3. Flow cytometry analysis of leucocytes in pulmonary lavages during *Aspergillus* infection. Leucocytes were recovered from lung lavages of chickens and turkeys at days 1, 2 and 3 post-infection. All samples were filtered and suspended in FACS buffer at 10^6 cells/100 μl concentration for further labeling and FACS analysis. Two experiments with either 7 birds (infected groups) or 3 birds (PBS control groups) were performed. CD45+ leucocyte distribution in a chicken sample is exemplarily shown in (A). Note the greater granularity of avian heterophils when compared with mononuclear cells (B). Representative flow cytometry plots before and after *Aspergillus* infection (1 dpi). Chickens showed higher modification of cell proportions than turkeys at all days after infection ($P < .005$). Chicken CD45+ cell proportions significantly increased from 1 dpi (C). CD8+ lymphocytes fraction at 1 dpi was higher in the infected chicken group but decreased at 2 dpi (D). Statistical analyses were performed after deduction of background. –: mean percentage of cytokine-expressing cells. Figures correspond to one representative experiment. *: $P < .05$ and **: $P < .005$ by Dunn's multiple comparisons test.

observed at 3 dpi (Fig. 4A). Interspecies comparison revealed an IL-1β upregulation at 2 dpi that differed significantly between infected chickens and infected turkeys.

IL-2, IL-6, and IL-10 displayed distinct gene transcription patterns at different times post-infection. A transient upregulation of IL-6 in chickens was observed at 2 dpi only, while in turkeys it was downregulated throughout the 3 days of infection (Fig. 4C). Interestingly, IL-2 transcription was downregulated during the whole infection in chickens (Fig. 4B). We also observed a steady overexpression of IL-10 in chickens at 1 dpi, 2 dpi, and 3 dpi, while in turkeys the same cytokine was downregulated starting from 1 dpi (Fig. 4C).

Mitochondrial RNA (mRNA) levels of CCL5 diverged between infected chickens and turkeys. In chickens, chemokine CCL5 was strongly upregulated from 1 dpi on, reaching up to a 130-fold increase at 2 dpi ($P < 0.001$), followed by a down-

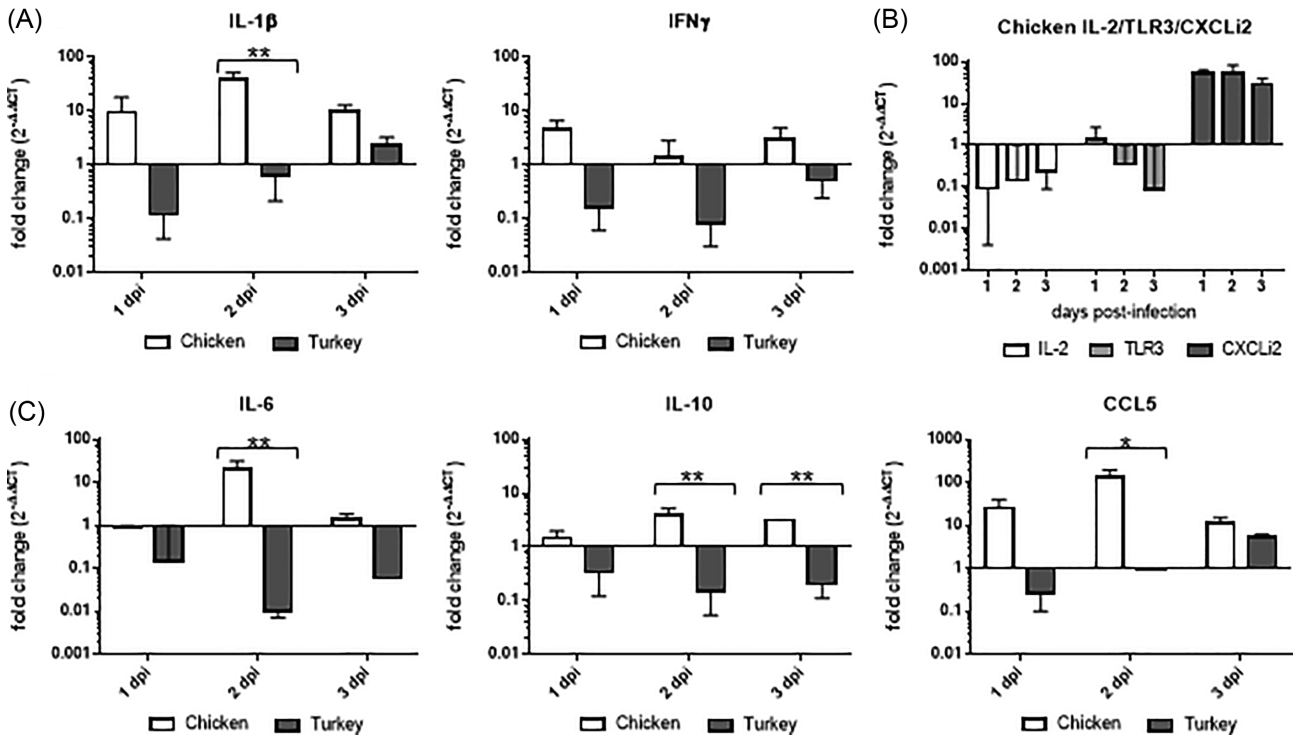

Figure 4. Relative expression of pulmonary cytokine genes of chickens and turkeys during acute aspergillosis. Differential expression of chicken and turkey cytokine genes from lungs at 1, 2 and 3 dpi. The y-axis of each graph represents the relative expression of the respective genes calculated using the ΔΔCt method and normalized against GAPDH mRNA. Upregulation of chicken IL1β and IFNy cytokines contrasts with downregulation of the same cytokines in turkeys (A). Chicken upregulation of CxCLi2 is also observed, while chicken Il-2 and TLR3 cytokines are downregulated (B). Comparative analysis of chicken and turkey IL-6, IL-10 and CCL5 cytokine genes depicting divergent profiles between chicken and turkey, with oscillating profiles of downregulation and upregulation during the experiment, especially for chicken IL-6 and IL-10 cytokines (C). Experiments were performed in triplicate with 3 noninfected birds and 5 infected birds in each group. Data are expressed as means and SEM. Statistically significant differences between groups are indicated (*$P < .05$; **$P < .005$).

regulation at 3 dpi. On the other hand, CCL5 in turkeys was initially downregulated at 1 dpi, but on the following 2 days it increased and reached a comparable upregulation to chickens at 3 dpi (Fig. 4C).

Quantification of TLR3 gene transcription in chickens displayed a baseline expression in the infected group at 1 dpi, followed by a downregulation trend from 2 dpi onward (Fig. 4B).

Discussion

In this article, we have provided comparative and novel information on how the two most commercially relevant galliform species—chickens and turkeys—respond to experimentally controlled aspergillosis in terms of differential clinical presentation and innate immune responses. The experimental infection model with *Aspergillus* CBS.144.89 strain by aerosol inoculation ensured equal conditions of infection for chickens and turkeys and induced a strong inflammatory response and lung damage, as already seen both in mammal and avian models.[5,30,35,36]

We used 7-day-old birds because most acute aspergillosis cases are reported soon after hatching, when a poult's respiratory system is naive and has not yet interacted with *Aspergillus* conidia from its environment.[2,16,37] In addition, the carefully chosen inoculation dose allowed us to survey the onset of clinical signs and lung lesions without sudden deaths. With these experimental conditions, our results are in accordance with previous studies[5,30] and confirm the higher susceptibility of turkeys to *Aspergillus* infection.

Infected turkeys were found to develop more severe clinical signs and showed higher mortality, but their mean weight loss was not statistically significant compared to noninfected turkeys. Earlier mortality in turkeys together with the heterogeneous weight of the PBS control group throughout the study may explain this ambiguity. Furthermore, although mean lung fungal loads tended to remain high in both avian species during the 3 monitored days, only infected turkeys displayed a severe infiltration of macrophages and lymphocytes as soon as 1 dpi. This may have owed to a flawed leukocyte recruitment that exacerbated tissue inflammation and necrosis but was unable to control *Aspergillus* proliferation. Femenia et al.[38] have suggested that the theoretical absence of resident macrophages and the enzymatic activity of heterophils are responsible for the increased susceptibility of birds, in which an exacerbated inflammation together with an increased fungal burden would lead to lung damage and

a bad disease prognosis. However, the results of the present study suggest that the infiltration of leukocyte subpopulations varies between turkeys and chickens, accounting for the discrepant profiles of lung damage and *Aspergillus* control.

To confirm the role of leukocyte recruitment in aspergillosis pathogenesis, we recovered respiratory lavages and characterized leukocyte subpopulations in chickens and turkeys. In spite of the low leukocyte concentrations expected and ultimately found in the birds' samples,[39] we were able to detect and analyze the proportions of leukocyte subpopulations. Aside from infected chickens at 1 dpi, lung leucocyte proportions remained comparable between infected and control groups. This could be interpreted as an early activation of the innate immune response in infected chickens that helped limit lung fungal colonization and the onset of clinical signs. This has already been seen in chickens inoculated with as much as 2×10^6 ufc, which did not develop clinical signs before 3 dpi.[40]

The early increase of CD8+ T cell proportions in chickens at 1 dpi could be interpreted as a consequence of the modulation of this cytotoxic lymphocyte subset by elevated IFN-γ levels and by early priming by Toll-like receptors such as TLR3.[41,42] In mammals, a transient expansion of CD8+ T cells has previously been described after sensitization with *Aspergillus*,[43] while fungal clearance long after infection has been found to be impaired in CD8+ T cell-depleted mice.[42] In this sense, low CD8+ T cell counts in infected turkeys correlate with a poor disease outcome, as has also been described for immunocompromised human patients.[44] Further research regarding the interaction between CD8+ T cells and Toll-like receptors in turkeys is needed to unveil the early mechanisms of fungal recognition and control of inflammation.

Avian heterophils share immunological properties with mammalian neutrophils, such as phagocytic ability, oxidative burst activity, and extracellular trap formation.[45–47] Although the higher proportion of heterophils in lung lavages found in chickens and turkeys supports the importance of their presence against inhaled pathogens such as *Aspergillus*,[40] we did not observe differences in the proportions of this cell subtype between infected and control birds during the three days of infection. Further research is needed to unveil the mechanisms of chicken and turkey heterophil recruitment and activation during *Aspergillus* infection.

We also analyzed the activation of several cytokines and chemokines known to be secreted soon after *Aspergillus* infection, such as CXCLi2 (the avian ortholog of mammalian IL-8) and IL-1β, which are produced by lung epithelial cells and activated phagocytic cells, respectively.[48,49] In chickens, both cytokines were strongly upregulated throughout the 3 days of infection, whereas in turkeys IL-1β expression only increased at 3 dpi. Although the expression of these cytokines has already been confirmed in chicken lung epithelial cells,[50,51] this is the first description of CXCLi2 and IL-1 β production after *Aspergillus* conidia infection; moreover, the delayed expression of IL-1 β in turkeys at 3 dpi may suggest a defective recruitment of phagocytic cells, potentially associated with poor PRR interaction in lung epithelial cells.[52]

Furthermore, early stages of pulmonary invasive aspergillosis in mammalian species are characterized by the stimulation of IL-6 expression in epithelial cells, which enhances pro-inflammatory and immunomodulatory responses.[11,27] Lung lymphocytes and alveolar macrophages also participate by secreting pro-inflammatory mediators such as IL-2 and IFN-γ.[12,27] These cytokines, in concert with CCL5 (recruitment of lymphocyte subsets) and the upstream control of TLR3, can participate in fungal clearance by promoting a protective cytotoxic T cell response.[42,48,53,54] However, dysfunction in their expression and/or interaction with immune cells may impair host protection[42] or lead to persistent infections.[55] In this study, chicken TLR3 remained downregulated at 2 dpi and 3 dpi. Although early TLR3 upregulation triggers pro-inflammatory cytokine production and controls fungal dissemination, the inhibition of its expression in chickens may be a strategy to reduce *Aspergillus* recognition and to avoid the overproduction of pro-inflammatory cytokines.[26,56] The downregulation of pro-inflammatory cytokine IL-2 in chickens has already been described,[40] but its role in avian Th17 cell response against *A. fumigatus* infection remains elusive.

Divergent IFN-γ, IL-6, and IL-10 mRNA regulations were observed between infected chickens and turkeys. As previously described, the upregulation of IFN-γ and IL-6 in chickens may ensure a better and faster host immune response[40] and be concomitant with a CD8+ T cell-related inflammation,[57] while their inhibition in turkeys—just as in patients with chronic sinusitis—may be associated with a Th17-like cytokine regulation.[58] The role of IL-10 as a negative regulator of inflammation during aspergillosis is less known in birds than in mammals.[28,59] Our model revealed opposed IL-10 expression between infected chickens and turkeys, indicating that the conserved function of this cytokine in chicken may help control aspergillosis-related inflammation.[60,61] The direct role of *A. fumigatus* chitin and conidia germination in the activation of host Th1- or Th2-like immune response has also been postulated for mammals and may additionally be true for birds.[62] Chicken epithelial cells produce CCL5,[51] a pro-inflammatory chemokine developed soon after *Aspergillus* stimulation that may be enhanced by anti-inflammatory IL-10[63,64] and be responsible for exacerbating the disease. Our results confirmed a divergent CCL5 regulation between infected chickens and turkeys at 1 and 2 dpi as well as a subsequent upregulation at 3 dpi in both avian species. Whereas early CCL5 upregulation in chickens may derive from lung epithelial cells, late expression in turkeys may be associated with natural killer (NK) cells' antifungal response.[64]

A host's immune response and treatment efficacy against *A. fumigatus* infection may also been screened by means of the

detection of plasma acute-phase proteins (APPs). Unfortunately, as shown in quail and rabbit models,[65,66] APPs' profiles seem to depict different trends among birds and mammals, limiting the use of these host surrogate markers in the former. In the light of our results and given that the association between IL-8, IL-6, IFN-γ, and C-reactive protein and haptoglobin modulation has already been suggested in mammals,[67] it is essential to apply this interesting approach as a predictor of adverse outcomes in birds due to their inherent susceptibility to this fungal infection.

In conclusion, the clear differences in pro-inflammatory response identified here strongly suggest that compared to turkeys, chickens' faster immune response may entail an anti-inflammatory response in order to preserve lung parenchyma and physiology from *Aspergillus* germination and dissemination. However, this hypothesis needs further functional assays to support our gene expression results and to validate differential immunological profiles in galliform birds. Further research is also required to understand the mechanisms used by turkeys to overcome *Aspergillus* infection and to define the role of Th1 response in the control of acute aspergillosis in galliform birds.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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