Avian Macrophages: Regulators of Local and Systemic Immune Responses

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ABSTRACT Macrophages are key regulatory cells of the immune system involved in initiating and directing the innate and specific immune responses, the systemic acute phase response, tissue repair, and tissue remodeling. In the early stages of a challenge from invading microorganisms or from tissue injury, macrophages defend local and systemic homeostasis by initiating a complex series of cellular, biochemical, and behavioral events. These pathophysiological adjustments are mediated by an extensive variety of communication molecules, including: cytokines, cytokine inhibitors, endocrine hormones, eicosanoids, neurotransmitters, and reactive oxygen intermediates. The cytokines produced by macrophages (monokines) are not well characterized relative to their mammalian counterparts, but a variety of chemokine, pro-inflammatory, and colony-stimulating factor activities have been described. Although the sequence homology, and thus species cross-reactivity, between avian and mammalian cytokines is typically low, the functional characteristics appear to be generally similar. The pro-inflammatory cytokines are important initiators and regulators of the local immune response. They are also released in sufficient quantities during some infections to coordinate a systemic acute phase response that impacts the growth, reproduction, and well-being of poultry. An understanding of the mechanisms and molecules used by macrophages to regulate immune and inflammatory responses may permit the development of products, diets, or husbandry techniques to modulate immunity for the enhancement of the productivity of poultry.

(*Key words*: macrophages, regulation, cytokines, immunity, cell lines)

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INTRODUCTION

Macrophages are active effector cells that can detect, phagocytize, and kill extracellular microorganisms (Dietert and Golemboski, 1997; Qureshi, 1998). They are also the key regulatory cell of the immune system involved in initiating and directing the immune and inflammatory responses. In the early stages of a challenge from invading microorganisms or from tissue injury, macrophages defend local and systemic homeostasis by initiating a complex series of cellular, biochemical, and behavioral adjustments. Macrophages are especially suited for a regulatory role because they are widely dispersed throughout the bird's body fluids and within its tissues, and because of their capacity to secrete an extensive variety of communication molecules. The lineage of cell types is also dynamic, with functional capacities and regulatory characteristics changing during transitions between cell types. Monocytes are naive precursors with poorly developed effector and regulatory capacities. Upon stimulation, monocytes differentiate into macrophages that are more capable of mediating host defense. Macrophages can be characterized as inflammatory or cytocidal, depending on the spectrum of effector and communication molecules secreted (Henson and Riches, 1994). In many ways, the macrophage is also an immature cell and is capable of further differentiation into a variety of cells within tissues that are important in terminating the inflammatory response and mediating repair processes. These modified cells include "giant" cells that form a syncytium around an inflammatory site, and tissue resorbing cells, such as osteoclasts.

Regulatory actions of macrophages are important in determining the type and intensity of specific and innate immune responses. This role makes the macrophage an important determinant in resistance to infectious disease. Further, the communication between macrophages and other physiological systems markedly impacts the growth, reproduction, and well-being of poultry. These

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Abbreviation Key: 1,25-$(OH)_2D_3$ = 1,25-dihydroxycholecalciferol; 25-$(OH)D_3$ = 25-hydroxycholecalciferol; ACTH = adrenocorticotropin; CC = adjacent cysteines; CXC = cysteines separated by another amino acid; IL = interleukin; INF = interferon; LPS = lipopolysaccharide; MGF = myelomonocytic growth factor; MIP = macrophage inflammatory proteins; PGE = prostaglandin E; TGF = transforming growth factor; TNF = tumor necrosis factor.

important pathophysiological roles of macrophages compel a thorough understanding of their regulatory mechanisms and the molecules involved.

MACROPHAGE SOURCES AND CELL LINES

To facilitate study, macrophages can be isolated from virtually any tissue or body fluid. They are commonly separated from lymphocytes and other contaminants by density gradient centrifugation and adhesion to plastic. Peripheral blood monocytes can be isolated directly, or following attraction into the peritoneal cavity by sephadex or other inflammatory agents (Chu and Dietert, 1988). All of these populations are heterogeneous, as indicated by expression of cell surface proteins and functional characteristics. Their variable contamination with thrombocytes, fibroblasts, and other adherent cells can confound interpretation of experimental results. Additionally, the process of isolation partially activates the cells and begins their transformation into larger polynucleated cells of poorly characterized functionality.

Several chicken macrophage-like cell lines have been developed that provide a population of cells with more uniform functional characteristics than primary cultures. All of these cell lines are adherent, phagocytic, and express cell surface proteins characteristic of monocytic cells. They were developed by different modes of transformation that may have a bearing on their use. The HD11 cell line was transformed by a MC29 retrovirus, and expresses *v-myc* oncogene (Beug *et al*., 1979). The MQ-NCSU cell line was virally transformed by Marek's disease virus and is generally more active than the HD11 cells at many effector and regulatory functions (Qureshi *et al*., 1990, 1994). A cell line designated IN24 cells was established from a natural myelocytic leukemia (Inoue and Sato, 1988) and the cell line LSCC-NP1 was derived from the bursa of a chicken infected with lymphoid leukosis (Inoue *et al*. 1992). Although cell lines are convenient and permit greater experimental control and repeatability than primary cultures of macrophages, the shedding of virus or functional anomalies due to transformation may give artifactual results in some experimental paradigms.

REGULATORY MEDIATORS PRODUCED BY MACROPHAGES

Macrophages have the capacity to synthesize and secrete the largest number of communication molecules of any cell type, and rival hepatocytes in metabolic and functional capabilities (Oppenheim and Shevach, 1990). Communication molecules include cytokines, cytokine inhibitors, endocrine hormones, eicosanoids, neurotransmitters, and reactive oxygen intermediates. These molecules can act on the secreting macrophage (autocrine), on surrounding cells (paracrine), or they may enter the circulation and act systemically (endocrine). They regulate specific immunity, inflammation, the systemic acute phase response, tissue repair, and tissue remodeling.

Cytokines

The cytokines produced by macrophages and monocytes (monokines) are not well characterized relative to their mammalian counterparts (for reviews see Klasing, 1994; Kaiser, 1996). A variety of activities similar to those possessed by mammalian cytokines have been described for cytokines from chicken, turkey, and duck macrophages. A few of these cytokines have been characterized at the amino acid or genomic level. Although the sequence homology, and thus species cross-reactivity, between avian and mammalian cytokines is typically low, the functional roles appear to be generally similar.

Avian macrophages produce chemotactic cytokines (chemokines) of both macrophage inflammatory protein (MIP) families. The chicken MIP-1 and MIP-2 chemokines have the same amino acid motifs as mammalian chemokines: adjacent cysteines (CC) in the MIP-1 chemokine, and cysteines separated by another amino acid (CXC) in the MIP-2 family. The chicken MIP-2 family chemokine is currently designated as 9E3/CEF4. It has high homology to mammalian interleukin (IL)-8 and is abundantly expressed by activated peripheral blood monocytes (Bedard *et al*., 1987; Sugano *et al*., 1987; Barker *et al*., 1993). This chemokine is highly chemotactic for mononuclear cells and for heterophils. The MIP-1 family chemokine is highly homologous with mammalian MIP-1β, but its biological activities await characterization (Petrenko *et al*., 1995). The MIP-1 and MIP-2 chemokines, along with the transforming growth factor-β (TGF-β), have considerably higher homology with their mammalian counterparts than most other leukocytic cytokines described to date. Chickens also express a chemokine that is weakly homologous to mammalian lymphoactin. This protein has a novel CX_3C amino acid motif (Rossi *et al*., 1996). Presumably the three chemokines cloned from chickens are important in the recruitment of inflammatory cells during the initiation of the avian immune response.

Chicken macrophages produce cytokines with activities similar to the three primary pro-inflammatory monokines of mammals, IL-1, IL-6, and TNF-α (Bombara and Taylor, 1991; Qureshi and Miller, 1991; Qureshi *et al*., 1994, 1993; Klasing, 1995). The receptor for IL-1 has been cloned (Guida *et al*., 1992) and is expressed in monocytes (Peng and Klasing, 1995), indicating an autocrine action of this cytokine. The extracellular ligand binding domain of this receptor neutralizes the thymocyte co-stimulation activity of IL-1 (Figure 1). An avian IL-6 analog called chicken myelomonocytic growth factor (MGF) has been cloned (Leutz *et al*., 1989). This cytokine acts as a monocyte-specific colony-stimulating factor, but does not display many of the acute-phase inducing activities of mammalian IL-6. Chickens virally transfected with an MGF expression construct have markedly increased blood

monocyte numbers (York *et al.*, 1996). In turkeys, MGF is expressed by bone marrow macrophages following stimulation by lipopolysaccharide (LPS), and Type 1 interferon (INF) augments this expression (Suresh *et al.*, 1995). Chicken macrophages release another monokine that is also related to IL-6 and is a more active inducer of the acute phase response than MGF. This IL-6-like cytokine is similar enough to human IL-6 to be detected by commercially available antibodies (Samad *et al.*, 1993; Rath *et al.*, 1995) and by bioassays with murine cell lines (Higgins *et al.*, 1993). The pro-inflammatory cytokines are important initiators and regulators of the local immune response and they are also released in sufficient quantities during some infections to coordinate a variety of systemic responses characteristic of an acute phase response (see below).

Although MGF is the best characterized avian colony-stimulating factor, the chicken macrophage cell line MQ-NCSU produces a colony-stimulating factor with activity distinct from MGF. This cytokine induces embryonic bone marrow progenitor cells to differentiate into granulocytes, rather than macrophages (Qureshi *et al.*, 1994).

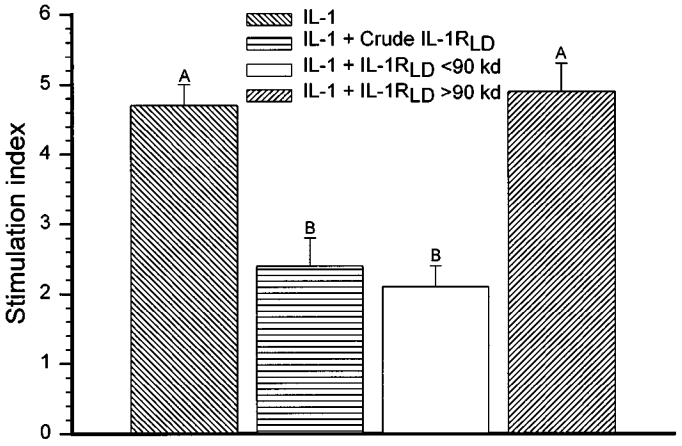

FIGURE 1. Chicken thymocytes were used to assess interleukin-1 activity by the co-mitogenesis assay described previously (Klasing, 1987). Treatments were: 1) IL-1: chicken interleukin-1 purified according to Klasing (1995); 2) IL-1 + CRUDE IL-1R$_{LD}$: chicken interleukin-1 plus the ligand binding domain of the type 1 interleukin-1 receptor. The CRUDE IL-1R$_{LD}$ represents the unpurified translation product of nucleotides 57 to 1014 (Guida *et al.*, 1992) expressed in *Pichia pastoris* as previously reported by Peng and Klasing (1995). 3) IL-1 + IL-1R$_{LD}$: chicken IL-1 plus IL-1R$_{LD}$ that was partially purified by size exclusion ultrafiltration. 4) IL-1 + CONTROL: chicken IL-1 + *P. pastoris* supernatant without IL-1R$_{LD}$.

Cytokine Inhibitors

Chicken macrophages stimulated with LPS or other immunogens release large amounts of cytokine inhibitors, including at least three that inhibit the co-mitogenic activity of chicken IL-1 (Klasing, 1995). Duck blood plasma contains a very potent inhibitor of human rIL-6 and rIL-1β (Higgins *et al.*, 1993). Presumably, inhibitors of pro-inflammatory cytokines are important regulatory products released by chicken macrophages as they differentiate following activation. These inhibitors may be analogues of the mammalian IL-1 receptor antagonist and soluble receptors that coordinate the resolution of the inflammatory response.

Eicosanoids and Nitric Oxide

Nitric oxide (NO) and eicosanoids are released by activated macrophages and have a number of effector and regulatory functions (see review by Golemboski and Dietert, 1997). Chicken INF-γ, but not Type I INF, induces the synthesis of NO (Lowenthal *et al.*, 1995; Schultz *et al.*, 1995). However, turkey Type 1 INF acts synergistically with LPS to induce NO release from bone marrow macrophages (Suresh *et al.*, 1995).

Hormones

Avian macrophages have the capacity to convert 25-hydroxycholecalciferol [25-(OH)D_3] to the active hormone, 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$], by the action of 25-(OH)D_3-1-hydroxylase. This intramitochondrial cytochrome P450 mixed function oxidase is not subject to regulation by the same regulatory factors (e.g., parathyroid hormone and Ca^{2+}) that modulate the renal 25-(OH)D_3-1-hydroxylase (Adams *et al.*, 1994). Instead, the macrophage 25-(OH)D_3-1-hydroxylase is activated by INF-γ, LPS, and IGF-I. The resulting active hormone down-regulates the proliferation of macrophages and induces their fusion into multinucleated giant cells (Woods *et al.*, 1995). Chicken leukocytes also produce ACTH (Hendricks *et al.*, 1995), which may be important in local regulation of the immune response as well as systemic endocrine-like actions. The release of 1,25-$(OH)_2D_3$ and ACTH would be expected to aid in the resolution of the inflammatory response and promote reparative activities.

SYSTEMIC EFFECTS OF MACROPHAGE ACTIVATION

Research on the *in vivo* systemic actions of cytokines produced by macrophages has been seriously hampered by the lack of sufficient amounts of purified cytokines. Several approaches have been employed to circumvent this problem. First, partially purified preparations have been utilized, however, the probable content of contaminating cytokines confounds interpretation. Second, commercially available mammalian cytokines can be utilized when there is sufficient cross-species bioactivity (e.g., IL-6, IL-8, TNF, TGF-β). In several cases, mammalian cytokines have little or no cross-reactivity in the avian system (e.g., INF-α, IL-2). Third, stimulants of macrophage cytokine release can be utilized instead of the actual cytokines. Most commonly, LPS is employed, although other inflammatory agents such as bacteria, sephadex, and Freund's adjuvant have utility. The advantage of this approach is that large amount of

inflammatory cytokines are released in a sustained fashion. Further, the synergistic actions of the multiple inflammatory cytokines released gives responses that are typically more robust than those seen following administration of individual cytokines. The obvious disadvantage is that the scientist has little control or knowledge of the amounts and types of cytokines released. The research described below illustrates the endocrine-like actions of the pro-inflammatory cytokines, but the results should be interpreted in light of the above limitations.

Acute Phase Responses

A variety of infections cause somnolence, lethargy, anorexia, and subdued social interactions in chickens. Most of these behavioral responses can be duplicated by the injection of purified chicken IL-1 or LPS (Klasing *et al*., 1987; Johnson *et al*., 1993). Fever is typical of most infectious diseases and is mediated by macrophage-produced cytokines. Injection of purified chicken IL-1, i.p. (Klasing *et al*., 1987), or human rIL-1β, i.c.v. (Macari *et al*., 1993) induces fever in chickens. The action of IL-1β is prostaglandin (PG) dependent and can be duplicated by the injection of PGE_2. Febrile temperatures augment the thymocyte mitogenic activity of chicken IL-1 (Klasing and Peng, 1987).

Chicken IL-1-like and IL-6-like cytokines from macrophages induce the hepatic secretion of a variety of proteins during the acute phase of an immune response (Amrani, 1990; Klasing, 1991). These acute phase proteins aid in nonspecific immunity (e.g., mannan-binding protein, transferrin, avidin, very low density lipoprotein, and C-reactive protein), cooperate in specific immunity (e.g., complement and fibronectin), and provide substrate for the coagulation of body fluids and walling off of pathogens (e.g., fibrinogen and fibronectin). Several acute phase proteins limit the damage that results from an immune response or from pathogen invasiveness. Thus, macrophages release noxious substances such as enzymes and reactive oxygen intermediates to kill invading pathogens and also stimulate the secretion of proteins that protect host cells from these effector molecules. For example, α_2-macroglobulin inhibits proteases so that clotting and complement cascades are not triggered at sights distal to that of the infection or wound. Heme and divalent cations released from damaged tissues can be pro-oxidants and are bound by the acute phase proteins hemopexin, haptoglobin, transferrin, and metallothionein. Several of the acute phase proteins are immunoregulatory and act by binding cytokines or by interacting with leukocyte receptors (e.g., α-1-acid glycoprotein and α_2-macroglobulin; Figure 2). Chicken IL-1 induces the release of corticosterone from the adrenals, which augments acute phase protein production (Amrani, 1990). Corticosterone release apparently requires the participation of the hypothalamic-pituitary axis and is deficient in birds susceptible to autoimmunity (Brezinschek *et al*., 1990, 1993).

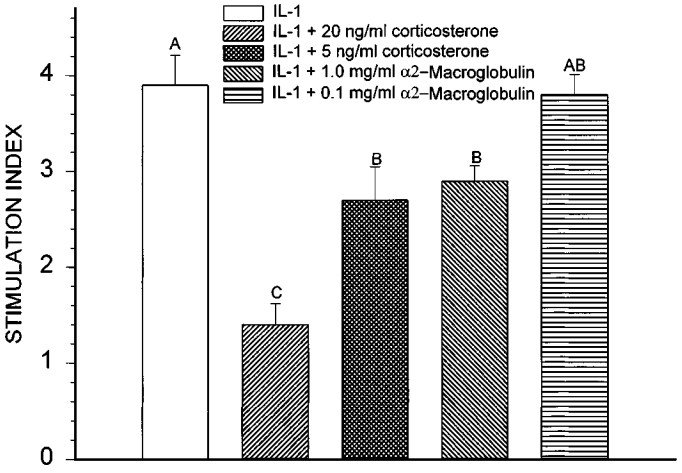

FIGURE 2. Chicken thymocytes were used to assess interleukin-1 activity by the co-mitogenesis assay described previously (Klasing, 1987). Treatments were: 1) IL-1: chicken interleukin-1 purified according to Klasing (1995); 2) Chicken IL-1 plus 20 ng/mL corticosterone; 3) Chicken IL-1 plus 5 ng/mL corticosterone; 4) Chicken IL-1 plus 1.0 mg/mL human α_2-macroglobulin; 5) Chicken IL-1 plus 0.1 mg/mL human α_2-macroglobulin.

Growth and Reproduction

When the macrophages that are attracted to a simulated peritoneal infection using sephadex or LPS are collected and the cytokines released during several hours of *in vitro* culture are re-injected into chicks, growth is stunted (Klasing *et al*., 1987; Klasing and Johnstone, 1991). The cytokine cocktail also induces most of the same behavioral, metabolic, and cellular alterations that are observed during a continuous *in vivo* challenge with LPS. Interleukin-1 purified from macrophages increases the rate of protein degradation in skeletal muscle and slows the growth of this tissue, permitting the diversion of nutrients to the liver for the synthesis of acute phase proteins and to cells of the immune system for their proliferation (for review, see Klasing and Korver, 1997).

The inhibition of follicular development and ovulation that accompanies infection is at least partly mediated my monokines. Soboloff *et al*. (1995) demonstrated that TNF-α acts directly on follicular cells from Single Comb White Leghorn hens by a calcium-dependent pathway to suppress differentiation. The injection of hens with cytokines produced by LPS-stimulated macrophages blocks egg laying for several subsequent days (Klasing, unpublished observations).

MODULATION OF CYTOKINE RELEASE

Cytokine release by virally infected macrophages is often impaired. For example, infection of splenic or bone marrow macrophages with chicken anemia virus depresses their release of IL-1 (McConnell *et al*., 1993). Avian erythroblastosis virus infection of HD11 macrophages diminishes IL-1 release to undetectable levels (Romach *et al*., 1993). Presumably this downregulation

contributes to the immunosuppression that accompanies many viral infections. Conversely, bacteria and coccidia stimulate cytokine release from macrophages, at least initially. For example, LPS and *Staphylococcus aureus* are potent inducers of IL-1, TNF, MGF, and 9E3/CEF4 release from monocytes and macrophages (Klasing and Peng, 1987; Bombara and Taylor, 1991; Qureshi and Miller, 1991; Barker *et al*., 1993; Romach *et al*., 1993; Samad *et al*., 1993; Suresh *et al*., 1995). Macrophages from chickens infected by coccidia have markedly greater production of IL-1 and TNF-α activities than those isolated from uninfected controls (Brynes *et al*., 1993). These observations may explain the adjuvant-like action of LPS and other microbial products.

Cytokines originating from macrophages, or from other cell types, also regulate macrophage activity and cytokine release. Splenic lymphokines markedly enhance LPS-stimulated release of a TNF-like factor (Qureshi and Miller, 1991). Interferon may be one of the responsible lymphokines as macrophages are activated by INF-γ, as indicated by increased expression of major histocompatibility class II antigens (Kaspers *et al*., 1994), and NO release (Digby and Lowenthal, 1995; Lowenthal *et al*., 1995). Turkey type-1 INF is synergistic with LPS in activating macrophages as indicated by NO release (Suresh *et al*., 1995). However, neither turkey nor chicken INF can activate macrophages unless a trigger such as LPS is present (Schultz *et al*., 1995; Suresh *et al*., 1995). The state of activation of macrophages is also enhanced by cMGF, as indicated by augmented phagocytosis and NO production in response to INF-γ or LPS (York *et al*., 1996).

In mammals, macrophages are strongly influenced by hormones (Weigent and Blalock, 1995). However, only a few observations have been made in birds (Marsh., 1992). In the normal course of an inflammatory response, corticosterone is released from the adrenals. Corticosterone release is induced by IL-1 (Klasing *et al*., 1987) and ACTH from stimulated leukocytes (Hendricks *et al*., 1995) and is an important component of an inhibitory feedback mechanism that modulates the immune system and the inflammatory response. For example, corticosterone inhibits the release of IL-1-like activity from macrophages (Klasing, 1987 and Figure 2).

Epinephrine appears to activate macrophages by enhancing phagocytic capacity, Fc receptor expression, and the release of a TNF-like factor (Ali *et al*., 1994). Likewise, cyclic adenosine monophosphate, which acts as a second messenger for a number of hormones including epinephrine, augments the release of IL-1 by macrophages (Bombara and Taylor, 1991).

Several nutrients effect macrophage function through modulating cytokine release. Vitamin E increases the release of PGE_2 and decreases IL-1 release by avian erythroblastosis virus-infected HD11 macrophages in a dose-dependent manner (Romach *et al*., 1993). Similarly, the ratio of n-3 to n-6 fatty acids modulate IL-1 and PGE release by chicken macrophages (Korver and Klasing, 1995). Modulation of IL-1 release by IL-1 or vitamin E may be secondary to changes in the amount and types of PG synthesized.

PRACTICAL IMPLICATIONS

An understanding of the mechanisms and molecules used by macrophages to regulate immune and inflammatory responses may permit the development of products, diets, or husbandry techniques to modulate immunity for the enhancement of the productivity of poultry (Klasing, 1996). Specific rationales for modulating macrophage function in poultry include: 1) providing enhanced or sustained immune response to infectious organisms; 2) enhancement and direction of vaccination responses; 3) mitigation of immunosuppression arising from infectious diseases, dietary toxins, or stress; 4) accelerating the development and maturation of the immune system; 5) inducing tolerance to nonpathogenic environmental immunogens; and 6) mitigating the catabolic consequences of an immune response.

The goal of enhancing the immune system to minimize infectious disease is laudable for both economic and animal welfare reasons. The important regulatory roles of the macrophage make it an attractive target for immunomodulation. However, regulation of the immune response is very complex and requires the integrated interplay of dozens of cell types and communication molecules. Simple changes to a biological system with extraordinary redundancy and pleiotropy are difficult to affect. For this reason, initial applications of immunomodulation may best be directed toward the correction of a dysfunctional situation created by immaturity, stress, immunosuppressive disease, or genetics. Enhancement of macrophage activity beyond normal physiological levels may result in decreased productivity due to the catabolic nature of the monokines that are elaborated. From a practical point of view, these relationships must be appreciated before immunomodulation becomes a routine management tool.

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