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REVIEW

Cytokines of Birds: Conserved Functions—A Largely Different Look

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ABSTRACT

Targeted disruptions of the mouse genes for cytokines, cytokine receptors, or components of cytokine signaling cascades convincingly revealed the important roles of these molecules in immunologic processes. Cytokines are used at present as drugs to fight chronic microbial infections and cancer in humans, and they are being evaluated as immune response modifiers to improve vaccines. Until recently, only a few avian cytokines have been characterized, and potential applications thus have remained limited to mammals. Classic approaches to identify cytokine genes in birds proved difficult because sequence conservation is generally low. As new technology and high throughput sequencing became available, this situation changed quickly. We review here recent work that led to the identification of genes for the avian homologs of interferon-α/β (IFN-α/β) and IFN-γ, various interleukins (IL), and several chemokines. From the initial data on the biochemical properties of these molecules, a picture is emerging that shows that avian and mammalian cytokines may perform similar tasks, although their primary structures in most cases are remarkably different.

INTRODUCTION

Over the last 20 years, it became increasingly clear that cytokines are central regulators of the immune system. Whereas a growing number of these molecules were cloned and functionally characterized in mammals, knowledge of avian cytokines lagged far behind. A strong interest in these immunoregulatory molecules in birds arose from different lines of research. Lack of appropriate growth factors to maintain chicken T cell cultures and to generate antigen-specific T cell clones initially stimulated work on the chicken homolog of interleukin-2 (IL-2). More recently, researchers investigating infectious diseases in poultry became interested in the regulation of immune responses. In particular, the question whether Th1-type or Th2-type immune responses can be defined in the chicken became a major focus of discussion. With the cloning of interferon-γ (IFN-γ) and IL-18, as well as a number of proinflammatory cytokines, the existence of a Th1-like cytokine network could be demonstrated, although firm evidence for Th2 responses in birds is still missing.

The availability of recombinant cytokines and methods to express cytokines in birds initiated work on their potential use as immune response modifiers to stimulate innate immunity or as immune adjuvants to enhance the efficacy of vaccines. This knowledge is now used increasingly to investigate well-established disease models, including Rous sarcoma virus-induced tumors, the duck model for hepatitis B, several infectious diseases (Marek's disease, Newcastle disease, salmonellosis and coccidiosis), as well as autoimmune disease models (Hashimoto thyroiditis, scleroderma, autoimmune vitiligo).

Here, we review recent work on chicken IFN, IL, and chemokines. With the exception of chicken myelomonocytic growth factor, hematopoietic growth factors are not covered in this paper. For a recent review on these molecules, the reader is referred to Siatskas and Boyd.[1]

AVIAN CYTOKINE GENES AND THEIR PRODUCTS

Interferons

Type I IFN. From a historical point of view, the type I IFN of chickens occupies a privileged position in cytokine research, as it was the first IFN to be discovered. Isaacs and Lindenmann[2] discovered it during their seminal studies on a previ-

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ously unknown soluble factor that interfered with influenza A virus replication in allantoic membranes from embryonated eggs. It was only after IFN from mammals had been characterized at the molecular level that biochemical procedures became available that, for the first time, yielded substantial amounts of purified chicken IFN (ChIFN) from virus-induced embryonated chicken eggs[3] and virus-induced embryonic chicken cells.[4,5] Purified type I ChIFN was found to be a glycoprotein of approximately 25 kDa whose antiviral activity showed a remarkably high degree of resistance to acid treatment and exposure to heat. These biochemical properties contrasted with those of another factor with antiviral activity secreted by concanavalin A (ConA)-treated chicken spleen cells,[6] which was known as type II ChIFN, now referred to as IFN-γ.

The first cDNA for type I ChIFN was identified by Sekellick et al.[7] by an RT-PCR approach in which mRNA from virus-induced chicken embryo cells was analyzed with primers from conserved regions of mammalian IFN-α and IFN-β genes. The protein encoded by this novel chicken gene showed a barely significant degree of similarity to mammalian IFN-α and IFN-β (<25% identity at amino acid level). As the precise relationship of this molecule to the various mammalian type I IFN was not immediately clear,[8] it was initially designated "chicken IFN1" by some authors.[9,10] Recent functional studies indicate that it most likely represents the functional homolog of mammalian IFN-α.[11] Subsequent work revealed the existence of about 10 closely related intronless IFN-α genes (Table 1) in the chicken that form a cluster[9] on the short arm of the chicken Z chromosome.[12] The primary translation product of ChIFN-α mRNA is a 193-residue polypeptide of which the N-terminal 31 amino acids seem to represent a signal peptide. Secreted ChIFN-α (molecular mass ~19,000) contains four potential N-linked glycosylation sites. Recombinant ChIFN-α (rChIFN-α) produced in either COS cells or *Escherichia coli* is a potent antiviral agent that has a specific activity of about 10^8 U/mg protein,[13] a value comparable to that of human IFN-α2.

Low-stringency hybridization of recombinant lambda phages resulted in the identification of a chicken DNA fragment that carries an additional intronless type I IFN gene whose product was only 57% identical to ChIFN-α and <25% identical to mammalian IFN.[9] Functional studies indicate that this gene, initially designated "chicken IFN2" by some authors,[9,10] most likely codes for the chicken homolog of mammalian IFN-β[11] (Table 1). The primary translation product of ChIFN-β mRNA consists of 203 amino acids, including a 28-residue signal peptide. Thus, secreted ChIFN-β is 14 residues longer than ChIFN-α and is composed of 176 amino acids. It has a calculated molecular mass of 20,372 and features two potential N-linked glycosylation sites. In contrast to IFN-α, there is a single gene in the chicken that codes for IFN-β.[9] It maps to the short arm of the chicken Z chromosome, like the IFN-α genes.[12] A polyclonal antiserum that neutralizes the antiviral activity of ChIFN-α failed to neutralize ChIFN-β, indicating that (reminiscent of the situation in mammals) the chicken has two serologically distinct type I IFN.[9] rChIFN-β from *E. coli* or transfected monkey COS cells is active, although its specific antiviral activity is at least 20-fold less than that of ChIFN-α.[9]

The existence of two serologically distinct families of type I IFN in the chicken is of great interest from an evolutionary point of view. Phylogenetic analyses indicated that the mammalian IFN-α and IFN-β genes originated by duplication of a progenitor gene after the divergence of birds, most probably about 250 million years ago.[8] The same type of phylogenetic analysis further indicated that the ChIFN-α and ChIFN-β genes originate from a separate duplication event that occurred independently at a later time.[8] However, several experimental findings argue against this view and suggest rather that the critical gene duplication actually took place before the avian and mammalian lineages diverged.

First, ChIFN-α and ChIFN-β genes are strongly expressed in response to viral infection of monocyte-derived macrophages[10] or embryo fibroblasts (Fig. 1A), whereas only the IFN-α but not the IFN-β genes are expressed in spleen (Fig. 1B) and thymus[10] of chickens treated orally with the immune-modifying imidazoquinoline S-28463.[14] This differential regulation of IFN-α and IFN-β genes in response to the two different inducing agents resembles that previously observed with human cells.[15] Second, the promoter of the ChIFN-β gene contains a cluster of putative binding sites for transcription factors NF-κB and IFN regulatory factor (IRF) that is essential for virus inducibility.[10] A similar set of regulatory elements is present at the corresponding position in the promoters of mammalian IFN-β but not IFN-α genes.[16] Like their mammalian counterparts, the ChIFN-α genes further lack binding sites for NF-κB.[10] Third, as in most mammals, we find a large IFN-α gene family and a single IFN-β gene in the chicken. It thus appears most unlikely that two independent gene duplications in birds and mammals could have resulted in genetic setups that resemble each other to this great extent.

IFN-α genes of other birds (Table 1) have been cloned by employing homology screening approaches. Identity to ChIFN-α at the amino acid level varies from 82% in turkey[17] to 50% in the duck.[18] The cloned duck gene was recognized to be a member of a family of approximately 10 related genes,[11] suggesting that IFN-α gene clusters may exist in all birds. No IFN-β gene of birds other than that of chickens have been described to date. This raises the question whether IFN-β plays a role in the innate immune response of birds. Infection experiments of the type shown in Figure 1A clearly demonstrate that the ChIFN-β gene is strongly expressed in virus-infected cells, suggesting that its product may play a role in virus defense similar to that of mammalian IFN-β.

Little is known about type I IFN receptors and receptor-associated proteins in birds. A comparative genomic analysis of genetic loci located next to the *GART* gene in humans and chickens revealed two conserved genes, *IFNAR-1* (accession number AF082664) and *IFNAR-2* (accession number AF082665), that seem to code for the two subunits of the type I IFN receptor.[19] Other studies further indicated that the Jak-Stat signaling pathway, which mediates IFN signal transduction in mammals, is also operative in cells from birds.[20–23] Taken together, these findings indicate that most elements of the mammalian type I IFN system have been conserved in birds.

IFN-γ. IFN-γ represents the major macrophage-activating factor (MAF), exhibits antiviral activity, and drives Th1 immune responses (for reviews see refs. 24, 25). Before the first ChIFN gene was cloned, it appeared that birds might possess an archetype IFN species that combines the known properties

Table 1. List of Cloned Avian Cytokine cDNA

Cytokine	*Accession number*	*References*
Chicken		
Interferons		
IFN-α family members	U07868, X92476, X92477, X92478	7, 9
IFN-β	X92479	9
IFN-γ	U27465, X99774	28, 29
Interleukins		
IL-1β	Y15006	50
IL-2	AF000631	59
IL-6	AJ309540, AI982185	88
IL-15	AF139097, AF152927	
IL-16	AJ399362	
IL-18	AJ277865	105
Chemokines		
CXC chemokines		
cCAF (9E3/CEF4)	M16199	112
K60	Y14971	113
JSC	AF285876	
CXCL DT40	AJ393586	
CXCL pat	AI981378	
MDV vIL-8	AF065430	117
CC chemokines		
MIP-1β	Q90826	176
CCL	AI980712	
K203	Y18692	113
TARC	AI980713	
RANTES	AI982103	
TCA	L34552	175
FPV-116	AAF44460	
FPV-121	AAF44465	
C chemokines		
Lymphotactin (Ltn)	AF006742	118
Others		
cMGF	M85034	89
TGF-β	M31154, M31160	177, 178
CD40L	AI982044	
BAFF/BlyS	AJ397416	
Turkey		
Interferons		
IFN-α	U28140	17
IFN-γ	AJ000725	34
Interleukin		
IL-2	AJ007463	60
Duck		
Interferon		
IFN-α	X84764	18
IFN-γ	AF087134, AF100929	32, 33
Interleukin		
IL-18	AF336122	
Pheasant		
Interferon		
IFN-γ	AJ001289	34
Quail		
Interferon		
IFN-γ	AJ001678	34
Guinea fowl		
Interferon		
IFN-γ	AJ001263	34

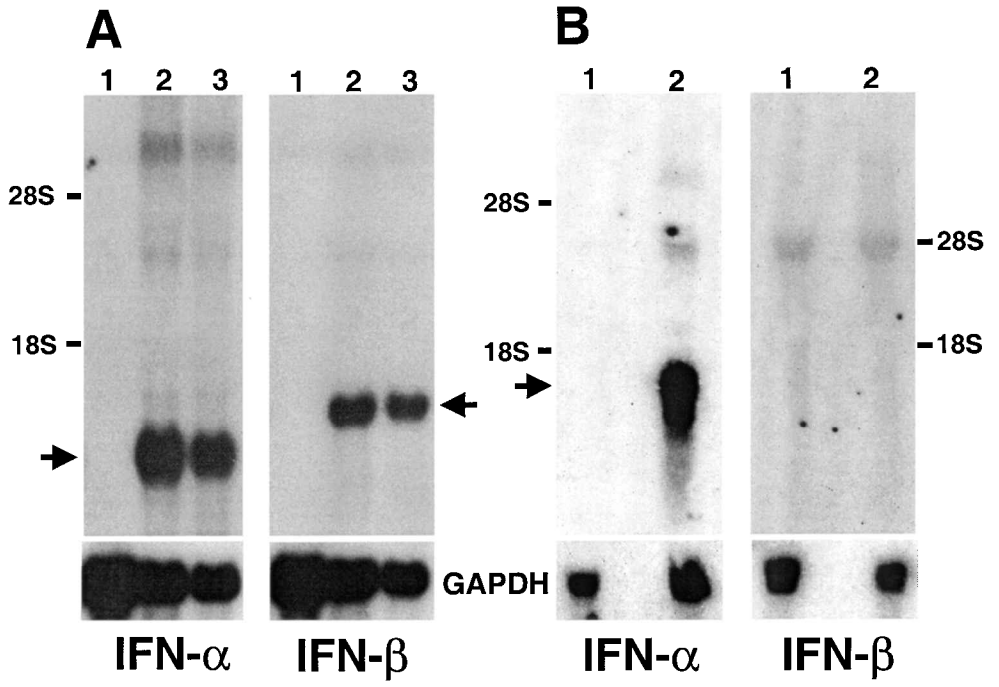

FIG. 1. Differential induction of ChIFN-α and ChIFN-β genes in response to viruses and the imidazoquinoline S-28463. **(A)** Cultures of primary chicken embryo cells were either left untreated (lane 1), infected with a mutant of influenza A virus lacking the gene for nonstructural protein 1 (NS1) at a multiplicity of infection of 1 (lane 2), or infected with Newcastle disease virus at a multiplicity of 10 (lane 3). RNA was prepared 24 h later, and samples were analyzed by Northern blotting for the presence of transcripts derived from IFN-α and IFN-β genes. **(B)** Three-week-old chickens were treated orally with either 2.5 mg S-28463 per kg body weight (lane 2) or mock-treated with distilled water (lane 1). The animals were killed 75 min later, and the spleens were removed and snap-frozen in liquid nitrogen before RNA was prepared. Samples were analyzed by Northern blotting for the presence of transcripts derived from IFN-α and IFN-β genes. Reprobing of the membranes with chicken GAPDH cDNA demonstrated that comparable amounts of RNA were loaded into the various lanes.

of mammalian IFN-α, IFN-β, and IFN-γ in a single molecule. This view was mainly based on results of experiments performed with ConA-stimulated chicken spleen cells. Mammalian spleen cells exclusively release IFN-γ under these induction conditions that is highly sensitive to heat and acid treatment. Surprisingly, chicken spleen cell supernatants exhibited not only MAF activity but also antiviral activity with the physiochemical properties of mammalian type I IFN, for example, resistance to heat and acid treatment.[26] This concept was disturbed by the observations that rChIFN-α is a potent antiviral factor that lacks MAF activity[27] and that a monoclonal antibody (mAb) to rChIFN-α (designated 8A9) neutralized most of the antiviral activity present in the supernatants of ConA-stimulated chicken spleen cells without affecting the MAF activity.[28]

cDNA encoding the chicken homolog of mammalian IFN-γ (Table 1) were cloned independently by two laboratories using different functional screening approaches.[28,29] Both groups expressed cDNA libraries prepared from chicken T cells in transfected monkey COS cells and screened the resulting supernatants for antiviral and nitric oxide (NO)-inducing activity[29] or for an activity that induced expression of the guanylate-binding protein (GBP) gene in CEC-32 cells.[28] ChIFN-γ mRNA has an open reading frame (ORF) that codes for a polypeptide of 164 amino acids whose sequence shows <34% identity to mammalian IFN-γ. The N-terminal 19 amino acids serve as a signal peptide. Secreted ChIFN-γ consists of 145 amino acids (calculated molecular mass ~18,000). The IFN-γ gene has been mapped to chicken chromosome 1.[30] rChIFN-γ expressed in either monkey COS cells or *E. coli* is biologically active. Like mammalian IFN-γ, it is sensitive to heat and acid treatment.[29] It potently induces the expression of genes known to be inducible by IFN-γ in mammals, including IRF-1, GBP, NO synthase (NOS)-2, and genes for MHC class II antigens, suggesting that most elements of this system are conserved as well. ChIFN-α and ChIFN-γ act synergistically,[31] demonstrating another feature shared by the mammalian and avian IFN systems. A polyclonal antiserum against rChIFN-γ neutralized the MAF activity in supernatants of ConA-treated spleen cells but had little effect on the antiviral activity,[28] confirming the view that most of the antiviral activity in such supernatants is probably due to the presence of IFN-α. Thus, unlike mammalian spleen cells, spleen cells from chickens seem to produce a mixture of type I and type II IFN in response to stimulation by mitogen. By homology screening, IFN-γ cDNA of other birds have been cloned recently (Table 1). IFN-γ from turkey, pheasant, and quail show a high degree of identity to ChIFN-γ, whereas IFN-γ from ducks is only 67% identical.[32–34] Nevertheless, duck IFN-γ showed antiviral activity on chicken fibroblasts, albeit ~16-fold less than on duck fibroblasts. Interestingly, recombinant duck IFN-γ strongly

blocked the replication of duck hepatitis virus in primary duck hepatocytes.[32]

Many poxviruses code for secreted soluble factors with IFN-γ binding activity that block the interaction between cytokine and cellular receptor (for review, see ref. 35). The protein encoded by the *B8R* gene of vaccinia virus binds IFN-γ of most mammals. Interestingly, it can also bind to ChIFN-γ, thereby neutralizing its GBP-inducing and NO-inducing activity.[36] These results indicate that in spite of the low conservation of primary sequence, the tertiary structures of chicken and mammalian IFN-γ must be quite similar.[157]

Interleukins

IL-1. Mammalian IL-1 was identified initially as a macrophage-derived factor with lymphocyte costimulating activity.[37] Subsequent work showed that IL-1 acts not only on T lymphocytes and thymocytes but also on a wide variety of other cells, thereby regulating the host response to infection and injury. The pleiotropic activities of IL-1 include its T cell comitogenic property, induction of fever, activation of the hypothalamic-pituitary axis and glucocorticoid secretion, triggering of the acute-phase response, and activation of the vascular endothelium. Many of the activities of IL-1 are mediated through its ability to induce other cytokines, such as the proinflammatory cytokines IL-6 and tumor necrosis-factor-α (TNF-α), and several chemokines (for reviews, see refs. 38, 39). Three structurally related members of the IL-1 cytokine family have been described in mammals. IL-1α and IL-1β are biologically active cytokines that are specifically inhibited by the third family member, termed IL-1 receptor antagonist (IL-1Ra).

In the chicken, an IL-1-like biologic activity was first demonstrated in supernatants of lipopolysaccharide (LPS)-stimulated adherent spleen cells.[40] Chicken thymocytes were stimulated with a suboptimal dose of mitogen that led to a weak proliferative response, which was strongly enhanced on addition of preparations containing crude IL-1. Using this assay it became clear that *Staphylococcus aureus*, LPS, phorbol esters, and silica all induced IL-1 secretion in primary chicken macrophages and in the macrophage cell lines HD-11 and MQ-NCSU.[40–43] Induction of ChIL-1 secretion was shown to be dependent on protein kinase C (PKC), calmodulin-dependent protein kinase,[42] and calcium.[44] A number of additional biologic activities, including induction of fibrinogen and metallothionein synthesis in hepatocytes,[45,46] induction of fever and anorexia,[47] and increased weight gain and feed intake,[48] were all attributed to an IL-1-like factor. IL-1-like bioactivity was also found in supernatants of ConA-stimulated chicken splenocytes. The protein exhibiting this activity was partially purified by immunoaffinity chromatography using a rabbit antihuman IL-1α/β antibody.[49] The enriched protein preparation induced the release of corticosterone *in vivo*.

Isolation of a cDNA encoding ChIL-1β (Table 1) was achieved by expression cloning using a cDNA library prepared from LPS-stimulated HD-11 cells.[50] The bioassay employed in this work relied on the fact that IL-1 strongly activated a CXC chemokine gene (*K60*) in target cells. The primary translation product of ChIL-1β cDNA consists of 267 amino acids and shows 25% similarity to human IL-1β (HuIL-1β). Like its mammalian counterparts, ChIL-1β lacks a signal peptide and seems to be synthesized as an inactive precursor molecule. Interestingly, the characteristic caspase-1 protease cleavage site is not conserved in chicken pro-IL-1β. A fragment corresponding to putative mature ChIL-1β that comprises amino acids 106–267 was expressed in *E. coli* and shown to be biologically active. It induced the synthesis of *K60* mRNA in an avian fibroblast cell line and it strongly upregulated corticosterone secretion *in vivo*.[50] cDNA for the chicken homolog of IL-1α or IL-1Ra have not been identified to date.

In mammals, two distinct IL-1 receptors (IL-1R1 and IL-1R2) have been described. Ligand binding to IL-1R1 leads to pronounced cellular responses. In contrast, IL-1R2 acts as a decoy receptor that binds ligand without transducing an activating signal. ChIL-1R1 cDNA has been cloned.[51] It shows 60% amino acid similarity to murine and human IL-1R1 (MuIL-1RI and HuIL-1R1). Recently, Klasing and Peng[52] expressed the entire extracellular region of ChIL-1R1 in the yeast *Pichia pastoris*, which resulted in the synthesis of an IL-1 binding protein that partially inhibited the biologic activity of natural preparations of ChIL-1. This recombinant protein was used to generate a neutralizing antiserum. Both soluble receptor and antireceptor antibodies will be valuable tools for future work aimed at elucidating the *in vivo* function of ChIL-1.

IL-2 and IL-15. IL-2 and IL-15 are two structurally related cytokines with overlapping biologic functions (for recent reviews, see refs. 53–56). Following several unsuccessful attempts to clone chicken IL-2 using either partial protein purification or cross-hybridization,[57,58] IL-2 cDNA (Table 1) was identified by COS cell expression cloning using a bioassay.[59] The 747-bp ChIL-2 cDNA has a 429-bp ORF that codes for a protein of 14.5 kDa with a predicted signal peptide of 22 amino acids. The 298-bp 3′-UTR contains five repeats of the RNA instability motif ATTTA. Interestingly, turkey IL-2 and ChIL-2 share only 70% amino acid identity, although they cross-react in functional assays.[60]

The overall amino acid identity to mammalian IL-2 and IL-15 is only ~24%. ChIL-2 harbors four cysteine residues that can potentially form two intrachain disulfide bonds, which is a feature of mammalian IL-15 but not IL-2. For this reason, it was initially thought that ChIL-2 might represent an ancestral form of a cytokine that may combine features of mammalian IL-2 and IL-15.[59] This view is probably incorrect, as evidenced by recent results of genomic analyses of the ChIL-2 gene and the identification of a ChIL-15 cDNA in an expressed sequence tag (EST) database (see below). ChIL-2 and ChIL-15 both contain structural features characteristic of four α-helix bundle cytokines.

The ChIL-2 gene is located on chromosome 4 linked to annexin 5 with synteny to mouse chromosome 3 and human chromosome 4, respectively.[61] A 3.8-kb fragment of the ChIL-2 locus (accession No. AJ224516) contains four exons and three introns with structures similar to mammalian IL-2 genes. The promoter sequence contains a TATAAA box and several conserved potential regulatory sequences, including NF-AT, AP-1, and CD28 response elements. The NF-κB and octamer binding sites of mammalian IL-2 promoters are less well conserved. Analyses of eight inbred chicken lines suggested that IL-2 is a single copy gene without detectable polymorphism.[61]

IL-15 was identified in an EST database from chicken liver[62] (Table 1). The 857-bp ChIL-15 cDNA encodes a 187-

residue polypeptide that shows about 38% identity to HuIL-15. Identity of this protein to ChIL-2 is very low apart from the four conserved cysteines. It has been demonstrated in mammals that several upstream AUG of the 5′-UTR and an unusual, long signal peptide impede effective translation of IL-15 mRNA.[63] The ChIL-15 mRNA has only three upstream AUG as opposed to 12 in the human counterpart. However, the signal peptide of ChIL-15 is longer, consisting of 63 amino acids.

Mammalian IL-2 and IL-15 receptors are composed of three subunits. Both cytokines have their own specific α chains but share the β chain. The common γ chain is used as signal transducing molecule by a variety of other cytokine receptors, including those for IL-4, IL-7, IL-9, and IL-21. Mutational analysis of ChIL-2 showed that an N-terminal asparagine and the entire C-terminus are critical for IL-2 function, indicating that these regions of the ligand might be essential for receptor triggering.[64] An mAb designated INN-CH-16, reacts with a 48–50-kDa antigen expressed on activated T lymphocytes and inhibits the proliferation of ConA blasts in response to supernatants of stimulated T cells. These features led to the hypothesis that INN-CH-16 may recognize the ChIL-2R.[65,66] A candidate cDNA clone for the ChIL-2R α chain recently was deposited in the database (accession number AF143806). It is unclear at present if this protein is recognized by INN-CH-16. The predicted ChIL-2R α chain shows about 30% amino acid identity to the mammalian counterparts and contains a typical Sushi domain, but, interestingly, it lacks the entire cytoplasmic domain. A partial sequence of the IL-15R α chain has been deposited in the database (accession number AI980376). Because of the shortness of the cloned cDNA fragment, its identification as IL-15R α chain must remain preliminary.

Several chicken EST clones with homology to the common γ chain have been isolated.[62] Although the different clones do not contain the complete ORF, they cover a highly conserved extracellular domain (~40% amino acid identity to mammalian counterparts), including a 19-residue signal peptide, a fibronectin-like domain, and a 20-amino acid transmembrane region. The presence of four conserved cysteines and the canonical WSXWS motif clearly identify this protein as a member of the cytokine I superfamily of receptors.[67] Taken together, these preliminary analyses in the chicken allow the conclusion that the IL-2/IL-15 receptors most likely have a mammalian-type structure.

Before rChIL-2 was available, crude supernatants of ConA-stimulated splenocytes or partially purified IL-2 were used to study various aspects of the biology of this cytokine. These experiments have been summarized elsewhere.[68] They still need to be validated using rIL-2 preparations and neutralizing mAb. In spite of these technical limitations, a number of different experiments clearly indicated that ChIL-2 induces proliferation of T cells and natural killer (NK) cells and enhances their cytolytic activity.[69] IL-2 bioactivity traditionally has been measured in proliferation assays using splenocytes that are activated with ConA for 48 h.[59] These T lymphocyte blasts respond very well to rIL-2. However, this assay is not very sensitive, and minute amounts of secreted IL-2 cannot be measured accurately because of the high background levels of preactivated cells. Moreover, it is likely that the ConA blasts will also respond to a variety of other soluble factors, which may have led to inaccurate conclusions. Unfortunately, alternative assay procedures have not been described to date, and the various cell lines previously classified as "IL-2 sensitive" were tested without success for proliferative responses to rIL-2. As the ChIL-15 cDNA sequence has been deposited in the database only recently, rChIL-15 is only beginning to be produced in several laboratories, and no information on its biologic activity is available. As for IL-2, it will be essential to develop a reliable and sensitive bioassay for IL-15 in order to elucidate its function in the chicken immune system.

IL-6. IL-6 in mammals is a multifunctional cytokine produced by T and B lymphocytes, endothelial cells, monocytes/macrophages, and fibroblasts.[70] The huge diversity of biologic activities of IL-6 is exemplified by a long list of alternative names (B cell differentiation factor [BCDF], B cell stimulation factor [BSF-2], hepatocyte-stimulating factor [HSF], IFN-β2).[71] IL-6 is a potent B cell hybridoma growth factor[72] and regulator of immunoglobulin synthesis.[73] It was long known for its role in the initial activation of T lymphocytes.[74] It has been shown to promote the development of Th2 immune responses by inducing IL-4 synthesis[75] and to inhibit Th1 differentiation by upregulating the suppressor of cytokine signaling 1 (SOCS-1).[76] The important role of IL-6 as an immunoregulator is further underscored by its ability to switch monocyte differentiation from dendritic cells to macrophages.[77] It also enhances the proliferation of multipotential hematopoietic progenitors. One of the major biologic effects of IL-6 is the induction of an acute-phase response. IL-6 released in response to infections, injuries, malignant tumors, and immunologic disorders profoundly modulates the protein secretion pattern of the liver, the fever response, and secretion of corticosteroids.[78]

Early work in chickens focused on the demonstration of IL-6-like biologic activities in various assay systems. Amrani et al.[79] showed that LPS-stimulated macrophages secreted an HSF that induced fibronectin production by cultured hepatocytes. HSF was also secreted by LPS-treated fibroblasts and could be neutralized by an antiserum to rHuIL-6. Furthermore, rHuIL-6 was biologically active on chicken hepatocytes,[80] strongly indicating the existence of a highly conserved IL-6 system in birds. Further evidence in support of this view came from studies that demonstrated an IL-6-like activity in chicken serum[81] and chicken ascites fluids[82] that strongly induced proliferation of certain murine hybridoma cell lines. The ascites fluid activity could be neutralized by an antiserum to HuIL-6.[82] Antibodies raised against mammalian IL-6 were also reported to detect ChIL-6 in thymic tissue sections.[83] Several bioassays for detection of ChIL-6 have been established that are based on the production of fibronectin by a chicken hepatocyte cell line[84] or the induction of proliferative responses in murine hybridoma cell lines, including B9[82] and 7TD1.[85] Employing these assay systems, IL-6-like activities were demonstrated in serum of *Eimeria*-infected birds,[85] in turkey poults suffering from poult enteritis and mortality syndrome,[86] and in supernatants of LPS-stimulated chicken heterophil cultures.[87]

Using suppression subtractive hybridization technology, Schneider et al.[88] identified an mRNA in spleens of imidazoquinoline-treated chickens that showed <35% identity at the amino acid level to mammalian IL-6 (Table 1). In addition, a cDNA fragment of the same gene was identified in an EST database generated from ConA-treated chicken T lymphocytes (Table 1). The 1.5-kb ChIL-6 mRNA harbors eight ATTTA

RNA instability motifs in the 3′-UTR that are typically found in cytokine mRNA. Its translation product features an unusually long signal peptide of 47 amino acids. ChIL-6 shares a common motif consisting of four cysteine residues with mammalian IL-6, granulocyte colony-stimulating factor (G-CSF), and chicken myelomonocytic growth factor (cMGF). The mature secreted form of ChIL-6 appears to consist of 194 amino acids, with a calculated molecular mass of ~22,000. However, the molecular weight of natural ChIL-6 is approximately 35 kDa,[82] indicating extensive posttranslational modification. rChIL-6 produced in *E. coli* induces proliferation of murine 7TD1 cells at similar concentrations and to a similar extent as rHuIL-6.[88] Thus, IL-6 seems to be exceptional among ChIL and ChIFN, as it exhibits biologic activity across species boundaries. The *in vivo* biologic activity of rChIL-6 was further demonstrated by showing that it can induce serum corticosterone accumulation after intravenous application to chickens.

cMGF was found to be distantly related to mammalian IL-6, G-CSF, and ChIL-6. In their original work Leutz et al.[89] arrived at the conclusion that cMGF is most likely not the avian homolog of mammalian IL-6. Nevertheless, some authors who used cMGF as a marker to monitor cytokine responses in experimental infections of chickens continue to call this cytokine IL-6.[90,91] As *bona fide* ChIL-6 has now been cloned, this confusing nomenclature should no longer be used.[88]

IL-8. Chemokines that may represent the chicken homolog of IL-8 are discussed under the heading of *Chemokines.*

IL-16. IL-16 is an immunomodulatory cytokine first described in 1982 as a T cell chemoattractant factor.[92,93] IL-16 is synthesized by a variety of immune and nonimmune cells as a biologically inactive precursor molecule of approximately 70–80 kDa. Like IL-1β and IL-18, IL-16 lacks a signal peptide. In mammals, pro-IL-16 is processed into the mature form by caspase-3. Secreted IL-16 aggregates to form biologically active homotetramers, which then bind to CD4. New data indicate that CD4 is not the only receptor for IL-16.[94] IL-16 has pleiotropic functions. It exhibits chemoattractant activity toward T cells, monocytes, and eosinophils, it activates proliferation of T cells, and it induces in target cells the expression of HLA-DR, IL-2Rα chain, and various cytokines genes, including IL-1β, IL-6, IL-15, and TNF-α (for reviews, see refs. 95, 96).

Analysis of BLAST search results from a chicken bursal EST database identified a cDNA clone that contains sequence information for ChIL-16 (Table 1). It contains information on the C-terminal moiety of ChIL-16, including the complete sequence of the putative mature form of this cytokine. ChIL-16 shows a surprisingly high similarity to mammalian IL-16. It shows 64% identity to HuIL-16 (Fig. 2). If conservative amino acids are taken into account, similarity is ~75%. No functional data are available at present.

IL-18. IL-18 was originally purified from livers of mice treated with *Propionibacterium acnes*, followed by LPS stimulation.[97] It enhances the cytotoxic activity of CD8 T cells[98] and NK cells[99] and promotes Th1 or Th2 differentiation depending on its cytokine milieu.[100–102] A characteristic property of IL-18 is its ability to induce in target cells the secretion of IFN-γ. For this reason, IL-18 was originally designated IFN-γ-inducing factor (IGIF).[97] IL-18 synthesis is regulated at the protein level. It is synthesized as a precursor molecule that is activated by caspase-1 cleavage after a conserved aspartate residue.[103,104]

A cDNA for ChIL-18 was identified by screening the BLAST search results of an EST database generated from a chicken B cell line (Table 1). ChIL-18 mRNA contains an ORF of 597 nucleotides (nt) that codes for a protein of 198 amino acid residues that shows ~30% identity to IL-18 from mammals.[105] Sequence alignments identified a conserved aspartate residue that is probably recognized by caspase-1. After proteolytic cleavage, the putative mature ChIL-18 comprises 169 amino acids. This molecule was produced in *E. coli* and subsequently was shown to be biologically active in an IFN-γ induction assay using chicken spleen cells[105] (Table 2).

A recent database entry (Table 1) provides sequence information on IL-18 of ducks. The putative mature forms of duck IL-18 and ChIL-18 show a high degree of identity (~88%) (Fig. 3). No information is available to date on the biologic activity of duck IL-18.

In mammals, induction of IFN-γ by IL-18 is dependent on IL-12. The synergism between these cytokines is the result of IL-12-mediated upregulation of the IL-18 receptor.[106] IL-18 and IL-12 further activate complementary sets of transcription factors that regulate the activity of the IFN-γ promoter.[107] ChIL-18 showed good activity in the splenocyte assay in the absence of exogenous IL-12,[105] indicating that avian IL-18 differs from its mammalian counterpart[106] in that it requires no costimulatory signal. However, we recently found interesting differences between splenocytes from B19 inbred chickens and splenocytes from an outbred chicken line. In cells from the latter animals, rChIL-18 was inactive unless a costimulatory signal was provided by T cell receptor (TCR) cross-linking using an anti-α/β TCR mAb (TCR-2) (unpublished observations).

The genomes of several poxviruses code for IL-18-binding proteins that are believed to play a role in the viral evasion strat-

```
chicken //KDGEKS--HNVSIGSSTSSVASDASQESTTEETICTITLDKTAAGLGFSLEGGKGSIHGDKPIIINRIFKGT 113
human   //TPEAMPDLNSSTDSAASA.A...V.V...A.A.V..V..E.MS.....L.....LT.......A 569

chicken   SLEQSSPVQPGDELLQVHTTALQGLTRFEAWNIIKALPDGPITAIIKRKNPSSVTKKASETL      199
human     AS...ET......I..LGG..M.....V.IV.R..SLQ.KETT.AGDS                   631
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FIG. 2. High similarity of chicken and mammalian IL-16. Alignment of the two amino acid sequences was done using the Jotun-Hein method. The N-terminal parts of the propeptides located upstream of the putative caspase-3 cleavage sites are not depicted. Identical amino acids are represented by periods. The GLGF motif shaded in gray is needed for tetramerization of IL-16 monomers. The arrow points to a conserved aspartate residue that marks the caspase-3 cleavage sites in mammalian IL-16. Overall identity between putative mature ChIL-16 and HuIL-16 is 64%.

TABLE 2. BIOASSAYS VALIDATED FOR BOTH RECOMBINANT AND NATURAL CHICKEN CYTOKINES

Cytokine	*Bioassay*	*Reference*	*Neutralizing antibodies/reagents*	*Reference*
IL-1	Thymocyte comitogenesis assay CXC chemokine (K60) induction assay	41, 180 50	Soluble IL-1 receptor (sIL-1R_I) and anti-IL1R_I both neutralize natural IL-1 activity	52
IL-2	Splenocyte proliferation assay	69, 180	Anti-ChIL-2 mAb 10E7 or 4F12	179
IL-6	IL-6-dependent proliferation of murine hybridoma cell lines (B9 or 7TD1)	82, 88		
IL-18	IL-18-induced IFN-γ secretion by chicken spleen cells (see also IFN-γ assay)	105		
IFN-α	Antiviral assay	7, 9, 180	Anti-IFN-α mAb 8A9	171
IFN-β	Antiviral assay	7, 9, 180		
IFN-γ	Induction of NO synthesis in HD-11 cells	29, 180	Anti-ChIFN-γ mAb 1E12	181
cMGF	Proliferation of E26-transformed myeloblasts	120		
cCAF	Chorioallantoic membrane assay	112		

egy.[108,109] The product of the fowlpox virus gene *FPV-073* has structural features that make it a good candidate IL-18-binding protein.[110] It still remains to be demonstrated that this viral protein in fact exhibits such activity.

Chemokines

Chemokines are a group of small (~8–14 kDa), structurally related proteins that regulate trafficking of various types of leukocytes through interactions with seven-transmembrane, G protein-coupled receptors (for a recent review, see ref. 111). About 40 chemokines have been identified to date in humans. They are divided into subfamilies on the basis of the arrangement of the conserved N-terminal cysteine residues, CXC, CC, C, and CX3C. Several genes of the chicken and of avian viruses encoding CXC, CC, and C chemokines have been identified recently (Fig. 4).

CXC chemokines. The best known chicken CXC chemokine is a protein designated cCAF (Table 1 and Fig. 4). It is encoded by the *9E3/CEF4* gene and exhibits both chemotactic and angiogenic factor activity (for a recent review, see ref. 112). cCAF is secreted as a ~9-kDa molecule by a variety of cell types in response to various forms of stress. It is then cleaved near the N-terminus by plasmin to produce a smaller form (~7 kDa) that binds to the extracellular matrix. On the chorioallantoic membrane, cCAF is chemotactic for monocyte/macrophages and lymphocytes at low concentrations. At higher concentrations, cCAF no longer shows chemotactic activity but rather stimulates sprouting and growth of blood vessels.

Very little is known about the other four chicken CXC chemokines of which cDNA recently have been cloned and sequenced (Table 1 and Fig. 4). They all show a similar low degree of identity (<50% at amino acid level) to mammalian CXC chemokines. Most information on these chicken proteins resulted from random sequencing of EST clones derived from stimulated chicken immune cells. The exception is the chemokine K60, which was identified by differential gene expression analysis of HD-11 cells.[113] K60 mRNA is absent in nonstimulated HD-11 cells but accumulates rapidly in response to LPS, IL-1β, and IFN-γ. In fibroblasts, it is induced by IL-1β but not IFN-γ. It is of interest to note that cCAF and K60 both feature a conserved ELR motif located immediately upstream of the first cysteine residue, whereas the other known chicken CXC chemokines do not. The ERL motif is found in all mammalian CXC chemokines that target neutrophils.[114] Cell migration studies with purified recombinant proteins are required to understand the biologic activities of these molecules in more detail. We would like to stress that information on primary cDNA structure alone is an insufficient basis to decide which of the various chicken molecules known to date represents the bona fide homolog of mammalian CXC chemokines.

Two recent reports indicate that the primary structures of CXC chemokine receptors are conserved to a higher degree between birds and mammals than their ligands. A chicken gene

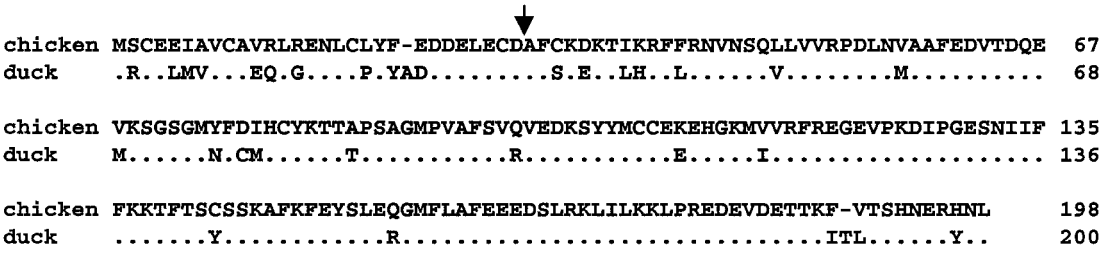

FIG. 3. High similarity of ChIL-18 and duck IL-18 precursors. Alignment of the amino acid sequences was done using the Jotun-Hein method. The putative caspase-1 cleavage site is marked by an arrow. Identical amino acids are represented by periods; alignment gaps are indicated by dashes. Overall identity is 81.8%. The putative mature forms of the two proteins are about 88% identical.

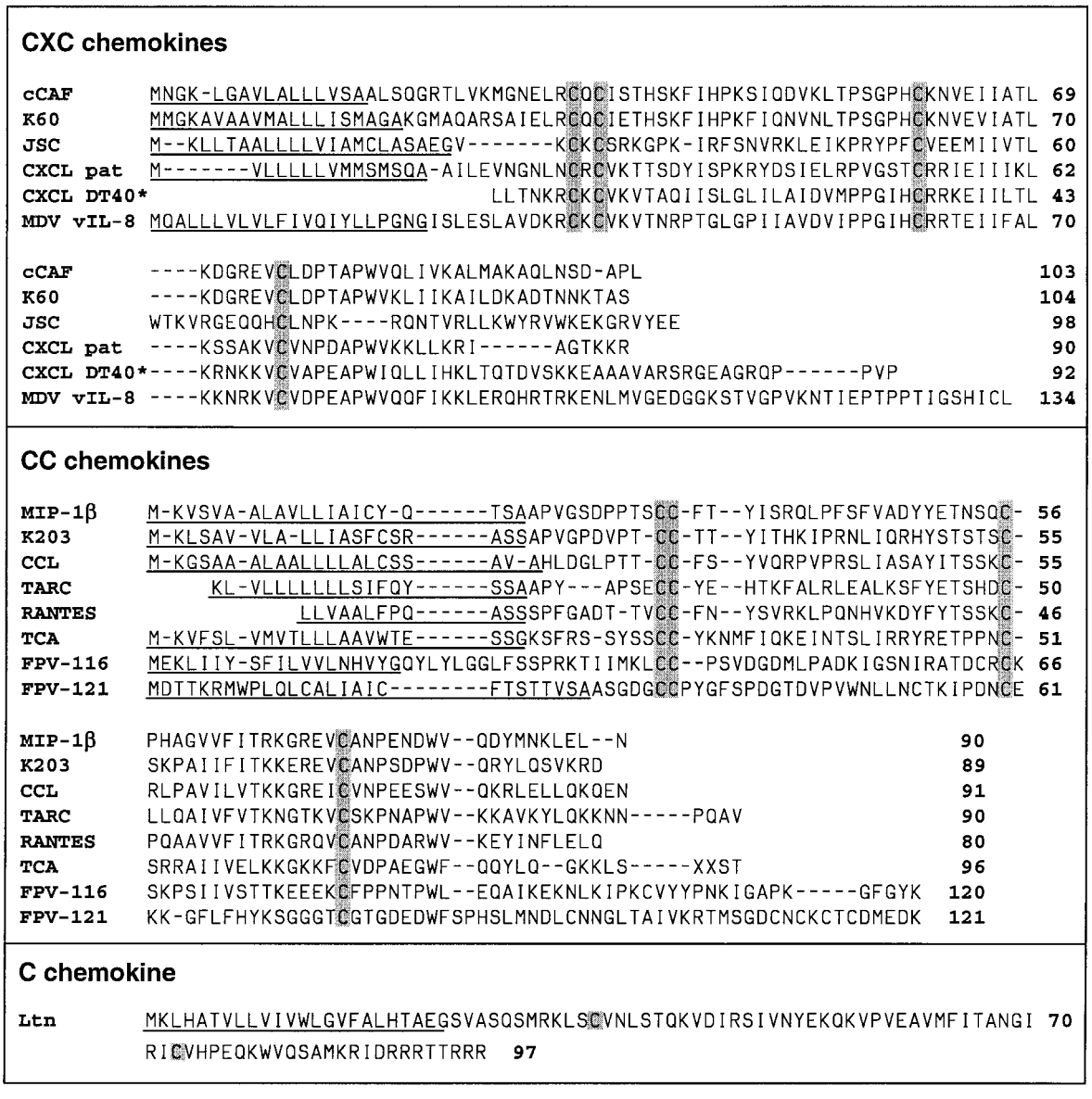

FIG. 4. Chemokines of chicken, Marek's disease virus, and fowlpox virus. Amino acid alignments were done using the Clustal method. The conserved cysteine residues are shaded, and the putative signal peptides are underlined. Alignment gaps are indicated by dashes. Sequences are identified by their common names shown in Table 1. The asterisk indicates that the listed protein sequence differs from that in the database. We corrected a sequencing error in the C-terminal moiety of the cDNA. Information on the structure of the extreme N-terminal regions of CXCL DT40, TARC, and RANTES is not available.

encoding a seven-transmembrane domain protein showing all structural characteristics of a chemokine receptor (accession number AF294794) has 67% and 65% sequence identity to human CXCR1 and CXCR2, respectively.[115] Furthermore, a chicken cDNA encoding a 359-residue polypeptide with 82% identity to human CXCR4 (accession number AF227961) was able to confer responsiveness to the human CXC chemokine SDF-1 in transfected CHO cells, demonstrating that it is functional.[116]

Marek's disease virus (MDV) codes for a CXC chemokine, designated vIL-8 (Table 1 and Fig. 4). The *vIL-8* gene is expressed late in the viral multiplication cycle. It consists of three exons that map to the repeats flanking the unique long region of the viral genome.[117] Baculovirus-expressed vIL-8 attracts peripheral blood mononuclear cells (PBMC) but not heterophils. As mammalian IL-8 has a different target cell specificity, the name of this viral chemokine is misleading. A recombinant MDV mutant strain with a deletion of the *vIL-8* gene showed a decreased level of lytic infection, but it retained its oncogenicity, albeit at a greatly reduced level. It was hypothesized that vIL-8 might be involved in leukocyte target recruitment or else might play a role in viral immunoevasion.[117] It is of interest to note that vIL-8 shows an unexpectedly high degree of similarity to a chicken CXC chemokine (Fig. 4) (pro-

visionally designated CXCL DT40), suggesting that MDV hijacked this gene from its host.

CC chemokines. Five different chicken CC chemokine cDNA have been cloned and sequenced to date (Table 1 and Fig. 4). They show <50% identity at the amino acid level to mammalian CC chemokines. Interestingly, the identity of these chemokines with each other also is not much greater, indicating that they may serve distinct functions. Most information on chicken CC chemokines resulted from random sequencing of EST clones derived from stimulated chicken immune cells. K203 was identified by differential gene expression analysis of HD-11 cells.[113] Expression of the *K203* gene is upregulated strongly in various cell lines in response to LPS, IL-1β, and IFN-γ,[113] as well as in lymphoid organs of chickens treated with the imidazoquinoline S-28463 (unpublished observations). As mentioned, sequence information alone is not sufficient for accurate predictions about the biologic activities of these molecules. Thus, although Figure 4 lists molecules designated MIP-1β and RANTES, their true identity needs to be verified by appropriate cell migration assays.

The products of four genes in the fowlpox virus genome have structural features reminiscent of CC chemokines.[110] Our sequence alignments (Fig. 4) indicated that FPV-116 and FPV-121 (Table 1 and Fig. 1) might represent virus-encoded CC chemokines. No information is available to date on whether these molecules are able to attract immune cells or interfere with the activity of cellular CC chemokines via receptor competition.

C chemokines. A cDNA for a chicken C chemokine (Table 1 and Fig. 4) related to mammalian lymphotactin has been described. This molecule shows the typical cysteine pattern, but the long C-terminal tail found in mammalian lymphotactin is replaced by a short extension that is rich in arginine residues.[118] Amino acid sequence identity to human and mouse lymphotactins is only 28% and 33%, respectively. Interestingly, recombinant chicken lymphotactin produced in transfected COS cells preferentially induced migration of spleen B cells (rather than T cells) in classic transwell plate assays. The chicken lymphotactin gene is constitutively expressed in spleens of chickens.[118]

Other cytokines

Myelomonocytic growth factor. cMGF was the first chicken cytokine to be fully characterized on the molecular level. Initially, cMGF was purified to homogeneity from LPS-activated HD-11 cell culture supernatants. Based on protein sequence data, cMGF was cloned from a myeloblast cDNA library. It is a secretory protein consisting of 201 amino acids with a calculated molecular mass of ~20,000.[89] Posttranslational glycosylation and sulfation result in a variety of cMGF isoforms.[119] However, glycosylation is not required for its biologic activity, as protein expressed in bacteria induced myeloblast proliferation at low concentrations.[89] The cMGF cDNA (Table 1) shows a weak structural relationship to mammalian IL-6 and G-CSF over its entire length. In particular, four conserved cysteines are present that are also found in ChIL-6. cMGF induces the growth of macrophage colonies and, to a lesser extent, of granulocyte colonies from bone marrow.[120–122] It further enhances osteoclast differentiation[123] and stimulates the growth of v-*myb* or v-*myc* transformed chicken myeloid cells.[124,125] Binding of cMGF to myeloid progenitor cells causes rapid Stat5 phosphorylation, followed by cell proliferation and differentiation.[126] Infection of birds with a recombinant fowlpox virus expressing cMGF significantly increased the number of circulating monocytes.[127]

cMGF is secreted by lectin-activated lymphocytes and LPS-stimulated myeloid cells. In addition, infection of myeloid cells with retroviruses carrying kinase-type oncogenes causes cMGF production. Gene expression is stimulated by NF-M, a myeloid-specific transcription factor that binds to the cMGF promoter.[128,129] More recently, cMGF gene expression was shown in response to infectious bursal disease viral infection[90] and MDV infection.[91]

Transforming growth factor-β (TGF-β). Members of the TGF-β family are important regulators of cell growth and differentiation (for a review, see ref. 130). In chickens, four distinct isoforms, TGF-β1 through 4, have been described that share several structural features and show up to 82% identity at the amino acid level.[131] Most studies in the chicken have focused on the role of TGF-β in embryonic development[131–135] and hematopoiesis.[136] TGF-β is known to play an important role in immunologic tolerance in mammals. Little is known about the putative immunoregulatory properties of ChTGF-β. Interestingly, rHuTGF-β1 strongly inhibits proliferation of chicken T and B cells *in vitro* and was shown to support the development of suppressor T cells.[137] Furthermore, HuTGF-β1 was found to enhance the expression of an integrinlike antigen on intestinal T cells[138] and to regulate T cell differentiation in the chicken thymus.[134] Increased TGF-β4 mRNA expression was demonstrated after coccidial infection in two recent studies.[131,139]

TNF family members. TNF-α is a potent inflammatory cytokine with pleiotropic functions.[140] Various biologic activities, including NO release, induction of MHC class II antigens by macrophages, and effects on granulosa cell proliferation, have been reported for unpurified or partially purified supernatants of stimulated primary macrophages or macrophage cell lines believed to contain the chicken homolog of TNF-α.[141–144] Although some database entries claim that certain sequences represent fragments of the ChTNF-α gene, to our knowledge, nucleic acids encoding this chicken cytokine have not been identified to date. The molecular identification of ChTNF-α and related genes will be essential to further characterize its functions, especially as, in mammals, these cytokines play key roles in the development of lymphoid organs.[145]

Two EST clones have been described (Table 1) that seem to contain sequence information for the chicken homolog of CD40 ligand and the TNF family member BAFF/BlyS, respectively.

POTENTIAL USE OF AVIAN CYTOKINES

Important intrinsic features of commercial rearing of birds include a very short life span and a low value of individual animals, which greatly limit any therapeutic strategies aimed at treating diseased flocks. The goal, therefore, is to have prophylactic approaches in place that help to enhance pathogen resistance of the animals. In the near future, the latter strategy might greatly profit from the availability of recombinant cyto-

kines. First attempts to demonstrate the feasibility of these novel concepts are discussed.

Cytokines as vaccine adjuvants

Because cytokines are proteins that control the type and the extent of immune responses after infection or vaccination, they have the potential to serve as vaccine adjuvants. A recent study in the mouse system showed that IFN-α/β is a powerful adjuvant when coinjected with a soluble antigen.[146] Using sheep red blood cells (RBC) as model antigens, Lowenthal et al.[147] showed that the immune response in chickens was enhanced when the vaccine contained rChIFN-γ. Higher levels of specific antibodies were achieved in the presence of cytokine, and lower antigen doses were effective. Similarly, significant immunoadjuvant activity of rIFN-γ, rIFN-α/β, and rIL-1β was observed in 3-week-old chickens immunized with tetanus toxoid as a bacterial model antigen.[148] Unlike oil-based adjuvants, the specific enhancement of B cell responses mediated by cytokines was associated with no or only minimal adverse reactions. Interestingly, no significant adjuvant effect was recorded when cytokines were applied in combination with inactivated infectious bursal disease viral antigen or when 1-day-old chickens were used.[148] An adjuvant effect on the antibody response was also seen when soluble protein antigens were injected i.m. together with IFN-γ and a synthetic lipopeptide but not with cytokine alone.[149] Antibody and T cell proliferative responses to immunization with MDV glycoprotein B were strongly enhanced when latex microspheres coated with rIL-2 were coinjected with antigen into 3-week-old chickens.[150]

Few studies have assessed the immunomodulatory activity of plasmid-expressed cytokines. Coinjection of DNA constructs encoding chicken cytokines boosted the immune response to tetanus toxoid.[148] However, this enhancing effect was mostly if not exclusively due to the intrinsic adjuvant effect of plasmid DNA. Interestingly, the tetanus toxoid-specific primary antibody response was selectively suppressed by plasmid-encoded IFN-γ, suggesting that this cytokine may actually inhibit antibody production under certain conditions. A similar inhibitory effect of plasmid-expressed IFN-γ was observed in turkeys vaccinated with DNA constructs expressing a *Chlamydophila psittaci* antigen.[151] In contrast, coadministration of an *Eimeria acervulina* antigen with plasmids encoding either IFN-γ or IL-2 was reported to induce a more robust immunity than administration of protozoal antigen alone.[152] It should be noted, however, that in this study, no control immunizations were performed with equal amounts of empty plasmid vector. Plasmid-encoded IL-2-enhanced antibody responses to MDV glycoprotein B in 3-week-old chickens had no effect on T cell proliferation.[150]

The vaccine efficacy of a recombinant fowlpox virus coexpressing a Newcastle disease virus (NDV) antigen and IFN-γ was evaluated in turkeys.[153] The antibody response after *in ovo* vaccination developed earlier, and a larger proportion of birds was protected from challenge with NDV when the IFN-γ gene was present in the vector. Interestingly, chickens immunized with a recombinant fowlpox virus coexpressing ChIFN-α showed no enhanced immunity.[154] The decreased vaccine potency of this cytokine-producing fowlpox virus vector was probably due to its attenuated phenotype in the chicken. Infection of chickens with a recombinant fowlpox virus expressing cMGF was shown to cause a marked and sustained increase in the number of circulating blood monocytes as well as an increase in their state of activation.[127] Whether such animals would show enhanced vaccine responses is unknown.

Use of cytokines to enhance innate immunity

The IFN-α/β system of mammals contributes to innate immunity toward a wide range of viral pathogens, suggesting that it may serve a similar function in birds. Marcus et al.[155] found that oral administration of IFN-α ameliorated Newcastle disease in treated chickens. Some but not all chickens that received high amounts of IFN-α via the drinking water resisted virus-induced weight loss and showed strongly reduced pathology of the tracheal tissue. Treatment of chickens with high doses of rIFN-α in the drinking water also decreased the number of virus-infected splenocytes after infection of the birds with MDV.[156] Interestingly, the NK cell activity of splenocytes from IFN-treated chickens was decreased rather than increased, indicating that oral application of type I IFN may induce complex changes in the immune response in chickens.

A series of intriguing experiments suggest that IFN-γ has potent growth-promoting properties in chickens that may result from cytokine-induced enhancement of the innate immunity. Injection of IFN-γ into chicks or infection of chicks with recombinant avian adenovirus expressing IFN-γ resulted in enhanced weight gain of the birds.[157,158] Furthermore, IFN-γ-treated animals showed better endurance to challenge with a parasite.[159,160] It is of great interest to determine the mechanism of this unexpected beneficial effect of IFN-γ, which may have an important impact on the broiler industry.

Cytokine profiling to assess the immune response and the immune status of birds

Investigation of the cellular immune response in chickens during infectious diseases had been hindered for a long time by the lack of appropriate assay systems. From studies in mammals, it is well known that analysis of cytokine expression during the course of an infection can provide important information on the host immune status and on mechanisms involved in resistance or susceptibility to diseases. The various cloned cDNA for avian cytokines permitted establishment of methods for quantification of the corresponding mRNA as well as for their translation products. Assaying for translation products rather than for gene transcripts is of particular importance for cytokines that are regulated at the posttranscriptional level, for example, IL-1, IL-15, and IL-18.

Several techniques have been applied to study cytokine expression during coccidial parasite infection. Using standard RT-PCR analysis, significantly increased IFN-γ mRNA levels were observed in the gut of infected birds.[161] These results were confirmed independently by quantitative RT-PCR.[162] More detailed studies revealed a complex picture of cytokine,[162,163] chemokine,[162] and TGF synthesis[131] during *Eimeria* infection, which mirrors the induction of a highly inflammatory immune response. IFN-γ gene expression in response to coccidial parasite infection could also be demonstrated at the protein level[164] (for a review, see ref. 165). Lynagh et al.[85] measured IL-6-like bioactivity in the sera of *Eimeria*-infected chickens.

They observed a significant induction of this cytokine during the first 2 weeks after infection.

In MDV infection models, cytokine expression profiles were established by semiquantitative RT-PCR. A strong induction of IFN-γ mRNA was observed in spleens of infected birds as early as 3 days after infection and up to day 15 postinfection, suggesting a major role for IFN-γ in the pathogenesis of Marek's disease.[91] Using microarray technology to investigate differential gene expression in MDV-infected chicken embryo fibroblasts, Morgan et al.[166] observed the induction of several cytokines and cytokine-regulated immune response genes.

Cytokine expression in response to infection of chicken fibroblast cultures with different *Salmonella* serotypes was monitored by quantitative RT-PCR and biologic assays. Again, a complex expression profile emerged, indicating that pathogenic serotypes may limit the inflammatory response and thereby cause clinical disease.[167]

A recent report[168] suggests that transient immune suppression, which might be caused by such infectious agents as infectious bursal disease virus or MDV, can be measured accurately by assessing the animal's ability to respond to inactivated NDV with enhanced IFN-α and IFN-γ gene expression.

Cytokines as tools for basic research in avian immunology and virology

As cDNA for duck IFN-α[18] and IFN-γ[32] have been cloned, the duck model of hepatitis B virus (HBV) infection can now be used to address open questions about the therapeutic use of IFN in humans. Experiments with primary hepatocyte cultures showed that both types of IFN effectively inhibit HBV replication.[18,32,169] Treatment of ducks with IFN-α had some inhibitory effect on HBV replication in the liver.[21] Furthermore, when an IFN-α-expressing HBV vector was used to direct expression of this cytokine in liver cells of intact birds, an inhibitory effect on wild-type HBV replication was observed.[170]

A different experimental setup was used to show that IFN-α can inhibit tumor formation induced by a transforming retrovirus.[171] Intravenous injection of mAb 8A9, which neutralizes IFN-α (Table 2), resulted in enhanced tumor development after Rous sarcoma virus infection of strain CB chickens that are usually highly resistant to tumor formation. Furthermore, repeated intravenous injections of recombinant IFN-α into tumor-susceptible chickens of strain CC conferred a significant degree of resistance to Rous sarcoma virus-induced tumor formation.

Advanced knowledge of the avian cytokine system initiated studies aimed at answering questions about immune evasion strategies of viruses, with economic impact on the poultry industry. As discussed, the genomes of fowlpox virus and MDV seem to code for chemokines as well as cytokine-binding proteins (Fig. 4). The CXC chemokine encoded by MDV has interesting chemoattractant properties and appears to function as a virulence factor.[117] These initial data suggest that it may be possible to alter the virulence of MDV vaccine strains by simple genetic manipulation of the viral CXC chemokine gene or by replacing it with genes encoding wild-type or mutant forms of its cellular counterpart.

Using the available novel tools, the roles of cytokines in the autoimmune disease of a strain of obese chickens was analyzed. These birds spontaneously develop a Hashimoto thyroiditis-like disease. Thyroid glands of obese birds were found to contain elevated levels of IL-2 and IL-15 mRNA in the initiation phase of the disease in comparison to glands of CB control birds.[172] In a second study, increasing levels of IFN-γ mRNA were found while the disease progressed. The functional relevance of IFN-γ in this autoimmune disorder was demonstrated by *in vivo* neutralization studies with specific polyclonal antiserum. Antiserum treatment reduced the severity of the disease to nearly 50%.[173]

DISCUSSION

From the work on avian cytokines discussed in this paper, it becomes increasingly clear that a highly developed cytokine network is present not only in mammals but also in nonmammalian species. This is most clearly illustrated by the presence of major components of the Th1 cytokine system in the chicken. Studies on the phylogeny of cytokines can now be extended approximately 350 million years back to the time when the avian and the mammalian lineage diverged. However, significant gaps in our knowledge on chicken cytokines still exist. In this context, it is of interest to note that no avian homolog of typical Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) has been molecularly identified to date (Table 1). This suggests either that birds lack these cytokines altogether or that cloning of chicken cytokine cDNA was done with mRNA from cells that mainly expressed Th1 cytokine genes. Evidence in favor of the latter possibility is that chickens possess an IL-10R gene (accession number AF082666) that shows 42% identity at the amino acid level to HuIL-10R2.[19] Furthermore, a recent *in vivo* study demonstrated that a preferential Th1-type inflammatory immune response can be induced with malyelated antigens, whereas a more antibody-dominated Th2 type response is observed with nonmalyelated control antigens.[174] It should be noted, however, that biologic activities of typical Th2 cytokines have not been demonstrated to date in chicken cell culture systems. Thus, it may be argued that classic Th2-type cytokines do not exist in birds and that cytokines with moderate Th2 characteristics, such as IL-6, IL-18 and TGF-β, may downregulate proinflammatory responses, indirectly favoring Th2-type immune responses.

We assume that answers to these highly relevant questions, as well as additional information on cytokines, cytokine receptors, and cognate cell signaling molecules, may come soon from the application of advanced molecular techniques. EST databases have already proved to be valuable sources of sequence information on chicken cytokines. Further progress may come from studying syntenic gene loci and from sequence information derived from the ongoing chicken genome projects.

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