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Th1/Th2 polarization by viral and helminth infection in birds

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Abstract

Mammals developed an immune system able to functionally polarize into so-called type 1 or type 2 immune pathways, to resolve infections with intracellular and extracellular pathogens, respectively. In the well-studied avian immune system of the chicken, however, no evidence for polarized immunity could be found, as yet. To investigate whether these two major arms of mammalian immunity, regulated by a T helper (Th)1/Th2 cytokine balance, evolved similarly in birds, chickens were exposed to a prevalent intracellular (viral) or extracellular (helminth) infection. By using semi-quantitative RT-PCR analysis we provide evidence that polarization of Th1/Th2 type immunity extends beyond mammalian species, and, therefore, has been evolutionary conserved for more than 300 million years, when the lineages of mammalian and avian vertebrates are assumed to have segregated.

Keywords: Th1/Th2 polarization; Chicken; Mammalian; Semi-quantitative RT-PCR; ChIL-13; ChIFN-γ

1. Introduction

Mammals developed an immune system able to functionally polarize into so-called type 1 or type 2 immune pathways (Mosmann et al., 1986), to resolve infections with intracellular and extracellular pathogens, respectively (Janeway, 1992). The polarization of acquired immune reactions is largely regulated by antigen-specific Th cells (Abbas et al., 1996). Naïve Th cells can differentiate into either Th1 or Th2 type cells, driving counterbalanced cell-mediated and humoral immune reactions, respectively (Mosmann and Sad, 1996). Th1 type cells typically produce IFN-γ, crucially driven by early IL-12 and IL-18, and are associated with inflammatory cytolytic responses; generally necessary for destroying cells infected by viruses and other intracellular microbes. By contrast, synthesis of IL-4, IL-5 and IL-13 form hallmarks of Th2 cells, which are facilitated by IL-4 or IL-13 and are associated with antibody production, anti-helminth reactions and IgE synthesis. Polarization of cytokine production and

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cellular responses by T helper cells was later extended to CD8 + α/β T cell receptor (TCR) cells, γ/δ TCR cells (Mosmann and Sad, 1996) and to dendritic cells (Liu et al., 2001; Kapsenberg, 2003). The ultimate source of Th1/Th2 phenotype bias remains largely unknown. Th1 responses likely involve recognition of conserved microbial motifs by innate immune cell receptors (Kapsenberg, 2003), while Th2-dominated reactions may result form default responses and non-microbial adjuvants (Schnare et al., 2001).

Whether the two major arms of immunity, regulated by a T helper (Th)1/Th2 cytokine balance, evolved similarly in birds is unknown. In the well-studied chicken (*Gallus gallus*) immune system immunoglobulin (Ig) E or IgG subtypes are missing. Also, Th2-associated allergies are unknown for birds. Chickens possess few eosinophils, basophils and mast cells—all hallmarks of mammalian Th2 responses. As yet, only Th1 type cytokines, including chicken (Ch) interferon (IFN)-γ (Digby and Lowenthal, 1995) and Ch interleukin (IL)-18 (Schneider et al., 2000), have been identified, with amino acid identities of only 30–35% relative to their mammalian orthologues (Secombes and Kaiser, 2003). The functional homologue of the long-searched-for ChIL-12, driving ChIFN-γ synthesis, was identified only very recently (Degen et al., 2004). Birds evolved delayed-type hypersensitivity reactions and produce a single IgG equivalent, known as IgY, possibly guided by a regulatory pathway of Th1 cytokines only. Recently, however, the first non-mammalian (chicken) Th2 cytokine gene sequences, with ChIL-5 identified as a pseudogene, were identified (Avery et al., 2004). This allowed us to examine whether avian Th2 type cytokine expression has been conserved over 300 million years, since the great radiation of birds and mammals (Kumar and Hedges, 1998). In the present study, we provide evidence that chickens are able to mount a typical Th1- or Th2-biased cytokine response to experimental viral and helminth parasite infections, respectively.

2. Materials and methods

2.1. Chickens

Three weeks old Normal White Leghorn SPF chickens were derived from the Intervet Animal Facilities and housed under SPF conditions. The animals received water and food ad libitum. All experiments were carried out according to protocols approved by the Intervet Animal Welfare Committee.

2.2. Microbial infection

Groups of three-week-old SPF White leghorn layer chickens ($n = 5$ per group) were infected with paramyxoviral NDV strain Herts 33/56 (1×10^4 ELD50/0.2 ml, intramuscularly) or embryonated *Ascaridia galli* (strain Liederbach) worm eggs (1000 per dose, orally). At several time points post-infection chickens were sacrificed, spleen and ileal lymphoid tissues extracted, and total RNA isolated from these tissues.

2.3. RNA isolation

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Grand Island, NY) as described by the manufacturer. RNA quality was evaluated on 1.2% agarose gels. RNA samples were used subsequently for semi-quantitative RT-PCR (semi-Q-RT-PCR) or stored at −70 °C.

2.4. Semi-quantitative RT-PCR

Two micrograms of total RNA was reverse-transcribed into cDNA using the Superscript II RT protocol (Invitrogen Life Technologies) in a 20 μl reaction. One microliter of cDNA was mixed with 0.5 μl of 1 unit/μl Supertaq (HT Biotechnology, Cambridge, U.K.), 1 μl of 10 ng/μl of each primer, 1.6 μl of 2 mM dNTPs, and 2 μl of 10× ST PCR buffer (HT Biotechnology) in a final volume of 20 μl. The reaction cycling conditions were: 5 min 94 °C, variable cycles (30 s 94 °C, 1 min 55 °C, 1 min 72 °C), and 5 min 72 °C for a final extension. To determine the optimum number of PCR cycles required for a near linear relationship between the amount of RNA and amplified DNA band intensity, a variable number of cycles was performed for each marker. Internal primers used are described in Table 1. PCR products were separated on a 1.5% agarose gel and analysed. ChGAPDH was used for RT-PCR control and semi-quantitative comparison for ChIFN-γ, ChIL-4 and ChIL-13 mRNA expression.

Table 1
Semi-quantitative-RT-PCR (semi-Q-RT-PCR) primers

RNA	Internal primer sequences	Fragment size (bp)	Cycles	Account number
IFN-γ	5′-GGACATACTGCAAGTAGTCT-3′ 5′-GGCCAGGTCCATGATATCTT-3′	308	35	X99774
IL-4	5′-GCGTCAAGATGAACGTGACA-3′ 5′-GTCTGCTAGGAACTTCTCCA-3′	230	35	AJ621249
IL-13	5′-CACCCAGGGCATCCAGAA-3′ 5′-TCCGCAGGTAGATCTCAT-3′	243	35	AJ621250
GAPDH	5′-GGCCGCCTGGTCACCAGGGCTGCC-3′ 5′-GGAGGAGTGGGGGAGACAGAAGGG-3′	1099	25	K01458

2.5. Analysis of the relative cytokine mRNA expression levels

Agarose gel images were scanned and analysed to determine the optical density (in arbitrary units) of individual PCR bands using the Molecular Analist™ version 1.5 software (Bio-Rad, Hercules, CA). Individual cytokine expression levels were calculated using the optical density values of individual ChGAPDH signals as a reference. They were subsequently used to calculate the relative cytokine mRNA ratios (infected/non-infected).

2.6. Statistical analysis

The significance of the differences between the relative cytokine mRNA ratios of Th1 type ChIFN-γ versus Th2 type ChIL-4 or ChIL-13 levels per time point was analysed using the Student's *t*-test. Differences were considered significant at a confidence level of 95% ($P < 0.05$).

3. Results

3.1. Th1-induced polarization in chicken

To investigate the existence of divergent cytokine reactions in birds, we first tested the presumed avian equivalent of a mammalian Th1-dominated cytokine response. Chickens were infected with Newcastle disease virus (NDV) a prevalent known mammalian Th1-inducing viral infection. At several time points post-infection, chickens were sacrificed, spleen and ileal tissue removed, and RNA isolated from these tissues. The RNA samples were analysed by semi-quantitative RT-PCR using internal PCR primers for ChIFN-γ, ChIL-4, ChIL-13 and ChGAPDH (Table 1). As shown in Fig. 1A and B, an acute viral NDV infection was characterized by an elevated expression of the signature cytokine ChIFN-γ in spleen and ileal immune tissue within 24 h, in line with the known Th1 cytokine constellation of viral infections in mammals, but without augmented Th2 cytokines. The increased ChIFN-γ expression in the spleen at 24 h was associated with diminished ChIL-13 expression.

3.2. Th2-induced polarization in chicken

To investigate whether chickens are capable to develop an avian equivalent of a prototypic mammalian Th2-pathway, dominated by augmented IL-4 or IL-13 levels, chickens were exposed to a prevalent helminth infection, known to evoke a mammalian Th2-response. Indeed, analysis by semi-quantitative RT-PCR revealed that chickens infected orally with embryonated *A. galli* worm eggs exhibited a typical Th2 type cytokine pattern at day 14 post-infection, with selectively augmented ChIL-4 and especially ChIL-13 mRNA expression in ileal immune tissue, but less pronounced in the spleen, with diminished ChIFN-γ expression in both tissues (Fig. 1C and D).

4. Discussion

Our results show a remarkable homology for infection-driven cytokine responses between evolutionary distant chickens and mice. In particular, ChIL-13 proved a sensitive Th2 marker. Mutually exclusive

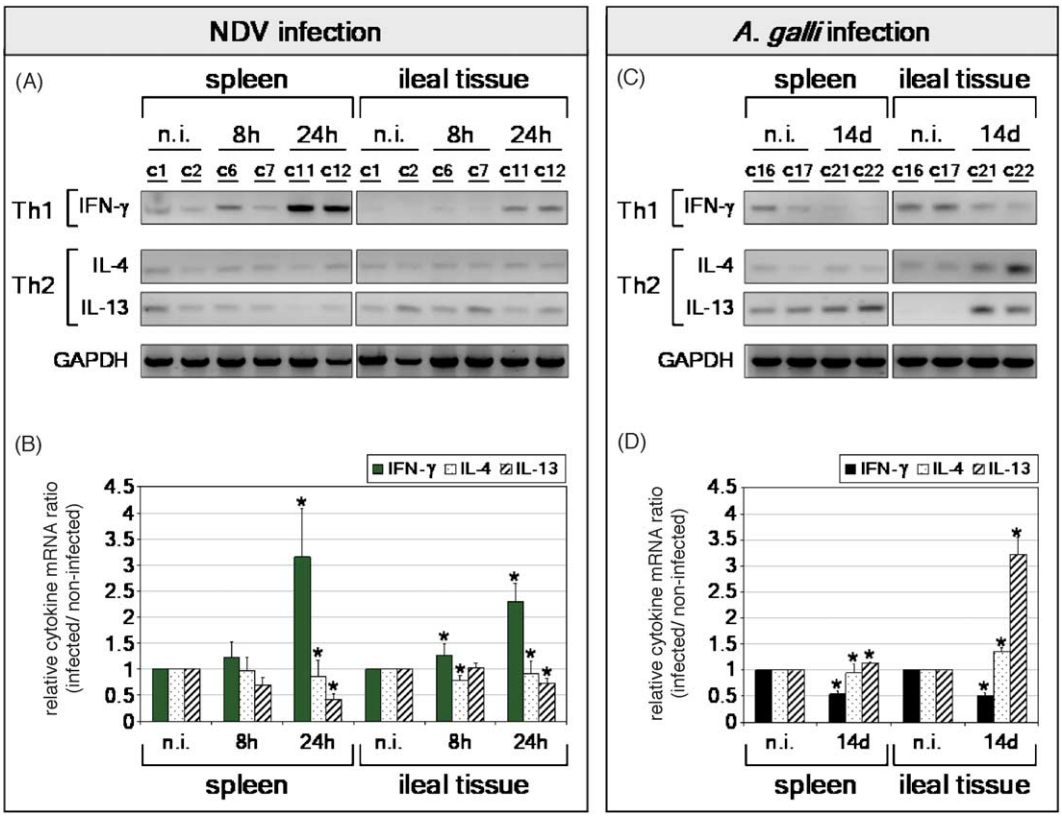

Fig. 1. Selective induction of Th1 characteristic ChIFN-γ and Th2-associated ChIL-4 and ChIL-13 mRNA expression by paramyxoviral NDV (strain Herts 33/56) (A and B), or embryonated *A. galli* (strain Liederbach) worm eggs (C and D) infection in SPF White leghorn layer chickens ($n = 5$ per group), respectively. [(A) and (C)] Ethidium bromide stained agarose gels; [(B) and (D)] relative cytokine mRNA expression ratio of [infected/non-infected] values. Means ± S.E.M, analysed by Student's *t*-test, are shown. $^{*}P < 0.05$ between Th1 and Th2 cytokine levels per time point. Non-infected (n.i.) birds served as controls; results are shown for two of the five chickens (c) in each group.

Th cell polarization, with associated cytokine diversity was first observed in mice (Mosmann et al., 1986); it results from cross-inhibitory transcriptional regulation of cytokine synthesis and receptor down-regulation (Ferber et al., 1999). Later, a similar skewing was found in man (Romagnani, 1991; Kapsenberg, 2003). After cloning and characterization of the first homologues of signature cytokines in swine, cattle and companion animals it became apparent that the cytokine-driven skewed immunity also holds for mammalian species of veterinary relevance (Schijns and Horzinek, 1997). Our data suggest a similar mechanism in chickens and provide evidence that polarization of Th1/Th2 type immunity extends beyond mammalian species (Schijns and Horzinek, 1997). Therefore, the capability to develop functionally distinct immune responses has been evolutionary conserved for more than 300 million years, when the lineages of mammalian and avian vertebrates are assumed to have segregated (Kumar and Hedges, 1998). Our results now permit in depth analysis at the molecular level of type 1- or type 2-biased reactions during microbial interference; for example, our approach may clarify why *A. galli*-infected birds exhibit impaired antiviral immunity (Horning et al., 2003). It will also allow investigations on the quality of infection-driven, vaccine adjuvant-guided or genotype-prone immune responses in chickens.

The conservation of two major immune pathways, instead of just one, in pre-mammalian species proves its vital importance in host defence against distinct types of microbes, such as viruses and worms. It

remains to be discovered whether such a dichotomy also arose in ''lower'' vertebrate ancestors of birds and mammals.

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