IMPACT OF DIETARY ARGININE ON IMMUNITY IN BROILER CHICKS

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Jennifer Lynn D'Amato

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COMMITTEE MEMBERSHIP

TITLE:	Impact of dietary arginine on immunity in broiler chicks
AUTHOR:	Jennifer Lynn D'Amato
DATE SUBMITTED:	October 2009
COMMITTEE CHAIR:	Dr. Brooke D. Humphrey, Associate Professor
COMMITTEE MEMBER:	Dr. Daniel G. Peterson, Associate Professor
COMMITTEE MEMBER:	Dr. Kirk C. Klasing, Professor

ABSTRACT

Impact of Dietary Arginine on Immunity in Broiler Chicks

Jennifer Lynn D'Amato

Arginine (ARG) is an immunologic modulator due in part to its role as a substrate for leukocytes. Therefore, the objective of these studies was to evaluate the impact of dietary ARG on ARG utilization in peripheral blood mononuclear cells (PBMC) and thymocytes in broiler chicks as they provide a model that is not confounded by *de novo* ARG synthesis. Several experiments were performed to measure: 1) ARG transporters as markers of ARG utilization by leukocytes; 2) thymocyte proliferation; 3) PBMC phenotype, and 4) ARG's impact on the acute phase response (APR). In experiment 1, chicks were fed diets containing 1.20% or 1.35% dietary ARG. On d7, PBMC CAT-1 mRNA abundance was 2-fold higher in chicks fed 1.35% ARG than in those fed 1.20% ARG ($P<0.05$). Between d3 and d14, 1.20% ARG increased thymocyte y^+LAT-2 mRNA abundance 2.5-fold ($P<0.05$) while 1.35% ARG did not. In experiments 2 and 3, chicks were fed diets containing 1.1%, 1.3%, and 1.5% dietary ARG. On d10, 1.5% ARG reduced thymocyte proliferation compared to 1.1% and 1.3% ARG ($P<0.05$). The percentage of peripheral blood B cells tended ($P=0.06$) to have an age by ARG interaction. On d14, but not on d10 or 17, the percentage of monocytes from chicks fed 1.5% was higher than from those fed 1.1% and 1.3% ARG ($P<0.05$). In experiment 3, chicks from four replicate pens per treatment were not injected (control) or injected subcutaneously with *Salmonella typhimurium* lipopolysaccharide (LPS; 1 mg/kg BW) to initiate the APR. At 2 h post LPS injection, there tended ($P=0.08$) to be a LPS by ARG interaction for TGF-β4 mRNA abundance. Therefore, these studies indicate that the

optimal level of dietary ARG supplementation for utilization by ARG transporters in leukocytes is higher than what is sufficient for growth and is both age and leukocyte dependent. Additionally, these studies indicate that dietary ARG levels above the NRC requirement may be beneficial for the abundance of PBMC sub-populations. These studies also indicate that the APR may be manipulated by dietary ARG.

DEDICATION

To my parents, Ralph and Linda D'Amato, who have always believed in me, and encouraged me. Without their support, I could never have faced the many challenges life has provided especially in my academic work. One could not ask for better parents. Thank you so very much for your love and encouragement.

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LIST OF ABBREVIATIONS

AL	argininosuccinate lyase
APR	acute phase response
ARG	arginine
AS	argininosuccinate synthetase
ATB^+	Na^+-dependent amino acid transporter
bp	base pair
bw	body weight
c	chick
CAA	cationic amino acid
CAT	cationic amino acid transporter
CPS I	carbamylphosphate synthetase I
d	day
g	gram
GLY	glycine
h	hour
HPRT	hypoxanthine phosphoribosyltransferase-1
IFN	interferon
IL	interleukin
NOS	nitric oxide synthase
LPS	lipopolysaccharide
LYS	lysine
NAA	neutral amino acid
NO	nitric oxide
NRC	National Research Council
OTC	ornithine transcarbamylase
PBMC	peripheral blood mononuclear cell
PHA-P	phytohemagglutinin-P
RNS	reactive nitrogen species
ROS	reactive oxygen species
TCR	T cell receptor
T_H	T helper
TGF	transforming growth factor
y^+LAT	y^+L type amino acid transporter

CHAPTER 1

Introduction

1.1. Arginine is Essential for the Growth of Chickens

1.1.1. Arginine Metabolism

Arginine (ARG) is a cationic amino acid (CAA) as it has both a positively charged R-group and guanidino group. It is polar, basic, and required for growth. It is both an essential and conditionally essential amino acid due to species differences in ARG metabolism. In uricotelic species, ARG is essential due to an incomplete urea cycle as ARG is not necessary to excrete nitrogenous waste (1). In chicks, dietary ARG must be provided due to a lack of mitochondrial carbamylphosphate synthetase I (CPS I) as well as low renal argininosuccinate synthetase (AS), argininosuccinate lyase (AL), and ornithine transcarbamylase (OTC) activity (1). The ARG requirement for chicks is among the highest documented in model species; however, ARG requirements can be spared by dietary citrulline supplementation (1). In ureotelic species with a functioning urea cycle, ARG is conditionally essential (young, growing animals) or nonessential (healthy adults) (1-2).

ARG metabolism begins with the ingestion and digestion of proteins in the gastrointestinal tract. In the small intestine, proteins are cleaved into amino acids and peptides by enzymes such as trypsin, carboxypeptidases, aminopeptidases, and aminodipeptidases. The component amino acids and peptides, particularly dipeptides, are absorbed from the intestinal lumen and transferred to the liver via the portal system (3). This allows the liver an opportunity to take up nutrients from circulation before other

tissues (3). The release of amino acids and peptides into the portal vein depends on dietary factors including the amount and biological value of the protein consumed, and other macronutrients consumed, and non-dietary factors including gut protein synthesis and physiologic state (4). In enterocytes, amino acids are transported by many transport systems including systems: L, B^0, asc, y^+L, y^+, and $b^{0,+}$ (5-6), and peptides are transported by PepT-1 (7). Of the amino acid systems, ARG is transported by systems: y^+, y^+L, and $b^{0,+}$ (5-10). In chickens, amino acids are mostly absorbed in the duodenum, but are also absorbed in the jejunum, and ileum (11). After absorption amino acids can be transaminated or deaminated, utilized for the synthesis of proteins, enzymes, hormones, and other metabolites, and their carbon skeletons can be oxidized for energy or gluconeogenesis (3). ARG is used for protein synthesis and is a precursor of a variety of compounds including ornithine, polyamines, proline, glutamate, urea, creatine, nitric oxide (NO), citrulline, and agmatine in reactions initiated by arginases, nitric oxide synthases (NOS), ARG:glycine (GLY) amidinotransferase, and ARG decarboxylase (8-9, 12-13). ARG is initially metabolized to ornithine, urea, NO, citrulline, and agmatine while additional enzymatic steps occur in the synthesis of polyamines, proline, glutamate, and creatine (12). For instance, ARG's guanidino group is transferred to GLY to produce guanidinoacetate which is later converted to creatine (12).

ARG can be catabolized by arginase into ornithine and urea (12-13). Ornithine is a precursor for polyamines which are ubiquitous low molecular weight aliphatic cations (14) and include putrescine, spermidine, and spermine (13). In mammals there are two types of arginase, arginase I and arginase II, encoded by different genes (12-13, 15). They differ in tissue and subcellular distribution (12-13, 15). Arginase I is in the cytosol

and is highly expressed in the liver as part of the urea cycle, whereas arginase II is in the mitochondrial matrix and is expressed in the kidney, brain, small intestine, muscle cell, mammary gland, and macrophage (8-9, 12, 16). In chicks, arginase activity is approximately 30 times higher in the kidney than in liver (1). ARG is also a substrate for nitric oxide synthases (NOS) which produce NO and citrulline (8, 12-13). There are three NOS enzymes, inducible NOS (iNOS), endothelial NOS (eNOS), neuronal NOS (nNOS) (8, 12-13). eNOS and nNOS are constitutively expressed and Ca^{2+}-dependent while iNOS is induced in response to bacterial endotoxin and proinflammatory cytokines and is Ca^{2+}-independent (9, 12). NO production is rate-limited by the availability of intracellular ARG (8). Arginase and NOS compete for ARG; therefore, in many cell types relative enzymatic activities (K_m values for arginase and NOS are 2-20 mM and 2-20 μM, respectively; the V_{max} of arginase is much higher than that of NOS) and the induction of NOS determine polyamine and NO production (2, 9, 15).

Endogenous ARG is predominately synthesized from citrulline through the intestinal-renal axis in ureotelic animals (2, 8-9, 15). Enterocytes produce citrulline from glutamine metabolism and release it into the blood where it is transported to proximal tubular cells in the kidney, converted to ARG, and released into the circulation (8-9, 12, 15). As citrulline is also a by-product of NO production, ARG can be synthesized through the citrulline-NO cycle if AS and AL are present in the cell; however, this pathway is inefficient compared to the intestinal-renal axis (8, 15). ARG is also synthesized in the liver within the hepatic urea cycle; however, there is little or no net synthesis due to the high hepatic arginase activity and the tight channeling of metabolites from enzyme to enzyme in the urea cycle (8-9).

ARG metabolism is regulated by many factors including diet, hormones, cytokines, bacterial endotoxins, and ARG availability (9, 15). For example, dietary changes in protein intake and subsequent alterations in amino acid nitrogen flux result in changes in the activities of urea cycle enzymes (15). In hepatocytes and enterocytes, glucocorticoids regulate ARG metabolism by upregulating arginase while downregulating NOS (9). Additionally, arginase and NOS are differentially expressed due to induction by different classes of cytokines (9, 13). In addition, ARG availability has been shown to impact gene expression of urea cycle enzymes and ARG transporters though many of the mechanisms by which this occurs have not been elucidated (2).

1.1.2. Arginine Utilization for the Growth of Chickens

Amino acid utilization for growth has been studied at the tissue level. Dean and Scott (17) developed an amino acid reference diet for chicks in a historic study that allows for the use of essential amino acid ratios to formulate and evaluate diets. From this study, the ideal protein concept was established as it aims to provide a precise ratio of essential amino acids to lysine (LYS) that meet the needs for protein accretion and maintenance while minimizing nitrogen excretion (18). Studies that followed this work aimed at determining these ratios to maximize growth. In the case of LYS and ARG, both are limiting cationic amino acids required for growth; however, it was determined that they can have an antagonistic relationship such that a growth depression occurs if LYS is provided in excess of ARG due to an increase in ARG catabolism through renal arginase (1, 19). In order to correct this antagonism, the ARG requirement is increased (19). In chicks, this antagonism has been reported with dietary LYS levels between 2%

to 3.5% or when the LYS to ARG ratio was 2.2-2.6:1 (1). In studies focusing on ARG utilization in chickens, dietary ARG supplementation above the National Research Council (NRC) requirement (20) increases feed intake, body weight, pectoralis weight, feed efficiency, carcass yield, and plasma ARG concentrations (21-24).

1.2. Arginine is Essential for Immunity of Chickens

1.2.1. Overview of the Immune System

The immune system consists of the innate and adaptive branches which are coordinated by cytokines and other cell signaling molecules to effectively respond to pathogens and other antigens. The innate system is constitutive, recognizes pathogen associated molecular patterns, and acts in the initial stages of infection (25-26). It has low receptor specificity and diversity, and includes natural killer cells, dendritic cells, monocytes/macrophages, and granulocytes which include neutrophils (heterophils in aves), eosinophils, and basophils (25, 27-28) (Table 1). Natural killer cells are involved in viral immunity (29) while dendritic cells and macrophages are phagocytic antigen-presenting cells (28). Granulocytes are also phagocytic, but unlike dendritic cells and macrophages, they have many granules containing lytic enzymes and antimicrobial molecules (28). Of the granulocytes, neutrophils respond early on in immune responses and are important in response to bacterial infections whereas eosinophils are involved in parasitc infections, and basophils mediate allergic responses (28). Effector molecules produced by innate immune cells include reactive nitrogen species (RNS), reactive oxygen species (ROS), defensins, cytokines, lysozyme and other hydrolytic enzymes (27-

28). Innate immunity is nutritionally expensive as the costs in mounting an immune response do not diminish with repeat exposure to a particular pathogen (25).

The adaptive immune system consists of cell-mediated and humoral immunity and is characterized by the generation of antigen-specific effector and memory T helper (T_H) cells, cytotoxic T lymphocytes and B cells. Both T_H cells and B cells are important in immune responses to extracellular pathogens while cytotoxic T lymphocytes are important in immune responses to intracellular pathogens (28). Lymphocyte effector molecules include cytokines and immunoglobulins. Adaptive immunity is less nutritionally expensive than innate immunity once a repertoire of antigen-specific B and T lymphocytes have been developed (25).

Additionally, the immune system is organized into primary and secondary lymphoid tissues. Lymphopoiesis occurs in primary lymphoid tissues which include the bone marrow (mammals) or bursa (aves) and thymus where B and T lymphocytes are produced, respectively, whereas lymphocytes encounter antigens in secondary lymphoid tissues which include the spleen, lymph nodes, and gut, bronchial, mucosal, and cutaneous associated lymphoid tissues (27).

1.2.2. Avian Leukocytes

Avian leukocytes include monocytes/macrophages, granulocytes which include heterophils, eosinophils, and basophils, and lymphocytes. Additionally, cell types similar to mammalian natural killer cells and dendritic cells have been identified. The natural killer cell candidate has been found in the thymus, bursa, spleen, and intestinal epithelium of chickens (28). Similar to their mammalian counterparts, avian

monocytes/macrophages originate from bone marrow, are phagocytic, and produce NO (30-31). Avian heterophils, like mammalian neutrophils, mediate acute inflammation, are important in bacterial infection, produce ROS, and are phagocytic (32-33). Avian lymphocytes include T_H cells, cytotoxic T lymphocytes, and B cells and are capable of chemotaxis as are mammalian lymphocytes (34). Both mammalian and avian T_H cells secrete cytokines and both B cells produce immunoglobulins; however, chicken B cells produce IgY, IgM, and IgA while mammalian B cells produce IgG, IgE, IgM, IgA, and IgD (28).

Nutrients and infection impact the numbers, locations, and functions of avian leukocytes. Avian macrophages and lymphocytes are impacted by several nutrients including vitamins, minerals, amino acids, and amino acid derivatives that can be supplemented in the diet. Examples of these include zinc, vanadium, vitamin A, vitamin D, vitamin E, isoleucine, valine, betaine, and β-hydroxy-β-methylbutyrate (31, 35). For instance, when zinc-methionine is supplemented in the diet, macrophages isolated from turkeys have enhanced *in vitro* phagocytosis of *Salmonella enteritidis* and increased *Escherichia coli* clearance from blood (31). Also, in chicks supplemented with dietary betaine, monocytes and macrophages have improved chemotactic abilities and NO production (35). In avian lymphocytes, the numbers of peripheral blood lymphocytes are decreased with feed restriction, malnutrition, and protein restriction (32, 35-36). In addition, protein restriction decreases splenocyte proliferation in response to the mitogen phytohemagglutinin (PHA) (35). Also, vitamin E dietary supplementation increases the percentages of thymic and splenic T_H cells (35) while dietary valine supplementation

above requirements for growth increases antibody production to Newcastle's disease virus (35).

Avian macrophages and lymphocytes are also impacted by different types of infections including bacterial and parastic infections (31), and by antigenic molecules that mimic infections such as lipopolysaccharide (LPS) from gram-negative bacterial cell walls. For instance, there are few resident macrophages in the avian respiratory tract, but if live *E. coli* or *Salmonella typhimurium* are introduced, macrophage phagocytic ability is enhanced and peripheral blood monocytes migrate to the site of infection (31). Also, LPS administration decreases the concentration of lymphocytes in peripheral blood shortly after exposure (32) and when exposed to LPS *in vitro* avian lymphocytes exhibit increased chemotaxis (34) and proliferation (37).

Avian granulocytes are impacted by nutrition and infection as well. Under varying degrees of feed restriction, the percentage of peripheral blood heterophils and basophils increase while the percentage of eosinophils decrease (36). In addition, dietary ascorbic acid supplementation, tended to decrease random migration and increase *Staphylococcus aureus* clearance by heterophils *in vitro* (38). Shortly after intravenous LPS administration, the concentration of heterophils in peripheral blood increases (32). Additionally, during infection with *S. enteritidis*, heterophils are recruited from circulation to sites of inflammation in the intestinal mucosa, and in chickens with induced heteropenia, *S. enteritidis* pathogenicity is increased (39).

1.2.3. Overview of the Interaction between Nutrition, Metabolism, and Immunity

Nutrition, metabolism, and the immune system are closely intertwined as metabolism determines the fate of nutrients for physiological processes while the immune system is reliant on these nutrients to function. For example, when lymphocytes are activated there is an increase in their metabolic rate and they rely on having adequate nutrients as they rapidly proliferate and produce antibodies, cytokines, and other cell signaling molecules (27, 40). Additionally, regulation of metabolism overlaps with regulation of the immune system as hormones, cytokines, and other factors act on both non-immune and immune cells and tissues. For instance, hormones such as insulin that regulate energy metabolism also act on lymphocytes which preferentially utilize glucose and glutamine for energy (40-41). In metabolism, insulin stimulates glucose uptake and utilization by tissues, and in activated T cells insulin promotes T cell responsiveness (27, 41).

Nutrition can be utilized to manipulate the immune system. Mechanisms by which nutrition modulates the immune system include altering signal transduction in leukocytes (polyunsaturated fatty acids, vitamins A, D, E), providing substrate for immune cells (amino acids, trace minerals) or pathogens (iron, biotin), protecting against immunopathology (antioxidants), and influencing gut microbial populations (fiber) and the hormonal environment (protein:energy ratio) (25-26).

1.2.4. Arginine Utilization for Immunity

ARG has a role in a mechanism of immunomodulation in part as a substrate for the immune system (22). ARG supplementation improves wound healing associated with surgery or trauma and improves survival in rodent and human tumor models (27, 42-44) which has resulted in many studies attempting to understand how ARG supplementation impacts the immune system. It has been determined that ARG utilization by immune cells has important implications regarding their function. For example, in B cells, an ARG deficiency impacts early B cell maturation in the bone marrow of transgenic mice at the pro- to pre-B cell transition and decreases the proportion of B cells in the lymph nodes, small intestine, and spleen, but does not impact mature B cell proliferation in activated B cells (45).

In T cells, ARG regulates development and function (Figure 1). The T cell receptor (TCR) complex includes the CD3ζ chain which is the primary signal transduction element of the TCR (46). In Jurkat $CD4^+$ T cells, the absence of ARG decreases CD3ζ chain expression *in vitro*; however this was reversed by replenishing ARG (46). Furthermore, CD3ζ chain mRNA abundance and half-life were decreased (46). In activated human peripheral blood T cells, the absence of ARG interferes with normal TCR cycling and CD3ζ chain re-expression *in vitro*; however, unlike in Jurkat T cells, this was not accompanied by a decrease in CD3ζ chain mRNA abundance (47). In addition, the absence of ARG results in a decrease in T cell proliferation and cytokine production *in vitro* (47) and has been associated with a decrease in T cell proliferation and cytokine production *in vivo* when extracellular ARG is diminished in response to surgery, trauma, or sepsis (46-48). Furthermore, in human peripheral blood T cells, the

absence of ARG blocks *in vitro* proliferation by arresting the cells in the G_0-G_1 phase of the cell cycle (48).

In macrophages (activated monocytes), ARG is a substrate for iNOS or arginase and generates NO, an important cytotoxic molecule, and citrulline or ornithine, a precursor for polyamines, and urea depending on cytokine signals (Figure 2). For example, in casein-elicited rat macrophages, there is an increase in iNOS protein as well as an increase in nitrite and citrulline production from radiolabeled ARG *in vitro* (49). Additionally, in the avian macrophage cell line, HD11, ARG and lipopolysaccharide (LPS) influence production of nitrite in a dose-dependent manner (30). ARG utilization in macrophages may also have a role in modulating T cells. In murine macrophages stimulated with T_{H2} subset cytokines (addressed below) *in vitro*, extracellular ARG is depleted when arginase I, but not arginase II or iNOS, is upregulated (50). Furthermore, when cocultured with Jurkat $CD4^+$ T cells this depletion results in a decrease in CD3ζ chain expression and inhibits proliferation (50).

1.2.5. Arginine Utilization for Immunity of Broiler Chickens

ARG utilization for immunity has been studied in broiler chicks as they provide a model that is not confounded by *de novo* ARG synthesis. It has been determined that dietary ARG levels 100% and 120% of the NRC requirement do not impact cutaneous basophil hypersensitivity which was used to assess cell-mediated immunity, sheep red blood cell antibody titer which was used to assess humoral immunity, relative lymphoid organ weights, or blood leukocyte counts (22). However, in another study dietary ARG supplementation to deficient diets improved cutaneous basophil hypersensitivity,

antibody titer to Newcastle disease virus, and relative thymic and spleen weights (51). In broilers vaccinated with a live infectious bursal disease virus, dietary ARG above the NRC requirement increases the proportion of T cells and B cells in peripheral blood (52). Also as dietary ARG increases, microbial infections detected during processing diminish (24). Therefore, even though dietary ARG supplementation in broilers has provided inconsistent results it is apparent that the immune system requirement for ARG warrants further study.

1.3. Arginine Transporters

Tissues and cells such as immune cells require nutrients to maintain physiological processes and have different nutrient priorities. For instance, within the immune system, nutrient priority is highest for macrophages and lowest for T cells while B cells fall somewhere between (26). Tissues acquire these nutrients through nutrient transporters which mediate transport across the plasma membrane (26). In the case of ARG, there are three gene families encoding four transport systems that are responsible for its transport (53-55). The system y^+ cationic amino acid transporter (CAT) family which imports CAA independently of Na^+ resulting in an influx of CAA (6, 12, 53, 55-58). The systems y^+L and $b^{0,+}$ glycoprotein-associated amino acid transporter (gpaAT) family (also referred to as the light chains of the heterodimeric amino acid transporters) in which y^+LAT-1 and -2 exchange Na^+ and large NAA for CAA resulting in efflux of CAA and $b^{0,+}$AT exchange large NAA and CAA independently of Na^+ resulting in an influx of CAA (5-6, 10, 12, 53, 55, 58). The system $B^{0,+}$ of the Na^+/Cl^- dependent transporter family in which $B^{0,+}$AT imports NAA and CAA in a Na^+/Cl^- dependent manner (12, 53, 55, 58) (Table 2).

1.3.1. System y^+

System y^+ includes glycosylated transmembrane transporters CAT1-4 (6, 58). CAT-1 and -3 are more closely related than CAT-4 (6, 58) while CAT-2A and CAT-2B are formed by the same gene by alternative splicing (6, 8, 56, 58).

In mammals, CAT-1 is ubiquitously expressed at varying levels with the exception of the liver (6, 8, 56, 58) and is the predominate system y^+ transporter in most cell types. CAT-1 expression is modulated by cell proliferation, growth factors, cytokines, and hormones and its expression is elevated during nutrient limitation (6). CAT-2A is present in the liver and has a low affinity while CAT-2B has a high affinity (6, 8, 12, 56). CAT-2B expression increases after cytokine or LPS stimulation and is often co-induced with iNOS in several cell types (6, 12, 58). The associated induction of CAT-2B and iNOS probably acts to increase ARG transport in support of elevated NO production as Nicholson et al. (56) indicated that CAT-2 is necessary for sustained NO production in macrophages. CAT-3 is found in the brain (8, 12), and in humans it is strongly expressed in the thymus (6, 58). CAT-4 is found in the brain, testis, and placenta, but its function is largely unknown (6). In humans an activation of transport through y^+ has been reported in T cells stimulated with PHA *in vitro* (59). System y^+ is a high affinity transport system (except for CAT-2A with a K_m of 2-5 mM) with K_m values ranging from 0.025 to 0.4 mM (6, 53-54, 57-58).

In broiler chicks, CAT isoforms are also expressed though their tissue distribution in some cases differs from that of mammals. For instance, unlike in mammals, CAT-1 is expressed in liver. CAT-1 is also expressed in gastrocnemius, pectoralis, and bursa but is

not expressed in heart, spleen, and thymus (53). Similar to CAT-1, CAT-2 is expressed in gastrocnemius, liver, pectoralis, and bursa, but unlike CAT-1, is also expressed in heart, spleen, and thymus (53). CAT-3 is expressed in pectoralis, bursa, heart, spleen, and thymus, but not in gastrocnemius and liver. CAT isoform expression is dependent on chick age. For instance, CAT-2 and -3 are not detectable in the thymus on d1 posthatch but are expressed on d7 posthatch (53). In lymphocytes CAT isoform expression differs some from bursa and thymus as CAT-1 and CAT-3 are expressed in bursacytes and thymocytes, but CAT-2 is not (55). An additional difference between mammals and broiler chicks in CAT isoform expression is in the alternate splicing of CAT-2. Similar to mammals, CAT-2A and CAT-2B have been identified in chickens; however, an additional CAT-2 isoform, CAT-2C has also been identified (60).

1.3.2. System y^+L and System $b^{0,+}$

Systems y^+L and $b^{0,+}$ includes unglycosylated transmembrane transporters $y^+LAT1/2$ and $b^{0,+}AT$ (6, 58). In these systems from the gpaAT family, cell-surface expression is dependent upon covalent association with a type II surface glycoprotein (4F2hc or rBAT) (5-6, 10). $y^+LAT1/2$ associate with 4F2hc while $b^{0,+}AT$ associates with rBAT (5, 10). These transporters are obligatory exchangers (5-6, 10, 58). y^+LAT-1 and $b^{0,+}AT$ are primarily expressed in epithelia with the highest mRNA abundance in the small intestine and kidney indicating that they are important in absorption and reabsorption of amino acids whereas y^+LAT-2 is rather ubiquitously expressed (5-6, 10, 58). System y^+L and system $b^{0,+}$ are high affinity transport systems with apparent K_m values between 5-10 μM and 20-80 μM, respectively (5-6). In humans, only minimal

transport of CAA through system y^+L has been reported in lymphocytes (59). In lymphocytes 4F2hc is barely detected in resting cells, but is strongly induced in proliferating cells presumably to provide amino acids necessary for synthesis of cell proteins in preparation of cell division (5-6, 10).

1.3.3. System $B^{0,+}$

System $B^{0,+}$ includes glycosylated transmembrane transporter $ATB^{0,+}$ (58). $ATB^{0,+}$ is a high affinity transporter with apparent K_m values between 0.10 and 0.15 mM (58). It is most abundant in the lung and salivary gland, but is also found in the mammary gland, pituitary gland, and stomach (53, 55, 58).

1.4. The Acute Phase Response and T_H Subset Cytokines

1.4.1. The Acute Phase Response

There are alterations in nutrient metabolism and nutrient partitioning when the immune system is activated (26). A specific instance of this alteration is demonstrated with the acute phase response (APR) which can be induced with LPS (61-62), and refers to the metabolic changes that occur after tissue injury or immune challenge resulting in an inhibition of growth, the production of hepatic acute phase proteins, and the recruitment and proliferation of immune cells (25, 61, 63). For instance, a metabolic change observed in chickens is an increase in the fractional synthesis rate of hemopexin, an acute phase protein, in LPS-injected chicks (63). Other markers associated with the acute phase response include: fever, anorexia, increased hormone and cytokine synthesis,

and leukocytosis (29, 54, 61). In broiler chicks, examples of these markers include elevated cloacal temperature at 3 h post-LPS injection and elevated plasma levels of IL-6 at 12 h post-LPS injection (64). During the APR, the alteration in protein metabolism is quite impressive as the immune system tends to use a large amount of available amino acids for host defense resulting in a decrease in muscle protein synthesis and an increase in muscle protein degradation (63, 65-68). For example, *E. coli* injection in chickens increased protein catabolism in the muscle, spleen, and thymus 43, 30, and 34%, respectively, but did not impact protein catabolism in the liver or bursa (69), whereas the fractional synthesis rate of protein increased in the liver and decreased in muscle (65). Additionally, there may be excessive muscle protein catabolism in order to supply the necessary amount of limiting amino acid needed for synthesis of acute phase proteins (67-68). For instance, some human acute phase proteins have high concentrations of phenylalanine, tryptophan, and tyrosine which results in the degradation of muscle protein in excess of the quantity of acute phase proteins synthesized (67).

1.4.2. Arginine and the Acute Phase Response

The alteration in metabolism during the APR has implications for ARG utilization by immune cells and tissues. During the APR, ARG is metabolized to produce NO in macrophages and to support increases in immune cell proliferation (21-22, 70). For instance, in broiler chicks fed ARG above the NRC requirement, there is an increase in plasma nitrite concentration and splenocyte proliferation in response to LPS injection (71). Similarly, in other species, such as rats, mice and pigs, dietary ARG

supplementation enhances immune responses to pathogens or bacterial endotoxins (9, 27) whereas ARG deficiency impairs nitrite production in response to LPS injection (72).

1.4.3. T_H Subset Cytokines

Cytokines are hormone-like peptides that coordinate immune responses (29, 73). They are secreted by immune cells, act locally, are antigen nonspecific, and are both pleiotropic and redundant (29). Cytokines can be divided into four main subsets: proinflammatory cytokines, T_{H1} subset cytokines, T_{H2} subset cytokines, and T_{H3} subset or anti-inflammatory cytokines. Proinflammatory cytokines initiate an innate immune response and generate localized inflammation and include interleukin (IL)-1β and IL-6 (73). IL-1β is produced by macrophages, mediates the acute phase response, increases *in vitro* CAT expression and activity in mammals, and stimulates neutrophil production (29, 54, 62, 74). IL-6 is produced by T cells and macrophages, stimulates acute phase protein production, elevates serum corticosterone levels, and induces B cell maturation (74-75). Both IL-1β and IL-6 induce antibody production and fever (29, 62). T_{H1} subset cytokines promote cell-mediated immunity and include IL-12, IL-18, and interferon (IFN)-γ (76). IL-18 is produced by activated macrophages and induces IFN-γ production by T cells and natural killer cells (74). IFN-γ induces secretion of NO in macrophages, and increases CAT expression and activity *in vitro* (54, 62, 74). T_{H2} subset cytokines promote humoral immunity (76) and include IL-3, IL-4, IL-5, IL-9, and IL-13. IL-4 is produced by T cells, stimulates growth and differentiation of B cells and suppresses T_{H1} cells (28, 74). IL-13 is produced by T cells and causes B cell growth while inhibiting inflammatory cytokine

production by macrophages (74). T_{H3} subset or anti-inflammatory cytokines include IL-10 and transforming growth factor-β (TGF-β). IL-10 is synthesized by T cells and macrophages and inhibits IFN-γ synthesis in activated lymphocytes (74, 77). TGF-β is immunostimulatory or immunosuppressive depending on the stimulus and target cell type such that it is immunosuppressive on activated lymphocytes, monocytes, and macrophages (62).

1.4.4. Avian T_H Subset Cytokines

Avian orthologs to mammalian cytokines have been identified (75, 78-82). Additionally, avian cytokines are also divided into proinflammatory, T_{H1}, T_{H2}, and anti-inflammatory subsets (78, 83). Like mammalian proinflammatory cytokines, avian proinflammatory cytokines include IL-1β and IL-6, elevate serum corticosterone levels, and are produced by macrophages (75, 80). Avian T_{H1} subset cytokines include IL-12, IL-18, and IFN-γ similar to their mammalian counterparts, and like mammals, IL-12 requires both subunits for functionality, induces IFN-γ production by splenocytes, and induces spleen cell proliferation (82), while IFN-γ induces nitrite production by macrophages (81). Avian T_{H2} subset cytokines include IL-3, IL-4, IL-5, IL-9, and IL-13 (78). This is similar to mammals except in chickens IL-5 is a pseudogene (78). IL-3, IL-4, and IL-13 are expressed in a variety of stimulated leukocytes and lymphoid and non-lymphoid tissues such as splenocytes, bursacytes, thymus, bursa, lung, and brain (78). IL-4 and IL-13 also stimulate B cell proliferation similar to these mammalian cytokines (78). Like mammalian anti-inflammatory cytokines, avian anti-inflammatory cytokines include IL-10 and TGF-β; however, in mammals TGF-β genes do not include TGF-β4 as

they do in chickens (84). It has been suggested that chicken TGF-β4 is actually chicken TGF-β1 as its established function is equivalent to mammalian TGF-β1 (84). Also, TGF-β4 is the most commonly expressed isoform in chickens (84).

Avian T_H subset cytokine profiles change in response to nutrition. For instance, serum concentrations of IL-1 increase in chickens injected with a series of immunogens including *E.coli* LPS, *S. typhimurium* LPS, and S. aureus when fed a methionine-sufficient diet compared to those fed a methionine-deficient diet (85). Likewise, plasma IL-1 levels increase in LPS-injected chicks fed a diet high in cysteine compared to diets low or medium in cysteine or deficient in sulfur amino acids (71). In addition, cytokine expression does not respond solely to dietary amino acids; for example, an increase in splenic IL-1β expression in LPS-injected chicks was impacted by dietary vitamin E (62), whereas dietary supplementation of n – 3 polyunsaturated fatty acids increased IFN-γ mRNA abundance in LPS-injected chickens (86).

Avian T_H subset cytokine profiles change in response to infection as well. For instance, of the proinflammatory cytokines, IL-1β expression is induced in response to LPS in macrophages (75) and splenocytes (61), and IL-6 expression in PBMC is decreased after oral exposure to *S. enteritis* (84), and increased in plasma in LPS-injected chickens (64). Of the T_{H1} cytokines, IL-12 expression is increased in splenocytes, an avian macrophage cell line, and an avian B lymphocyte cell line when stimulated with LPS *in vitro* (82), and IL-18 expression is increased in the spleen after S. enteritis exposure (77). Also, IFN-γ expression is increased in the spleen and ileum after exposure to Newcastle disease virus (83) and is increased in the spleen and splenocytes after exposure to *S. enteritis* and *S. typhimurium* LPS, respectively (61, 77). Of the T_{H2}

cytokines, ileal IL-13 expression is increased with *Ascaridia galli* infection (83). Of the anti-inflammatory cytokines, TGF-β2 splenocyte expression is increased when incubated with LPS *in vitro* (61), whereas TGF-β4 PBMC expression is decreased after *in vitro S. enteritis* exposure (84).

Many studies have addressed ARG's impact on the immune system; however, few have utilized broiler chicks as a model not complicated by *de novo* ARG synthesis to examine ARG's impact on the cellular level by considering markers of ARG utilization. Additionally, in broiler chicks, dietary ARG is provided to maximize growth; however, it is unknown if the NRC requirement for growth is optimal for immunity. Therefore, in the present study the following was determined: ARG transporter mRNA abundance as a marker of ARG utilization by peripheral blood mononuclear cells (PBMC); thymocyte proliferation as an indication of T cell function in response to dietary ARG; PBMC phenotype, and ARG's impact on the severity of the APR by considering PBMC phenotype and splenic cytokine profiles.

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Table 1. Cells of the innate immune system [1]

Cell Type	Express PRR	Phagocytose	Produce RNS/ROS	Present Antigen
Natural killer cells	No	No	No	No
Dendritic cells	Yes	Yes	Yes	Yes
Monocytes/macrophages	Yes	Yes	Yes	Yes
Granulocytes	Yes	Yes	Yes	No

[1] Abbreviations: PRR, pattern recognition receptors; RNS, reactive nitrogen species; ROS, reactive oxygen species.

Table 2. ARG transporter systems [1]

Transport System	Gene	Gene Products	Substrates	Function	Sodium Dependence	Site	Tissue Distribution
y^+	SLC7A1 SLC7A2-A SLC7A2-B SLC7A3 SLC7A4	CAT-1 CAT-2A CAT-2B CAT-3 CAT-4	LYS, ARG	Imports CAA, NAA, and Na^+; influx of CAA	Independent for CAA; dependent for NAA	BLM	Widespread, liver, skeletal muscle, macrophages
y^+L	SLC7A7 SLC7A6 SLC3A2	LC: y^+LAT-1 LC: y^+LAT-2 HC: 4F2hc	LYS, ARG, MET, GLN	Exchanges CAA for Na^+ and NAA; efflux of CAA; y^+LAT-2 may be an electroneutral ARG/GLN exchanger	Independent for CAA; dependent for NAA	BLM	Mainly in kidney, and small intestine; also in peripheral blood lymphocytes, liver, pancreas, testis, ovary, placenta, lung, thyroid, erythrocytes, brain, heart
$b^{0,+}$	SLC7A9 SLC3A1	LC: $b^{0,+}$AT HC: rbAT	LYS, ARG, LEU, Cystine, Ornithine	Exchanges NAA and CAA; influx of CAA	No	Apical	Kidney, small intestine, heart, liver, brain, lung, placenta, thymus
$B^{0,+}$	SLC6A14	$ATB^{0,+}$	ALA, LYS, ARG, VAL	Imports NAA and CAA	Yes	Apical	Trachea, stomach, glandular tissues

[1] Abbrevations: ALA, alanine; ARG, arginine; ATB^+, Na^+-dependent amino acid transporter; BLM, basolateral membrane; CAA, cationic amino acid; CAT, cationic amino acid transporter; GLN, glutamine; HC, heavy chain; LC, light chain; LEU, leucine; LYS, lysine; MET, methionine; NAA, neutral amino acid; SLC, solute carrier family; VAL, valine; y^+LAT, y^+L type amino acid transporter.

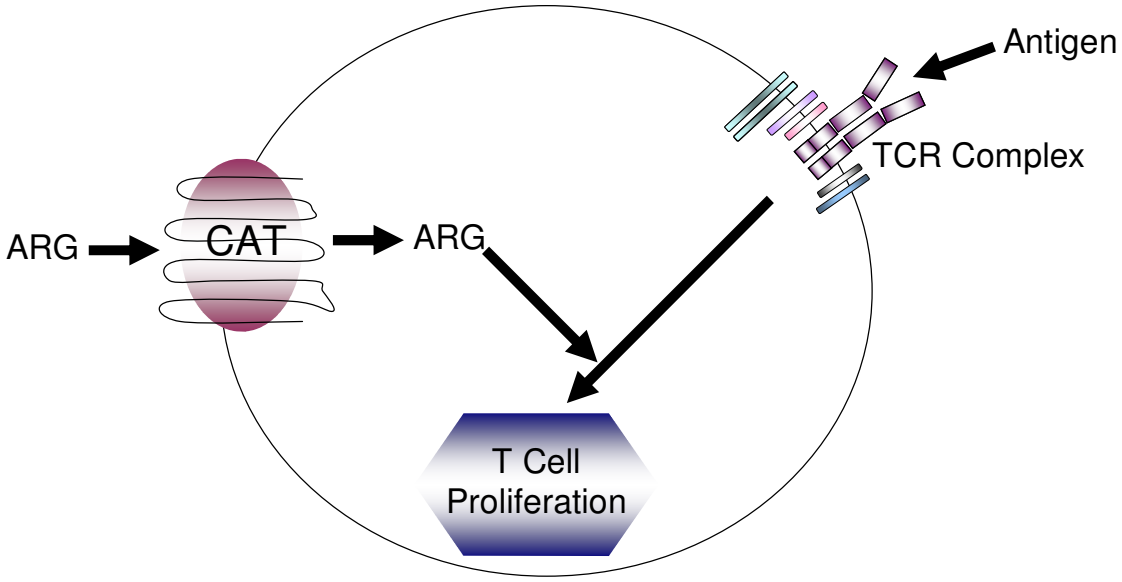

Figure 1

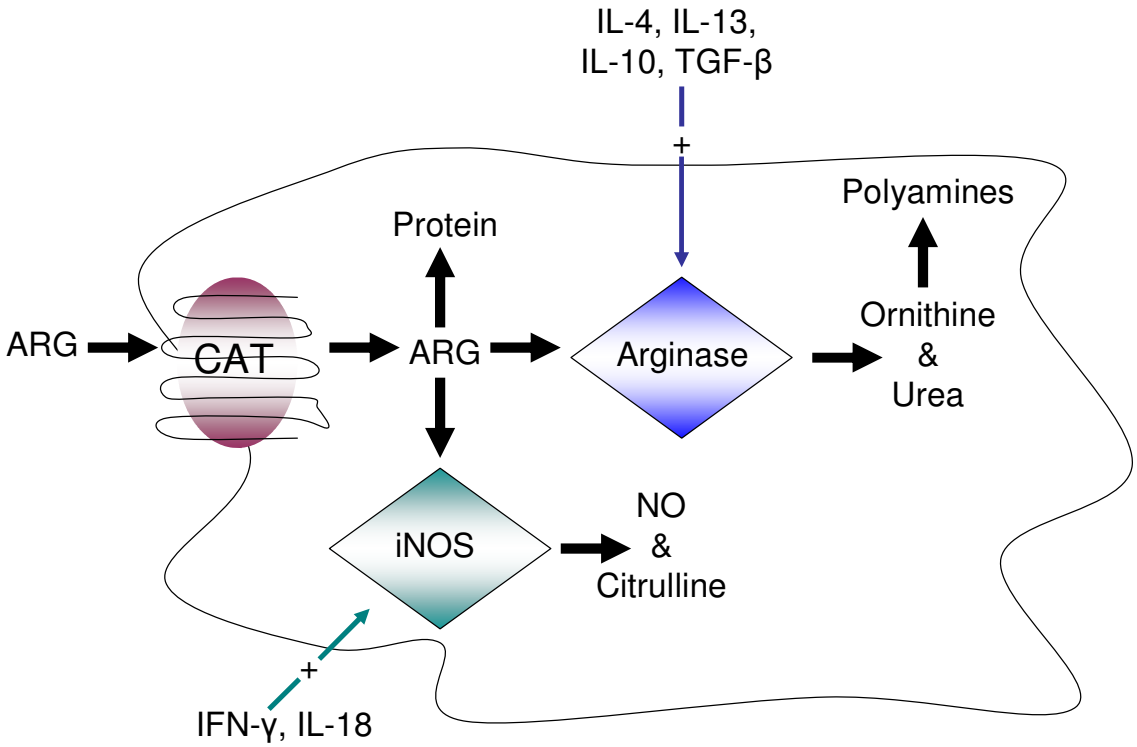
IL-4, IL-13,
IL-10, TGF-β
+
Polyamines
Protein
ARG
CAT
ARG
Arginase
Ornithine
&
Urea
iNOS
NO
&
Citrulline
+
IFN-γ, IL-18

Figure 2

Figure 1. ARG utilization in T cells. Abbreviations: ARG, arginine; CAT, cationic amino acid transporter; TCR complex, T cell receptor and CD3.

Figure 2. ARG utilization in macrophages. Abbreviations: ARG, arginine; CAT, cationic amino acid transporter; IFN, interferon; iNOS, inducible nitric oxide synthase; IL, interleukin; NO, nitric oxide; TGF, transforming growth factor.

CHAPTER 2

Dietary Arginine's Impact on Arginine Utilization for Immunity

Introduction

Nutrition can be utilized to manipulate immune responsiveness to pathogens by altering signal transduction in leukocytes, providing substrate for immune cells or pathogens, protecting against immunopathology, and influencing gut microbial populations and the hormonal environment (1-2). Nutrients such as vitamins, trace minerals, and amino acids are substrates for immunity (1); however, the optimal level of supplementation of these nutrients for an animal is often unknown (2). Therefore, to understand the impact of nutrient supplementation on immunocompetence, studies must relate nutrient supplementation with the magnitude of leukocyte pools and the function of peripheral leukocytes (3).

Arginine (ARG) is an immunologic modulator due in part to its role as a substrate for the immune system (4). ARG is an intermediate of the urea cycle and is metabolized either by nitric oxide synthase (NOS) to produce nitric oxide (NO) or arginase to produce polyamines. ARG supplementation improves wound healing and improves survival in rodent and human tumor models (5). ARG supplementation (2.8% dietary ARG) of wounded rats resulted in faster healing and less postoperative weight loss in comparison to those fed a standard commercial diet (1.8% dietary ARG) (6). In aves, ARG is an essential cationic amino acid (CAA) since they are uricotelic and cannot synthesize ARG *de novo* (4-5, 7); however, it is conditionally essential in mammals as it can be synthesized from citrulline in the adult kidney (8). This results in avian immune cells being dependent upon acquiring dietary ARG, whereas mammalian immune cells may

obtain ARG from either interorgan amino acid metabolism or the diet. Consequently, in addition to growth, dietary ARG levels for immunity must also be considered to ensure the immune system is acquiring the ARG it needs. Therefore, understanding ARG utilization by immune cells may help to better understand how ARG needs for immunity relate to dietary ARG levels that maximize growth.

ARG utilization by immune cells has important implications on their function. In T cells, ARG regulates development and function. For instance, a reduction in extracellular ARG in response to surgery, trauma, or sepsis has been associated with a decrease in T cell proliferation and cytokine production *in vivo* (9-11). In human peripheral blood T cells, the absence of ARG blocks *in vitro* proliferation by arresting them in the G_0-G_1 phase of the cell cycle (11). Also, the absence of ARG decreases T cell CD3ζ chain expression *in vitro* in Jurkat T cells (12) and interferes with CD3ζ chain expression with the T cell receptor (TCR) in activated human peripheral blood T cells (9). In B cells, an ARG deficiency impacts early B cell maturation in the bone marrow of transgenic mice at the pro- to pre-B cell transition and decreases the proportion of B cells in the lymph nodes and the spleen, but does not impact mature B cell proliferation (8). In monocytes that have become activated tissue macrophages, ARG is a substrate to generate effector molecules such as NO. For instance, in the avian macrophage cell line, HD11, production of nitrite was influenced by ARG and lipopolysaccharide (LPS) concentration in a dose-dependent manner with no nitrite produced in the absence of ARG (7). Therefore, functional aspects of T cells, B cells, and monocytes are reliant on ARG utilization which leads to implications for nutrient supply through nutrient transporters.

Nutrient transporters for ARG contribute to ARG retention and use in tissues. ARG is transported by three gene families (13). Transport proteins from systems y^+ and $B^{0,+}$ import ARG (13-15), while transport proteins from systems y^+L and $b^{0,+}$ export ARG (13-14, 16). In mammalian leukocytes, ARG utilization appears to be primarily mediated by gene products encoded for system y^+. For example, stimulated human peripheral blood T cells increase system y^+ activity, yet only have minimal system y^+L activity *in vitro,* (17) while macrophage NO production is dependent upon ARG uptake via cationic amino acid transporter-2B (CAT-2B) (15, 18). Additionally, compared to transport through system y^+, system y^+L transporter mRNA abundance and ARG flux in human umbilical vein endothelial cells stimulated with TNF-α and LPS was not significant (19); however in murine bone marrow-derived macrophages ARG transport was preferentially mediated by system y^+L when not activated (20). In chickens, high affinity CAT isoforms in the bursa are more highly expressed than in the thymus (13). Consequently, the number and type of ARG transporters expressed in cells and tissues helps to provide an understanding of ARG utilization. In chickens, the number and types of ARG transporters expressed in leukocytes in response to dietary ARG is unknown.

Therefore, the objective of these experiments was to evaluate the impact of dietary ARG supplementation on ARG utilization in peripheral blood mononuclear cells (PBMC) and thymocytes in young, growing broiler chicks as they provide a model that is not confounded by *de novo* ARG synthesis. Two experiments were performed to measure: 1) ARG transporters as markers of ARG utilization by PBMC and thymocytes; 2) thymocyte proliferation as an indication of T cell function in response to dietary ARG; and 3) PBMC phenotype.

Materials and Methods

2.1. Animals and Diets

Mixed sex Cobb 500 broilers were obtained from a commercial hatchery (Cedar Hatchery, Fresno, CA) and were raised in Petersime brooder batteries (Petersime Incubator Co., Gettysburg, OH) contained in an environmentally controlled room (27°C; 24 h light). In all experiments, chicks were provided *ad libitum* access to water and feed. In experiment 1, a wheat-soybean meal diet (Table 1) was formulated according to the National Research Council (NRC) (21) recommendations, except for arginine (ARG). The low ARG diet was analyzed to contain 1.20% ARG and the high ARG diet was analyzed to contain 1.35% ARG. In experiment 2, a corn-soybean meal diet (Table 2) was formulated according to NRC (21) recommendations for a young growing broiler chick, except for ARG. Experimental diets were analyzed to contain either 1.1% (low), 1.3% (adequate) or 1.5% (high) ARG. All experiments and procedures were approved by the California Polytechnic State University Animal Care and Use Committee.

2.2. Experimental Design

In experiment 1, mixed sex hatchlings (d1) were selected for uniform body weight (BW) from a larger population and twelve chicks were placed into one of six replicate pens per dietary treatment. On d3, 7, 14 and 21 posthatch, pen weights were measured and three chicks per pen were euthanized by CO_2 overdose for whole blood and organ collection. Whole blood from one chick per pen was collected via cardiac puncture into a syringe containing a 2% EDTA solution (Sigma-Aldrich, St. Louis, MO; #2854) and four

thymic lobes from the chick's left side were removed aseptically and stored in RPMI 1640 (MP Biomedicals, LLC, Solon, OH; #1260354) on ice. From whole blood and thymic lobes, respectively, PBMC and thymocytes were isolated to determine ARG transporter mRNA abundance. From the remaining two chicks per pen, four thymic lobes from the chick's left side were removed and weighed. Spleen and pectoralis major and minor were excised from three chicks per pen and weighed.

In experiment 2, mixed sex hatchlings (d1) were selected for uniform BW from a larger population and nine chicks were placed into one of eight replicate pens per dietary treatment. On d10, 14 and 17 posthatch, pen weights were measured and two chicks per pen were euthanized by CO_2 overdose for whole blood and organ collection. Whole blood from one chick per pen was collected into a heparinized (Hospira, Inc., Lake Forrest, IL; #0409-1402-31) syringe and six thymic lobes from the chick's left side were removed aseptically and stored in RPMI 1640 on ice. From whole blood, PBMC were isolated for phenotyping. From thymic lobes, thymocytes were cultured for a proliferation assay.

2.3. Peripheral Blood Mononuclear Cell Isolation

PBMC were isolated from approximately 3 mL of whole blood. Whole blood was overlaid onto Histopaque-1077 (Sigma-Aldrich, St. Louis, MO; #10771) and centrifuged at 350 x g for 15 min at 25°C. In experiment 1 only, the buffy coat was collected and overlaid on iso-volumetric Histopaque-1077 a second time to remove excessive red blood cell contamination. In both experiments, the buffy coat was reconstituted in RPMI 1640 and centrifuged at 500 x g for 5 min at 25°C. The pelleted cells were re-suspended in 1

mL RPMI 1640 and cells were enumerated with a hemocytometer (experiment 1) or ViaCount Reagent (experiment 2; Guava Technologies, Inc, Hayward, CA) per the manufacturer's instructions. PBMC from experiment 1 were aliquoted (4.5 x 10^6 cells) and centrifuged at 500 x g for 5 min at 25°C, snap-frozen in liquid N, and stored at -80°C until further analysis.

2.4. Peripheral Blood Mononuclear Cell Phenotyping

In experiment 2, PBMC phenotype was determined via flow cytometry using the EasyCyte Plus System (Guava Technologies, Inc, Hayward, CA) with a 488 nm argon laser. FITC-conjugated mouse anti-chicken CD4, PE-conjugated mouse anti-chicken CD8α, FITC-conjugated mouse anti-chicken Bu-1, and PE-conjugated mouse anti-chicken KUL01 (SouthernBiotech, Birmingham, AL; #8210-02, 8220-09, 8395-02, 8420-09) were used as markers for $CD4^+$, $CD8\alpha^+$, B cells, and monocytes, respectively. Approximately 10^6 cells were incubated with 0.2 µg (PE) or 1 µg (FITC) antibody for 30 min at 4°C. Samples were washed by centrifugation with wash buffer (250 µL PBS, 2% BSA, 0.1% NaN_3) at 4°C for 5 min at 500 x g. The cells were then re-suspended in wash buffer and diluted 1:10 for analysis. Lymphocyte and monocyte subpopulations were gated by forward and side-scatter characteristics and 5000 gated events were analyzed for FITC or PE fluorescence. The proportions of $CD4^+$, $CD8^+$, Bu-1^+, and $KUL01^+$ cell populations were expressed as a percentage of gated lymphocytes or gated monocytes, as appropriate.

2.5. Thymocyte Isolation

In experiments 1 and 2, thymic lobes were removed aseptically and placed into sterile 60 mm tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ; #353002) containing 1.5 mL RPMI 1640 as previously described (22-23). Briefly, thymic lobes were minced gently with forceps to release thymocyte populations into the media. The media from each sample was filtered through a sterile 70-μm nylon cell strainer (BD Falcon, Bedford, MA; #352350) and thymocyte concentration was determined with a hemocytometer (experiment 1) or ViaCount Reagent per the manufacturer's protocol (experiment 2). Thymocytes from experiment 1 were aliquoted (2×10^7 cells) and pelleted at 500 x g for 5 min at 25°C, snap-frozen in liquid N, and stored at -80℃ until further analysis.

2.6. Thymocyte Proliferation

Thymocytes (5×10^5 cells) were cultured in complete media (RPMI 1640, 5% FBS, 1% Penicillin/Streptomycin) in each well of two sterile 96-well flat bottom plates (BD Falcon, Bedford MA; #353072) with either 0 (control) or 30 μg/mL phytohemagglutinin-P (PHA-P; Sigma, St. Louis, MO; #L1668) as previously described (23). Thymocyte proliferation was determined after 48 h using Cell Proliferation ELISA, BrdU (colorimetric) (Roche Diagnostics, Indianapolis, IN; #11647229001) per the manufacturer's instructions. Data was expressed as absorbance at 370 nm.

2.7. Total RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from PBMC and thymocyte samples using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA; #740955.50) per the manufacturer's instructions. Samples were homogenized with a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA). Optical density at 260 nm was used to quantify RNA concentrations. PBMC and thymocyte total RNA (250 ng) was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad, Hercules, CA; #170-8891) per the manufacturer's protocol. Quantitative real-time PCR analysis of target gene mRNA abundance was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions utilized Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA; #4385610), 1 μL of RT product, and 10 μmol/L of each primer (Table 3). Thermal cycling parameters were 1 cycle of enzyme activation at 95°C for 20 s and 40 cycles of denaturing at 95°C for 3 s and annealing and extending at 60°C for 30 s. Melting curve analysis was performed after 40 cycles to confirm product specificity such that melt-curve analyses containing a specific product peak and primer dimer peak were excluded from analysis and considered to be below the limit for accurate detection. Melting curve analysis was performed at 95°C for 15 s, 60°C for 60 s, followed by a linear temperature increase of 0.5°C/s to 95°C while continuously monitoring fluorescence. The change in sample mRNA abundance was calculated using the Δ-Δ equation with modifications as previously described (22). Sample PCR amplification efficiencies were determined in the log-linear phase with LinRegPCR program (24). Target gene mRNA abundance was normalized by geometric averaging of raw non-normalized values of *β*-actin, HPRT, and TATA box binding protein using geNorm software (25). Data are presented as the

normalized fold-change in mRNA abundance relative to 1.20% ARG on d7 for thymocytes or d3 for PBMC.

2.8. Statistical Analysis

Dependent variables were analyzed by general linear model (JMP Software, SAS Inc, Cary, NC) using either a one-way or two-way analysis of variance (ANOVA). A one-way ANOVA was used to determine the effect of dietary ARG on BW, rate of gain, feed intake, and feed efficiency. A two-way ANOVA was used to determine the main effects of dietary ARG level, day, and their interaction on tissue weights, PBMC and thymocyte cell concentrations, PBMC phenotype, thymocyte proliferation, and ARG transporter mRNA abundance. When main effects or their interactions were significant ($P<0.05$), means were compared by student's T pairwise comparisons. PBMC phenotyping data was transformed using arcsine in order to meet conditions for ANOVA. Data are reported as nontransformed means and pooled standard errors.

Results

2.9. Experiment 1

2.9.1. Performance and Tissue Weights

Dietary ARG did not affect performance ($P>0.05$; Table 4) or tissue weights (Table 5); however, body weight (BW) and tissue weights increased with age ($P<0.05$). Thymus, spleen, and pectoralis weights increased from d3 to d21 posthatch ($P<0.05$). When organ weights were corrected for individual body weight (relative tissue weights)

from d3 to d21 ($P<0.05$), thymus, spleen, and pectoralis relative weights increased 84%, 49%, and 451%, respectively.

2.9.2. Peripheral Blood Mononuclear Cell and Thymocyte Concentrations

PBMC concentration increased approximately 145% from d3 to d7 ($P<0.05$; Table 6) and remained constant through d21. From d3 to d21 posthatch, thymocyte concentration was dependent upon the dietary ARG level ($P<0.05$; Figure 1). Between d14 and d21, both 1.20% and 1.35% dietary ARG increased the concentration of thymocytes; however, at d21, thymocyte concentration at 1.20% dietary ARG was 25% higher than at 1.35% dietary ARG ($P<0.05$).

2.9.3. ARG Transporter mRNA Abundance

In PBMC, the mRNA of several ARG transporters including CAT-1, CAT-3, y^+LAT-1, y^+LAT-2 and $ATB^{0,+}$, was detected while the mRNA of CAT-2A and CAT-2B was not detected and $b^{0,+}$AT mRNA was below the limit for accurate detection. When data were expressed relative to CAT-1 mRNA abundance on d7 from chicks fed 1.20% ARG, both 1.20% and 1.35% dietary ARG decreased CAT-1 mRNA abundance between d7 and d14; however, on d7 CAT-1 mRNA abundance at 1.35% ARG was 2-fold higher than at 1.20% ARG (Table 7). In PBMC, CAT-1 and y^+LAT-2 mRNA had the highest abundance while y^+LAT-1 mRNA had the lowest, regardless of dietary ARG level or age (Table 7).

In thymocytes, the mRNA of ARG transporters including CAT-1, CAT-3, y^+LAT-1, y^+LAT-2, and $ATB^{0,+}$ was detected while the mRNA of CAT-2A and CAT-2B

was not detected and $b^{0,+}$AT mRNA was below the limit for accurate detection. In thymocytes, y^+LAT-2 had the highest mRNA abundance while y^+LAT-1 had the lowest mRNA abundance, regardless of dietary ARG level or age (Table 8).

Dietary ARG did not change the mRNA abundance of CAT-1, CAT-3 and $ATB^{0,+}$ in thymocytes ($P>0.05$; Table 8); however, y^+LAT-1 mRNA abundance tended ($P=0.08$) to be lower in thymocytes from chicks fed 1.20% ARG. Thymocyte CAT-1, CAT-3 and y^+LAT-1 mRNA abundance decreased 47%, 51%, and 68%, respectively, from d3 to d21 ($P<0.05$; Table 8). Thymocyte y^+LAT-2 mRNA abundance was dependent upon the dietary ARG level ($P<0.05$; Table 8). Between d3 and d14, 1.20% ARG increased thymocyte y^+LAT-2 mRNA abundance 2.5-fold ($P<0.05$) while 1.35% ARG did not increase y^+LAT-2 mRNA abundance during this time period.

2.10. Experiment 2

2.10.1. Performance

Chicks fed 1.5% ARG had 6.4% higher BW compared to chicks fed 1.1% ARG ($P<0.05$; Table 9). Chicks fed 1.1% or 1.5% ARG consumed less feed than chicks fed 1.3% ARG ($P<0.05$). Feed conversion of chicks fed 1.5% and 1.3% dietary ARG was approximately 2.4% lower than that of chicks fed 1.1% dietary ARG ($P<0.05$).

2.10.2 Peripheral Blood Mononuclear Cell Concentration

The concentration of PBMC did not change in response to dietary ARG; however, the concentration of viable PBMC increased approximately 35% from d10 to d14

(P<0.05; Table 10). The concentration of apoptotic PBMC increased 97% from 5.28 x 10^5 cells/mL to 1.04 x 10^6 cells/mL between d10 and d17 (P<0.05; Table 10). The concentration of dead PBMC increased 51% from d10 to d14 (P<0.05; Table 10).

2.10.3. Peripheral Blood Mononuclear Cell Phenotyping

There was no significant effect of dietary ARG or age on percent $CD4^+$ PBMC (P>0.05; Table 11). The ratio of $CD4^+$:$CD8^+$ cells in peripheral blood was not impacted by dietary ARG (P>0.05; Table 11). The percentage of $CD8^+$ PBMC was highest on d17, increasing 42% from levels on d14 (P<0.05; Table 11). There tended (P=0.06) to be an age by ARG interaction for the percentage of peripheral blood B cells. On d17, the percentage of B cells was numerically greater from chicks fed 1.5% ARG compared to those fed 1.1% and 1.3% ARG. The percentage of monocytes in peripheral blood at a given age was dependent upon dietary ARG (P<0.05; Figure 2). On d14, the percentage of monocytes from chicks fed 1.1% and 1.3% ARG was lower than levels from chicks fed 1.5% ARG (P<0.05), but not on d10 or d17 (P>0.05).

2.10.4. Thymocyte Concentration

The concentration of thymocytes did not depend on dietary ARG levels (P>0.05) but did differ by day posthatch (P<0.05; Table 12). From d10 to d17, the concentration of viable and dead thymocytes increased 180% and 312%, respectively, while the concentration of apoptotic thymocytes increased 125% from d14 to d17 (P<0.05), but not from d10 to d14 (P>0.05).

2.10.5. Thymocyte Proliferation

Thymocyte proliferation was dependent upon dietary ARG at d10 posthatch (Table 13). On d10, 1.5% ARG reduced thymocyte proliferation compared to 1.1% and 1.3% ARG ($P<0.05$). PHA-P addition increased thymocyte proliferation 132%, 45%, and 19% on d10, 14, and 17, respectively, compared to controls ($P<0.05$).

Discussion

Dietary nutrient requirements for growth may differ from nutrient requirements for immunity. Therefore, in the case of ARG, an immune cell substrate, dietary levels were provided at 1.1%, 1.20%, 1.3%, 1.35%, and 1.5% to determine if the resulting differences in growth rates might impact ARG use by the immune system. To assess ARG use by the immune system, the genes coordinating ARG uptake (CAT1-3, $b^{0,+}AT$, and $ATB^{0,+}$) and ARG release (y^+LAT-1/2) were measured in quiescent (PBMC) and developing immune cells (thymocytes). In addition, leukocyte numbers, function, and type were also measured in order to determine the effect of dietary ARG level on the magnitude and characterization of leukocyte pools. Taken together, the results from these studies indicate that the immune system has a number of redundant pathways for ARG acquisition and retention and that the effect of dietary ARG on immunity is both age and leukocyte dependent.

Primary and secondary lymphoid organs play a critical role in the development and function of the immune system (26). In the present study, the thymus was selected as a marker for primary immune tissue while the spleen was selected as a marker for

secondary lymphoid tissues. Thymus and spleen weights are often times measured as indicators of health and immunological stress (5). Thymic weight is assumed to relate to the magnitude of developing T cells whereas spleen weight is assumed to relate to the proliferation of immune cells within this secondary lymphoid tissue during periods of infection (27-28). Lymphoid organ weights increased with age, though at a much slower rate than pectoralis, but lymphoid organ weights were not altered by dietary ARG levels examined in these studies. Kidd et al. (4) reported that feeding ARG at levels similar to (1.48%) and above (1.68%) those used in the present studies also did not alter thymus or spleen relative body weights. Taken together, these results indicate that at the tissue level dietary ARG levels from 1.20% to 1.68% may be in excess of the requirement for maximizing lymphoid mass. However, despite no change in thymus mass, 1.20% dietary ARG resulted in the greatest thymocyte concentration while 1.1% ARG resulted in the greatest thymocyte proliferation, suggesting that current NRC requirements for ARG are sufficient for developing T cells. Nonetheless, since dietary ARG's impact at the cellular level was not identified at the tissue level, lymphoid organ weights alone may not be a sensitive indicator of dietary ARG's needs by the immune system.

PBMC include T cells, B cells, and monocytes and their measurement allows for assessment of both adaptive and innate immunity. In contrast to developing T cells, the metabolic activity of PBMC are low since these cells are in the G_0 phase of the cell cycle and are not proliferating or synthesizing effector molecules (29). In healthy animals, PBMC concentrations do not indicate infection as in sick animals, but they do indicate the type of cells available for recruitment to the spleen, lymph nodes, or site of injury to respond to an immune challenge. Additionally, leukocytes require energy and nutrients

to support basal metabolism (30), so PBMC concentration may indicate aberrations in nutrient supply. For instance, in nutrient deficiencies such as protein-energy deficiencies, PBMC concentrations decline (31). Therefore, in the present studies, though the isolation procedure may underestimate the concentration of dead PBMC, PBMC concentration was determined to analyze ARG needs for maintaining circulating leukocyte concentrations. In the present studies, PBMC concentration was determined to analyze ARG needs for maintaining circulating leukocyte concentrations. PBMC concentrations were not responsive to any dietary ARG level examined and this may be due to low rates of protein synthesis for "housekeeping" functions in these cell types (30, 32-33). Therefore, the present studies indicate that dietary ARG levels as low as 1.1%, levels well below that needed for maximum growth, may be adequate for maintaining PBMC concentrations.

In broiler chicks, ARG transporters CAT-1, CAT-3, y^+LAT-1, y^+LAT-2 and $ATB^{0,+}$ were expressed in PBMC and thymocytes, but CAT-2 and $b^{0,+}$AT were below the limit of detection. The mRNA abundance for CAT-1 and y^+LAT-2 was highest in both of these cell types, suggesting that these are the primary transporters involved in ARG utilization by PBMC and thymocytes. In mammalian leukocytes, ARG utilization by monocyte/macrophages has major implications on NO production by these cell types. In both monocyte/macrophages and endothelial cells, ARG utilization for NO production is dependent upon the induction of the low-affinity, high velocity CAT-2 transporter (15, 34). Since the CAT-2 gene is only used for ARG uptake for NO synthesis, other transport proteins are presumably responsible for ARG uptake and use for other metabolic purposes, such as protein synthesis. In the case of y^+LAT-1and y^+LAT-2, their

associated heavy chain (4F2hc) has also been found on activated peripheral blood B cells, T cells, monocytes and tissue macrophages as a surface antigen (CD98hc) and is suggested to have a role in cellular activation activities such as T cell proliferation (35) which utilizes ARG (36). Furthermore, it is suggested that CD98hc directs the light chains (y^+LAT-1/2) to the plasma membrane and may have a role in amino acid sensing (35) which may indicate a role of CD98hc in unstimulated PBMC through its amino acid transport capacity. Since ARG is an essential amino acid in aves, the redundancy of ARG transporters in leukocytes may help to ensure that these cell types obtain adequate dietary ARG.

The mRNA abundance of the majority of ARG transporters measured in both PBMC and thymocytes decreased over the first three weeks posthatch, suggesting a decreased ability to acquire cationic amino acids during this life stage. This in contrast to pectoralis, gastrocnemius, heart, and liver tissues that increased CAT-1, CAT-2, and CAT-3 mRNA abundance over the first two weeks posthatch (13). Therefore, the decrease in mRNA abundance of ARG transporters in PBMC and thymocytes and increase in ARG transporter expression in skeletal muscle may result in these immune cells being at a disadvantage for obtaining dietary ARG in the first few weeks of life. Though the increase in ARG transporter expression in skeletal muscle is important to maximize protein synthesis for growth, if it is achieved at the expense of generating an immune cell repertoire there may be important implications for immunity; however, PBMC and thymocyte concentrations in the present studies were maintained or increased over this time period suggesting these cells are able to tolerate a decreased ability to acquire ARG compared to skeletal muscle.

In the present studies, dietary ARG levels in excess of the growth requirement increased the mRNA abundance of an ARG importer (CAT-1) in PBMC's and decreased the mRNA abundance of an ARG exporter (y^+LAT-2) in thymocytes. These changes in ARG importers and exporters suggest that more ARG may be available for utilization by PBMC and thymocytes when dietary ARG levels are in excess of the growth requirement. In comparison, dietary ARG's impact on ARG transporters in mammalian leukocytes is unknown. However, it is known that general amino acid supply regulates amino acid transport (37), and that mammalian CAT-1 translation is responsive to both a deprivation of a single essential amino acid or of total amino acids (38). Therefore, it would be expected that ARG supplementation would impact ARG transport in mammalian leukocytes thereby altering ARG availability for immunity. Consequently, this hypothesis could be tested experimentally in leukocytes since ARG transporters have been extensively characterized in other mammalian tissues.

Differences in the phenotype of leukocytes in peripheral blood in response to dietary nutrients may promote specific types of immune responses. When considering dietary ARG, the percentages of monocytes and B cells were both increased by 1.5% ARG, suggesting that dietary ARG levels may bolster both innate and humoral immune responses in healthy chicks. For monocytes, the mechanism by which ARG may regulate their percentage in peripheral blood may act as monocytes are released from bone marrow (39) and mature in circulation. For B cells, since they are released from the bursa in growing chicks (40) and since ARG deficiency arrests B cells in the pro- to pre-B cell transition (8), ARG may be regulating the percentage of B cells in peripheral blood by affecting B cell maturation. In the case of vaccinated broiler chicks, dietary ARG

supplementation at 2.2% ARG compared to 1.2% ARG increased the proportion of B cells in peripheral blood 9 d after vaccination (41) supporting the idea that dietary ARG level may affect humoral immunity. Consequently, PBMC phenotype in this study indicates that dietary ARG supplementation at 1.5% increases the abundance of monocytes and B cells in peripheral blood.

T cells are crucial in coordinating immune responses so nutrient supplementation that bolsters their populations in peripheral blood may influence this function; however, when supplemented with dietary ARG in healthy chicks, the percentage of $CD4^+$ T, $CD8^+$ T cells and the ratio of CD4:CD8 cells in peripheral blood were not altered. Vaccinated broiler chicks fed 1.2% or 2.2% dietary ARG also did not alter the percentage of $CD4^+$ T cells in PBMC's; however, $CD8^+$ T cells were numerically higher in birds supplemented with 2.2% ARG compared to 1.2% ARG (41). Taken together, this indicates that peripheral blood $CD4^+$ and $CD8^+$ T cell populations in healthy chicks are not responsive to ARG. However, during an immune response dietary ARG levels may promote T helper 1 responses due to elevated $CD8^+$ T cells which could be indicated by cytokine profiles in plasma or secondary lymphoid organs.

In summary, these experiments indicate that dietary ARG requirements for growth may not be sufficient for ARG utilization by the immune system for some parameters. In the case of ARG transporter mRNA abundance and leukocyte phenotype, ARG levels above the NRC requirement may be beneficial for ARG utilization and leukocyte abundance in peripheral blood. Additionally, ARG transporter mRNA abundance indicates that the immune system has redundant pathways for ARG

acquisition and retention in both quiescent (PBMC) and proliferating (thymocytes) leukocytes.

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Table 1. Composition of diets fed to growing broiler chicks in experiment 1

	Low Dietary ARG	High Dietary ARG
Ingredient (g/kg)		
Wheat	626.8	626.8
Soybean Meal	158.6	158.6
Safflower Meal	90.0	90.0
Vegetable Oil	66.1	66.1
Dicalcium Phosphate	18.3	18.3
Calcium Carbonate	16.2	16.2
L-Lysine-HCl	5.5	5.5
Salt	3.4	3.4
DL-Methionine	3.4	3.4
Vitamin-mineral premix[1]	2.5	2.5
L-Leucine	2.0	2.0
L-Arginine-HCl	-	2.0
L-Threonine	1.8	1.8
L-Cystine	1.6	1.6
Rice Hulls	1.3	1.3
L-Valine	1.2	1.2
Copper Sulfate	1.0	1.0
L-Isoleucine	0.3	0.3
L-Tryptophan	0.01	0.01
Analyzed Composition		
ME, kcal/kg (calculated)	3058	3058
Crude protein, %	18.86	19.35
Crude fat, %	7.41	8.50
Arg, %	1.21	1.34
Lys, %	1.21	1.22
Met, %	0.61	0.58

[1] Vitamins and trace minerals were provided as described in the NRC Standard Reference Diet for Chicks (21); Vitamin-mineral premix provided in g/kg of diet: choline chloride, 0.303; folic acid, 0.0004; niacin, 0.033; pantothenic acid, 0.011; riboflavin, 0.0044; thiamine-HCl, 0.0011; pyridoxine-HCl, 0.0022; menadione, 0.001; vitamin B12, 0.000009; Cu, 0.0051; I,0.0025; Fe, 0.045; Mn, 0.066; Se, 0.0002; Zn, 0.06; Ca, 0.375; P, 0.0003; Mg, 0.0033; K, 0.0026; S, 0.026; Cl^-, 0.0015; vitamin A supplement, 0.0025; vitamin D_3 supplement, 0.000083; vitamin E supplement, 0.0138.

Table 2. Composition of diets fed to growing broiler chicks in experiment 2

	Low Dietary ARG	Adequate Dietary ARG	High Dietary ARG
Ingredient (g/kg)			
Corn	483.7	483.7	483.7
Soybean Meal	139.5	139.5	139.5
Corn Gluten Meal	113.2	113.2	113.2
Alfalfa Meal	112.4	112.4	112.4
Wheat Millrun	58.1	58.1	58.1
Vegetable Oil	45.0	45.0	45.0
Dicalcium Phosphate	16.6	16.6	16.6
Limestone	12.6	12.6	12.6
L-Arginine-HCl	-	2.5	5.0
L-Lysine-HCl	6.5	6.5	6.5
Salt	4.1	4.1	4.1
DL-Methionine	1.4	1.4	1.4
Choline Chloride	4.8	4.8	4.8
Vitamin Premix[1]	0.7	0.7	0.7
Mineral Premix[1]	0.7	0.7	0.7
L-Threonine	0.6	0.6	0.6
Analyzed Composition			
ME, kcal/kg (calculated)	3080	3080	3080
Crude protein, %	22.24	22.96	23.68
Crude fat, %	6.01	6.01	6.01
Arg, %	1.12	1.31	1.53
Lys, %	1.35	1.35	1.34
Met, %	0.50	0.51	0.51

[1] Vitamins and trace minerals were provided as described in the NRC Standard Reference Diet for Chicks (21).

Table 3. Primer sequences for quantitative real-time PCR for chicken ARG transporters [1]

Target	Primer Sequence	PCR product (bp)	Genbank Accession Number
$b^{0,+}AT$	F 5'-TGAGTGAAGGAAAGGAGAAATTAAGAC-3'	309	
	R 5'-CCAGGATGACAGAAGTGTAGGC-3'		
$ATB^{0,+}$	F 5'-CATACACACTGATGTTGGCATTAGC-3'	241	
	R 5'-AAGCAATCTGACCACGGAAGC-3'		
y^+LAT-1	F 5'-GTTGGAGCCAGAGAAGGACATC-3'	165	XM_418326
	R 5'-AAGCCAGTAGTTGAAGCAGTAGTAG-3'		
y^+LAT-2	F 5'-TTGTTCTCTTATTCTGGTTGGGATAC-3'	100	XM_001231336
	R 5'-TTGGCATAGACACAGCAATAGC-3'		
CAT-1	F 5'-ACCTGCCATCGTCATCTCCTTC-3'	252	EU360441
	R 5'-AAGTCTTCAATGTGCCCACCTATG-3'		
CAT-2A	F 5'-TGCTTTGTCTACAAGTCTTCTCG-3'	165	EU360448
	R 5'-AATGCCATAATACCAGAGATGACC-3'		
CAT-3	F 5'-CCACGGGCACCAAACAGAAG-3'	150	XM_420204
	R 5'-CAGTCAGCACCACGCAGATG-3'		
β-actin	F 5'-ACCCCTGTGATGAAACAAAACCC-3'	265	NM_205518
	R 5'-GCGAGTAACTTCCTGTAACAATGC-3'		
HPRT	F 5'-GCCAGACTTTGTTGGATTTGAAG-3'	213	NM_204848
	R 5'-AGAGTTGAAGCCTGTGAGAGATAG-3'		
TATA BP	F 5'-TTTAGCCCGATGATGCCGTATG-3'	196	NM_205103
	R 5'-CTGTGGTAAGAGTCTGTGAGTGG-3'		

[1] Abbreviations: ARG, arginine; ATB^+, Na^+-dependent amino acid transporter; bp, base pairs; CAT, cationic amino acid transporter; y^+LAT, y^+L type amino acid transporter.

Table 4. Broiler performance in response to 1.20% or 1.35% dietary ARG fed from d1 to d21 posthatch [1,2]

	1.20% ARG	1.35% ARG	SEM	P-value
Body weight, *g*	285.50	297.05	49.64	0.8699
Feed intake, *g/c*d*	37.23	37.24	0.96	0.9924
Gain, *g/c*d*	33.25	35.25	0.95	0.1668
Feed conversion, *g Feed intake/g BW gain*	1.12	1.06	0.03	0.1540

[1] Values are means ± pooled SEM (n=6). A wheat-soybean meal diet formulated according to the NRC, except for ARG was fed.
[2] Abbreviations: ARG, arginine; BW, body weight; c, chick; d, day; g, gram.

Table 5. Tissue weights from broiler chicks fed 1.20% or 1.35% dietary ARG from d1 to d21 posthatch [1,2]

	1.20% ARG				1.35% ARG					P-value		
	d3	d7	d14	d21	d3	d7	d14	d21	SEM	ARG	Day	ARG X Day
Thymus, *g*	0.077	0.184	0.546	1.091	0.066	0.208	0.497	1.213	0.062	0.6547	<0.0001	0.5657
Thymus, *%BW*	0.088	0.113	0.133	0.146	0.079	0.125	0.120	0.160	0.010	0.9024	<0.0001	0.3901
Spleen, *g*	0.067	0.166	0.378	0.811	0.062	0.181	0.396	0.866	0.033	0.3772	<0.0001	0.8379
Spleen, *%BW*	0.078	0.099	0.094	0.109	0.071	0.113	0.094	0.111	0.008	0.6461	0.0001	0.6569
Pectoralis, *g*	1.78	11.74	37.06	85.77	1.87	11.97	36.71	89.73	1.73	0.4424	<0.0001	0.5853
Pectoralis, *%BW*	2.03	6.99	9.06	11.58	2.16	7.16	8.86	11.50	0.20	0.9473	<0.0001	0.7530

[1] Values are means ± pooled SEM (n=6).
[2] Abbreviations: ARG, arginine; BW, body weight; d, day; g, gram.

Table 6. Mean cell concentrations of PBMC isolated from broiler chicks fed 1.20% or 1.35% dietary ARG [1,2]

	1.20% ARG				1.35% ARG					P-value		
	d3	d7	d14	d21	d3	d7	d14	d21	SEM	ARG	Day	ARG X Day
PBMC (10^7/mL)	1.563	3.417	3.717	4.342	1.261	3.508	3.217	3.542	0.964	0.5539	<0.05	0.9692

[1] Values are means ± pooled SEM (n=6).
[2] Abbreviations: ARG, arginine; d, day; PBMC, peripheral blood mononuclear cell.

Table 7. Mean mRNA abundance of ARG transporters relative to CAT-1 in PBMC from broiler chicks fed 1.20% or 1.35% dietary ARG [1,2]

	1.20% ARG			1.35% ARG				P-value		
	d7	d14	d21	d7	d14	d21	SEM	ARG	Day	ARG X Day
CAT-1	1.000[b]	0.230[c]	0.297[c]	2.020[a]	0.515[c]	0.264[c]	0.146	0.0013	<0.01	<0.01
CAT-3	1.192	0.171	0.210	1.379	0.366	0.129	0.228	0.5948	<0.01	0.7888
y^+LAT-1	1.179	0.023	0.018	0.922	0.010	0.017	0.289	0.6884	<0.01	0.8812
y^+LAT-2	1.114	1.388	1.763	1.024	1.897	1.239	0.260	0.8689	0.1013	0.1642
$ATB^{0,+}$	1.109	0.218	0.163	0.697	0.171	0.050	0.231	0.3161	<0.01	0.7113

[1] Values are means ± pooled SEM (n=6). Data are expressed relative to CAT-1 mRNA abundance on d7 from chicks fed 1.20% ARG. Means within a row not sharing a common superscript differ (P<0.05).

[2] Abbreviations: ARG, arginine; ATB^+, Na^+-dependent amino acid transporter; CAT, cationic amino acid transporter; d, day; y^+LAT, y^+L type amino acid transporter; PBMC, peripheral blood mononuclear cell.

Table 8. Mean mRNA abundance of ARG transporters relative to CAT-1 in thymocytes from broiler chicks fed 1.20% or 1.35% dietary ARG [1,2]

	1.20% ARG				1.35% ARG					P-value		
	d3	d7	d14	d21	d3	d7	d14	d21	SEM	ARG	Day	ARG X Day
CAT-1	1.000	0.761	0.698	0.544	0.858	0.718	0.430	0.442	0.131	0.1310	<0.01	0.8514
CAT-3	0.992	0.588	0.618	0.457	0.820	0.450	0.592	0.425	0.111	0.2363	<0.001	0.8830
y^+LAT-1	0.999	0.437	0.307	0.520	1.913	0.686	0.544	0.424	0.250	0.0767	<0.001	0.2474
y^+LAT-2	2.352^{c}	3.317^{bc}	6.054^{a}	3.129^{bc}	3.858^{bc}	4.494^{ab}	3.152^{bc}	2.660^{bc}	0.722	0.7384	0.0806	<0.05
$ATB^{0,+}$	1.626	1.136	0.772	1.737	1.815	0.479	1.298	1.651	0.538	0.8205	0.2936	0.7721

[1] Values are means ± pooled SEM (n=6). Data are expressed relative to CAT-1 mRNA abundance on d3 from chicks fed 1.20% ARG. Means within a row not sharing a common superscript differ (P<0.05).

[2] Abbreviations: ARG, arginine; ATB^+, Na^+-dependent amino acid transporter; CAT, cationic amino acid transporter; d, day; y^+LAT, y^+L type amino acid transporter.

Table 9. Broiler performance in response to 1.1%, 1.3% or 1.5% dietary ARG from d1 to d17 posthatch [1,2]

	1.1% ARG	1.3% ARG	1.5% ARG	SEM	P-value
Body weight, *g*	524.88[b]	567.00[a]	560.75[a]	9.53	<0.05
Feed Intake, *g/c*d*	37.61[b]	38.95[a]	37.59[b]	0.41	<0.05
Gain, *g/c*d*	28.37[b]	30.84[a]	30.48[a]	0.56	<0.05
Feed conversion, *g Feed intake/g BW gain*	1.33[a]	1.27[b]	1.24[b]	0.02	<0.05

[1] Values are means ± pooled SEM (n=8). Means within a row not sharing a common superscript are significantly different (P<0.05).
[2] Abbreviations: ARG, arginine; BW, body weight.

Table 10. Mean cell concentrations of PBMC isolated from broiler chicks fed 1.1%, 1.3%, or 1.5% dietary ARG on d10, 14, and 17 posthatch [1,2]

	1.1% ARG			1.3% ARG			1.5% ARG				P-value		
PBMC (10^8/mL)	d10	d14	d17	d10	d14	d17	d10	d14	d17	SEM	ARG	Day	ARG X Day
Viable	0.823	0.931	1.162	0.814	1.109	1.299	0.837	1.288	1.230	0.085	0.1115	<0.0001	0.2587
Apoptotic	0.006	0.006	0.009	0.004	0.010	0.011	0.006	0.008	0.011	0.001	0.2563	<0.0001	0.1428
Dead	0.018	0.020	0.019	0.017	0.032	0.020	0.017	0.027	0.027	0.004	0.3302	<0.05	0.2466

[1] Values are means ± pooled SEM (n=8).
[2] Abbreviations: ARG, arginine; d, day; PBMC, peripheral blood mononuclear cell.

Table 11. PBMC phenotyping of cells isolated from broiler chicks fed 1.1%, 1.3%, or 1.5% dietary ARG on d10, 14, and 17 posthatch [1,2]

	1.1% ARG			1.3% ARG			1.5% ARG				P-value		
	d10	d14	d17	d10	d14	d17	d10	d14	d17	SEM	ARG	Day	ARG X Day
%$CD4^+$	10.18	8.39	8.77	10.51	9.71	9.66	8.81	9.74	10.21	1.20	0.7569	0.8375	0.7552
%$CD8^+$	4.78	3.74	4.84	3.98	3.91	5.54	3.42	3.27	5.13	0.69	0.5992	<0.05	0.6752
CD4:CD8	2.26	2.60	2.15	3.32	2.74	1.77	2.68	3.10	2.17	0.42	0.6123	<0.05	0.4656
%B cells	2.21	5.40	2.74	4.15	2.26	2.78	2.33	5.09	6.33	1.22	0.5627	0.2026	0.0605

[1] Values represent means ± pooled SEM (n=8). PBMC phenotype was determined via flow cytometry. Lymphocyte and monocyte subpopulations were gated by forward and side-scatter characteristics and 5,000 gated events were analyzed for FITC fluorescence. The proportions of $CD4^+$, $CD8^+$, and Bu-1^+ cell populations were expressed as a percentage of gated lymphocytes.
[2] Abbreviations: ARG, arginine; PBMC, peripheral blood mononuclear cell.

Table 12. Mean cell concentrations of thymocytes isolated from broiler chicks fed 1.1%, 1.3%, or 1.5% dietary ARG on d10, 14, and 17 posthatch [1,2]

	1.1% ARG			1.3% ARG			1.5% ARG				P-value		
Thymocytes (10^9/mL)	d10	d14	d17	d10	d14	d17	d10	d14	d17	SEM	ARG	Day	ARG X Day
Viable	0.581	0.969	1.763	0.743	1.224	1.725	0.670	1.237	2.080	0.126	0.1082	<0.0001	0.4666
Apoptotic	0.010	0.008	0.028	0.009	0.011	0.023	0.008	0.012	0.019	0.002	0.5530	<0.0001	0.1321
Dead	0.052	0.113	0.245	0.072	0.141	0.221	0.051	0.137	0.254	0.018	0.7667	<0.0001	0.4811

[1] Values represent means ± pooled SEM (n=8).

[2] Abbreviations: ARG, arginine; d, day.

Table 13. Mean absorbance of thymocyte proliferation of cells isolated from broiler chicks fed 1.1%, 1.3%, or 1.5% dietary ARG on d10, 14, and 17 posthatch [1,2]

		1.1% ARG		1.3% ARG		1.5% ARG			P-value		
Day	PHA-P	-	+	-	+	-	+	SEM	ARG	PHA-P	ARG X PHA-P
10		0.273	0.662	0.269	0.633	0.221	0.472	0.035	0.0011	<0.0001	0.1091
14		0.234	0.363	0.244	0.384	0.266	0.337	0.019	0.6848	<0.0001	0.1395
17		0.231	0.290	0.242	0.297	0.240	0.263	0.016	0.5028	<0.001	0.4603

[1] Values represent means ± pooled SEM (n=8).

[2] Abbreviations: ARG, arginine; PHA-P, phytohemagglutinin.

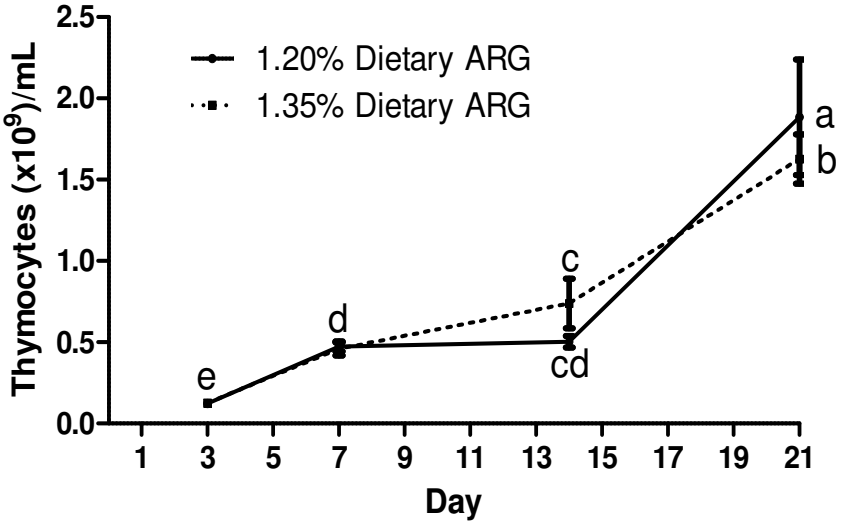
1.20% Dietary ARG
1.35% Dietary ARG
Thymocytes (x10^9)/mL
2.5
2.0
1.5
1.0
0.5
0.0
1
3
5
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Figure 1.

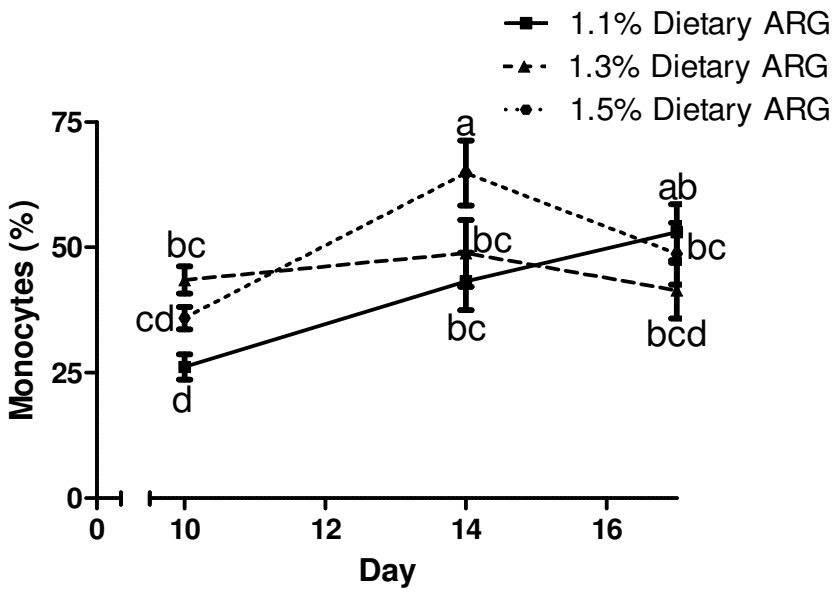
1.1% Dietary ARG
1.3% Dietary ARG
1.5% Dietary ARG
Monocytes (%)
75
50
25
0
0
10
12
14
16
Day
a
ab
bc
bc
bc
cd
bc
bcd
d

Figure 2.

Figure 1. Thymocyte concentration on d3, 7, 14, and 21 posthatch from broiler chicks fed 1.20% or 1.35% dietary ARG. Thymocytes were isolated and enumerated as described in section 2.5. Values represent means ± SEM (n=6). Means not sharing a common superscript differ ($P<0.05$). Abbreviations: ARG, arginine.

Figure 2. Peripheral blood mononuclear cell phenotyping of monocytes isolated from broiler chicks fed 1.1%, 1.3%, or 1.5% dietary ARG on d10, 14, and 17 posthatch. PBMC were isolated and phenotyped by flow cytometry as described in sections 2.3 and 2.4. Values represent means ± SEM (n=8). Means not sharing a common superscript differ ($P<0.05$). Abbreviations: ARG, arginine; PBMC, peripheral blood mononuclear cell.

CHAPTER 3: Dietary Arginine's Impact on the Acute Phase Response

Introduction

When the immune system is activated there are alterations in nutrient metabolism and nutrient partitioning (1). For instance, mounting an innate immune response such as an acute phase response (APR) initiated by gram negative bacterial lipopolysaccharide (LPS) (2) inhibits growth as the APR is nutritionally expensive due to the production of hepatic acute phase proteins and the recruitment and proliferation of immune cells (3, 4). Therefore, these alterations have implications for nutrient use, such as arginine (ARG), that are important substrates for both growth and immune functions. During the APR, ARG is metabolized by inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO) in macrophages and is necessary to support increases in immune cell proliferation (5-7). Consequently, ARG supply and utilization during the APR is of great importance to immune cells, especially avian immune cells, since aves are uricotelic and do not synthesize ARG *de novo* whereas mammalian immune cells obtain ARG from either interorgan amino acid metabolism or the diet. Since ARG and other nutrients must be obtained by leukocytes during the APR, LPS also induces immune cells to produce cytokines which alter metabolic and physiologic homeostasis (8).

The APR is generally characterized by proinflammatory cytokines (i.e. IL-1β and IL-6) (2, 9); however, many other cytokines help coordinate immune responses. Traditionally, in mammals the T_1-T_2 dichotomy in T_H cells is defined by two distinct sets of cytokines; T_{H1} subset cytokines (i.e. IL-18 and IFN-γ) which promote cell-meditated immunity and NO production in macrophages and T_{H2} subset cytokines (i.e. IL-4 and IL-13) which promote humoral immunity and antibody class switching to isotypes important

in allergy and antihelminthic responses; however, only relatively recently was this dichotomy expanded to other immune cells (T_{H3} subset cytokines (i.e. IL-10, TGF-β2, and TGF-β4)) and also identified in aves (2, 9-12). Therefore, cytokine profiles of immune tissues can be indicative of the type of immune response. In accordance with cytokine profiles, the shifting population of peripheral blood mononuclear cells (PBMC) is also indicative of the type and progression of an immune response.

PBMC include cells of both the innate (i.e. monocytes) and adaptive (i.e. B and T cells) immune system which circulate at maintenance until an immune response is provoked. During an immune challenge, PBMC are primarily recruited to lymph nodes, splenic germinal centers, and the site of infection (13). By determining the proportions of these cells in peripheral blood at different time points during an APR they can be used to characterize how nutrients such as ARG impact the APR.

Therefore, ARG's impact on the severity of the APR was evaluated by considering PBMC phenotype and splenic cytokine profiles. To study ARG's impact on the APR, broiler chicks were used as a model not confounded by *de novo* ARG synthesis allowing for dietary manipulation of ARG supply to immune cells and tissues.

Materials and Methods

3.1. Animals and Diets

Mixed sex Cobb 500 broilers were obtained from a commercial hatchery (Cedar Hatchery, Fresno, CA) and were raised in Petersime brooder batteries (Petersime Incubator Co., Gettysburg, OH) contained in an environmentally controlled room (27°C; 24 h light). Chicks were provided *ad libitum* access to water and feed. A corn-soybean

meal diet (Table 1) was formulated according to National Research Council (NRC) (14) recommendations for a young growing broiler chick, except for ARG. Experimental diets were analyzed to contain either 1.1% (low), 1.3% (adequate) or 1.5% (high) ARG. All experiments and procedures were approved by the California Polytechnic State University Animal Care and Use Committee.

3.2. Experimental Design

On d19, two chicks from four replicate pens per treatment were not injected (control) or injected subcutaneously with *Salmonella typhimurium* lipopolysaccharide (1 mg/kg BW; LPS; Sigma, St. Louis, MO; #L7261). At 2 h and 24 h post-injection, one chick per pen was euthanized by cervical dislocation for blood and organ collection. Whole blood from one chick per pen was collected into a heparinized syringe (Hospira, Inc., Lake Forrest, IL; #0409-1402-31) to isolate PBMC for phenotyping. Spleen from one chick per replicate pen was removed and frozen between aluminum plates in liquid N and stored at -80°C until further analysis. At 2 h and 24 h post-injection, pen weights of LPS injected chicks were measured.

3.3. Peripheral Blood Mononuclear Cell Isolation

PBMC were isolated from approximately 3 mL of whole blood. Whole blood was overlaid onto Histopaque-1077 (Sigma-Aldrich, St. Louis, MO; #10771) and centrifuged at 350 x g for 15 min at 25°C. The buffy coat was reconstituted in RPMI 1640 (MP Biomedicals, LLC, Solon, OH; #1260354) and centrifuged at 500 x g for 5 min at 25°C. The pelleted cells were re-suspended in 1 mL RPMI 1640 and were enumerated with

ViaCount Reagent (Guava Technologies, Inc, Hayward, CA) per the manufacturer's instructions.

3.4. Peripheral Blood Mononuclear Cell Phenotyping

PBMC phenotype was determined via flow cytometry using the EasyCyte Plus System (Guava Technologies, Inc, Hayward, CA) with a 488 nm argon laser. FITC-conjugated mouse anti-chicken CD4, PE-conjugated mouse anti-chicken CD8α, FITC-conjugated mouse anti-chicken Bu-1, and PE-conjugated mouse anti-chicken KUL01 (SouthernBiotech, Birmingham, AL; #8210-02, 8220-09, 8395-02, 8420-09) were used as markers for $CD4^+$, $CD8\alpha^+$, B cells, and monocytes, respectively. Approximately 10^6 cells were incubated with 0.2 µg (PE) or 1 µg (FITC) antibody for 30 min at 4℃. Samples were washed by centrifugation with wash buffer (250 µL PBS, 2% BSA, 0.1% NaN_3) at 4℃ for 5 min at 500 x g. The cells were then re-suspended in wash buffer and diluted 1:10 for analysis. Lymphocyte and monocyte subpopulations were gated by forward and side-scatter characteristics and 5000 gated events were analyzed for FITC or PE fluorescence. The proportions of $CD4^+$, $CD8^+$, $Bu\text{-}1^+$, and $KUL01^+$ cell populations were expressed as a percentage of gated lymphocytes or gated monocytes, as appropriate.

3.5. Total RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from spleen samples using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA; #740955.50) per the manufacturer's instructions. Samples were homogenized with a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA). Optical density at 260 nm was used to quantify RNA concentrations.

Spleen total RNA (1 μg) was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad, Hercules, CA; #170-8891) per the manufacturer's protocol. Quantitative real-time PCR analysis of target gene mRNA abundance was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions utilized Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA; #4385610), 1 μL of RT product, and 10 μmol/L of each primer (Table 2). Thermal cycling parameters were 1 cycle of enzyme activation at 95°C for 20 s and 40 cycles of denaturing at 95°C for 3 s and annealing and extending at 60°C for 30 s. Melting curve analysis was performed after 40 cycles to confirm product specificity such that melt-curve analyses containing a specific product peak and primer dimer peak were excluded from analysis and considered to be below the limit for accurate detection. Melting curve analysis was performed at 95°C for 15 s, 60°C for 60 s, followed by a linear temperature increase of 0.5°C/s to 95°C while continuously monitoring fluorescence. The change in sample mRNA abundance was calculated using the Δ-Δ equation with modifications as previously described (15). Sample PCR amplification efficiencies were determined in the log-linear phase with LinRegPCR program (16). Target gene mRNA abundance was normalized by geometric averaging of raw non-normalized values of β-actin, HPRT, and GAPDH using geNorm software (17). Data are presented as the normalized fold-change in mRNA abundance relative to 1.30% ARG at 2 h post-injection from uninjected chicks.

3.6. Statistical Analysis

Dependent variables were analyzed by general linear model (JMP Software, SAS Inc, Cary, NC) using either a one-way or two-way analysis of variance (ANOVA). A

one-way ANOVA was used to determine the effect of dietary ARG on rate of gain, feed intake, and feed conversion. A two-way ANOVA was used to determine the main effects of dietary ARG level, treatment with LPS, and their interaction on feed intake, PBMC cell concentrations, PBMC phenotype, and T_H subset cytokine mRNA abundance. When main effects or their interactions were significant ($P<0.05$), means were compared by student's T pairwise comparisons. PBMC phenotyping data was transformed using arcsine in order to meet conditions for ANOVA. Data are reported as nontransformed means and pooled standard errors.

Results

3.7. Performance

Feed intake and gain did not differ between dietary ARG levels ($P>0.05$; Tables 3 and 4). However, dietary ARG tended to impact feed conversion from 0-2 h ($P=0.06$; Table 4). Also, from 0-2 h, LPS decreased feed intake by 60% compared to controls ($P<0.05$; Table 3), but feed intake was similar to controls from 2-24 h post-LPS injection ($P>0.05$; Table 3).

3.8. Peripheral Blood Mononuclear Cell Concentration

The concentration of PBMC after LPS injection was not impacted by dietary ARG ($P>0.05$; Table 5). The concentration of viable PBMC at 2 h was 36% lower in chicks injected with LPS compared to controls ($P<0.05$). At 24 h post injection, the concentration of dead PBMC was 54% higher in LPS injected chicks ($P<0.05$). At both 2

and 24 h post injection, the concentration of apoptotic PBMC did not differ (P>0.05) in response to LPS injection.

3.9. Peripheral Blood Mononuclear Cell Phenotyping

LPS injection did not alter the percentage of $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes, and the ratio of $CD4^+$:$CD8^+$ T cells in peripheral blood in response to dietary ARG (P>0.05; Table 6). LPS decreased peripheral blood $CD4^+$ T cells, $CD8^+$ T cells, B cells, and monocytes by 46%, 52% 38%, and 82%, respectively, at 2 h post-injection (P<0.05; Figure 1), but this decrease was no longer evident at 24 h post injection (P>0.05; Table 6). LPS did not impact the ratio of $CD4^+$:$CD8^+$ T cells (P>0.05; Table 6).

3.10. Cytokine mRNA Abundance

Dietary ARG did not alter the mRNA abundance of IL-1β, IL-6, IL-18, IFN-γ, TGF-β2, and TGF-β4 after LPS injection at either 2 or 24 h (P>0.05; Table 7). At 2 h post injection, LPS administration increased IL-1β, IL-6 IL-18, IFN-γ, and TGF-β4 mRNA abundance (P<0.05), but this did not persist at 24 h (P>0.05) except in the case of IL-18 mRNA abundance (P<0.05). There tended (P=0.08) to be a LPS by ARG interaction for the mRNA abundance of TGF-β4 at 2 h post LPS administration. IL-4, IL-10, and IL-13 mRNA abundance was low or not detectable at both 2 h and 24 h post LPS administration as it was below the level needed for accurate quantification.

Discussion

Activation of the immune system, such as during the APR, results in changes in nutrient metabolism (1). Of the many nutrients metabolized by leukocytes during the APR, ARG is of great importance in NO production and leukocyte proliferation. Therefore, dietary ARG levels were provided at 1.1%, 1.3%, and 1.5% to determine if ARG impacts the severity of the APR initiated by *Salmonella typhimurium* LPS injection. To assess ARG's impact on the APR, PBMC concentration and phenotype as well as splenic cytokine profiles were determined. Taken together, the results from this study indicate that LPS injection results in a rapid APR that may be lessened in severity through dietary ARG's affect on TGF-β4 mRNA abundance.

During an immune response in growing animals, the decrease in feed intake accounts for most of the decline in growth rate while inefficient nutrient metabolism accounts for the remainder of the decline (1). Therefore, nutrient supplementation that alleviates this decline in growth is of great interest. In the present study, LPS injection resulted in decreased feed intake typical of the APR (8, 18-21), but the decrease was not mitigated by dietary ARG level. Similarly, Takahashi et al. (22) found that dietary ARG levels above (2.44%) and below (0.65%) those in this study did not impact feed intake following LPS injection. Taken together, this indicates that manipulation of dietary ARG level is unable to alleviate anorexia induced by the APR. However, early on in the APR, dietary ARG tended to offset negative feed conversion. Still, feed conversion is calculated from feed intake and gain and only accounts for weight loss attributed to decreased feed intake. Therefore, further studies are necessary to determine if this trend

has biological meaning concerning dietary ARG's impact on the growth depression associated with the APR.

Alteration of peripheral blood leukocyte concentration is often used as an indicator of health status in humans and animals (23). In the present study, the rapid change in concentration of viable and dead PBMC indicates that the immune system is reacting to the LPS injection and indicates that PBMC are quickly recruited from blood to the spleen or site of infection. Additionally, PBMC metabolize nutrients at a basal metabolic rate until recruitment and activation when their rate of metabolism and demand for nutrients increases (1). For instance, during the APR, demand increases for some amino acids (i.e. phenylalanine, threonine, cysteine) presumably due to acute phase protein synthesis, leukocyte proliferation, and mitigation of body nitrogen loss due to skeletal muscle catabolism (24-26). Therefore, since ARG is an amino acid metabolized by leukocytes, dietary ARG's impact on PBMC concentration was utilized to indicate if ARG reduces the severity of the APR by maintaining the concentration of PBMC. However, in the present study, dietary ARG did not impact PBMC concentration. Therefore, ARG may not reduce the severity of the APR by maintaining the concentration of PBMC.

In agreement with PBMC concentration, the percentages of PBMC subpopulations decreased in response to LPS injection, but were not impacted by dietary ARG as they were in healthy chicks in which peripheral blood monocyte and B cell populations were increased by 1.5% dietary ARG (D'Amato and Humphrey, unpublished). This differential affect of ARG may occur because of differences in nutrient priority in healthy chicks verses those mounting the APR. In healthy chicks,

ARG may be directed toward regulating the percentage of monocytes and B cells in peripheral blood during maturation and as they are released from bone marrow or bursa, respectively (27, 28) whereas in chicks mounting an APR, ARG may instead be directed toward production of effector molecules (29). In either case, ARG utilization by leukocytes is directed by cytokines.

LPS injection transiently increases splenic proinflammatory and T_{H1}, but not T_{H2}, subset cytokine mRNA abundance. The increase in proinflammatory and T_{H1} subset cytokines in response to inflammatory stimuli has been previously reported (3, 20, 30, 31). Since proinflammatory cytokines are in part responsible for the decline in growth associated with the APR (1), dietary ARG may impact splenic cytokine profiles and mediate this decline. However, in the present study, dietary ARG did not alter the mRNA abundance of proinflammatory cytokines. On the other hand, 1.3% dietary ARG did tend to increase the expression of TGF-β4 mRNA in LPS-injected chicks. TGF-β4 is the most commonly expressed TGF-β isoform in chickens (31) which may explain why this trend was only observed in TGF-β4 and not TGF-β2 mRNA abundance. TGF-β4 is anti-inflammatory and immunosuppressive on activated lymphocytes and monocytes/macrophages (2) and is important in suppression of the APR to avoid excessive inflammation. Since ARG tends to impact TGF-β4, dietary ARG supplementation may decrease the severity of the APR and alleviate its detriment to growth.

In summary, PBMC concentration and phenotype and splenic cytokine production demonstrated that lymphocyte and monocyte populations decreased rapidly following LPS injection suggesting emigration to the site of infection or secondary lymphoid

tissues. Also, since adequate dietary ARG levels tended to alleviate negative feed conversion and increase the splenic mRNA abundance of the anti-inflammatory cytokine, TGF-β4, dietary ARG supplementation may lessen the severity of the APR.

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Table 1. Composition of diets fed to growing broiler chicks

	Low Dietary ARG	Adequate Dietary ARG	High Dietary ARG
Ingredient (g/kg)			
Corn	483.7	483.7	483.7
Soybean Meal	139.5	139.5	139.5
Corn Gluten Meal	113.2	113.2	113.2
Alfalfa Meal	112.4	112.4	112.4
Wheat Millrun	58.1	58.1	58.1
Vegetable Oil	45.0	45.0	45.0
Dicalcium Phosphate	16.6	16.6	16.6
Limestone	12.6	12.6	12.6
L-Arginine-HCl	-	2.5	5.0
L-Lysine-HCl	6.5	6.5	6.5
Salt	4.1	4.1	4.1
DL-Methionine	1.4	1.4	1.4
Choline Chloride	4.8	4.8	4.8
Vitamin Premix[1]	0.7	0.7	0.7
Mineral Premix[1]	0.7	0.7	0.7
L-Threonine	0.6	0.6	0.6
Analyzed Composition			
ME, kcal/kg (calculated)	3080	3080	3080
Crude protein, %	22.24	22.96	23.68
Crude fat, %	6.01	6.01	6.01
Arg, %	1.12	1.31	1.53
Lys, %	1.35	1.35	1.34
Met, %	0.50	0.51	0.51

[1] Vitamins and trace minerals were provided as described in the NRC Standard Reference Diet for Chicks (14).

Table 2. Primer sequences for quantitative real-time PCR for chicken cytokines [1,2]

Target	Primer Sequence	PCR product (bp)	Genbank Accession Number
IL-1β	F 5'-CACCAACCAACCCGACCAG-3'	214	NM_204524
	R 5'-GCAACGGGACGGTAATGAAAC-3'		
IL-4	F 5'-AGACAAATAACAAAACTGAGC-3'	230	NM_001007079
	R 5'-TTGGTGGAAGAAGGTACG-3'		
IL-6	F 5'-GAAGTGGTCATCCCAGACTCG-3'	91	AJ309540
	R 5'-GGTGCATGGTGATTTTCTCTATCC-3'		
IL-10	F 5'-CATGCTGCTGGGCCTGAA-3'	448	AJ621614
	R 5'-CGTCTCCTTGATCTGCTTGATG-3'		
IL-13	F 5'-CACCCAGGGCATCCAGAA-3'	593	AJ621735
	R 5'-TCCGATCCTTGAAAGCCACTT-3'		
IL-18	F 5'-AGGTGAAATCTGGCAGTGGAAT-3'	94	AJ277865
	R 5'-ACCTGGACGCTGAATGCAA-3'		
IFN-γ	F 5'-GTGAAGAAGGTGAAAGATATCATGGA-3'	712	Y07922
	R 5'-GCTTTGCGCTGGATTCTCA-3'		
TGF-β2	F 5'-CCCTCCTACAGACTTGAGTCG-3'	84	NM_001031045
	R 5'-CTGCACATTCCTAAAACAATAGGC-3'		
TGF-β4	F 5'-AGGATCTGCAGTGGAAGTGGAT-3'	137	M31160
	R 5'-CCCCGGGTTGTGTTGGT-3'		
β-actin	F 5'-ACCCCTGTGATGAAACAAAACCC-3'	265	NM_205518
	R 5'-GCGAGTAACTTCCTGTAACAATGC-3'		
HPRT	F 5'-GCCAGACTTTGTTGGATTTGAAG-3'	213	NM_204848
	R 5'-AGAGTTGAAGCCTGTGAGAGATAG-3'		
GAPDH	F 5'-GGTGCTGAGTATGTTGTGGAGTC-3'	290	NM_204305
	R 5'-GTCTTCTGTGTGGCTGTGATGG-3'		

[1] Abbreviations: bp, base pairs; IL, interleukin; IFN, interferon; TGF, transforming growth factor.
[2] Published primers were used for IL-10 (32), IL-13 (10), IL-18 (33), IFN-γ (32), TGF-β4 (32).

Table 3. Feed intake of LPS injected chicks fed 1.1%, 1.3%, or 1.5% dietary ARG [1,2]

Feed Intake, *g*	1.1% ARG		1.3% ARG		1.5% ARG			P-value		
Time, *h*	- LPS	+ LPS	- LPS	+ LPS	- LPS	+ LPS	SEM	ARG	LPS	ARG X LPS
0 – 2	3.46	2.00	2.50	1.13	5.25	1.38	0.72	0.1584	<0.01	0.1706
2 – 24	50.88	51.50	58.75	45.00	59.00	55.75	8.02	0.6890	0.4006	0.6568
0 – 24	54.33	53.50	61.25	46.13	64.25	57.13	7.78	0.5831	0.2267	0.6611

[1] Values are means ± pooled SEM (n=4).

[2] Abbreviations: ARG, arginine; g, gram; h, hour; LPS, lipopolysaccharide.

Table 4. Performance at 2 h and 24 h post LPS injection in chicks fed either 1.1%, 1.3%, or 1.5% dietary ARG [1,2]

	1.1% ARG	1.3% ARG	1.5% ARG	SEM	P-value
Gain, *g/c*d*					
0 – 2 *h*	-8.00	-14.00	-11.75	2.14	0.1901
0 – 24 *h*	0.25	-8.50	12.50	8.87	0.2926
Feed conversion, *g Feed intake/g BW gain*					
0 – 2 *h*	-0.28	-0.08	-0.12	0.05	0.0570
0 – 24 *h*	0.30	-8.68	2.18	10.0	0.7224

[1] Values are means ± pooled SEM (n=4).

[2] Abbreviations: ARG, arginine; BW, body weight; c, chick; d, day; g, gram; LPS, lipopolysaccharide.

Table 5. Mean cell concentration of PBMC isolated from chicks fed 1.1%, 1.3%, or 1.5% dietary ARG at 2 h or 24 h post LPS injection [1,2]

		1.1% ARG		1.3% ARG		1.5% ARG			P-value		
PBMC (10^8/mL)	Time, *h*	- LPS	+ LPS	- LPS	+ LPS	- LPS	+ LPS	SEM	ARG	LPS	ARG X LPS
Viable											
	2	1.427	0.789	1.167	0.964	1.364	0.797	0.180	0.9719	<0.01	0.4476
	24	1.131	0.948	1.242	0.932	1.035	0.944	0.202	0.8827	0.2317	0.8633
Apoptotic											
	2	0.011	0.016	0.010	0.009	0.012	0.010	0.002	0.2431	0.6515	0.1828
	24	0.007	0.011	0.011	0.011	0.007	0.012	0.003	0.7875	0.2583	0.6886
Dead											
	2	0.022	0.037	0.027	0.026	0.028	0.023	0.007	0.8310	0.6525	0.3318
	24	0.017	0.022	0.017	0.042	0.015	0.043	0.010	0.5199	<0.05	0.4615

[1] Values are means ± pooled SEM (n=4).
[2] Abbreviations: ARG, arginine; h, hour; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell.

Table 6. PBMC phenotyping of cells isolated from chicks fed 1.1%, 1.3%, or 1.5% dietary ARG at 2 h and 24 h post LPS injection [1,2]

			1.1% ARG		1.3% ARG		1.5% ARG			P-value		
	Time, *h*	LPS	-	+	-	+	-	+	SEM	ARG	LPS	ARG X LPS
%$CD4^+$												
	2		11.71	3.92	12.89	7.29	8.64	6.16	1.33	0.2255	<0.01	0.1789
	24		12.66	10.87	10.54	10.21	10.69	9.98	1.04	0.2763	0.2314	0.7940
%$CD8^+$												
	2		5.26	2.25	5.42	2.43	3.99	2.29	0.76	0.6804	<0.01	0.6453
	24		4.04	5.71	3.92	3.19	4.33	2.81	0.79	0.2681	0.5561	0.1635
CD4:CD8												
	2		2.52	1.95	2.40	3.20	2.32	2.79	0.33	0.2970	0.4340	0.1336
	24		3.32	2.44	2.93	3.32	2.50	3.74	0.48	0.8638	0.5562	0.1122
%B cells												
	2		4.07	1.83	5.52	1.66	2.48	2.75	1.11	0.8556	<0.05	0.1263
	24		4.42	3.64	3.92	3.82	7.23	2.04	1.43	0.9756	0.0708	0.1402
%Monocytes												
	2		42.87	3.07	43.72	15.38	36.71	3.87	7.84	0.4056	<0.01	0.6626
	24		53.26	42.41	47.07	46.65	34.02	29.69	9.43	0.1816	0.4606	0.9060

[1] Values represent means ± pooled SEM (n=4).

[2] Abbreviations: ARG, arginine; h, hour; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell.

Table 7. Mean mRNA abundance of cytokines from spleens of chicks fed 1.1%, 1.3%, or 1.5% dietary at 2 and 24 h post LPS administration [1,2]

		1.1% ARG		1.3% ARG		1.5% ARG			P-value		
Gene	Time, *h*	- LPS	+ LPS	- LPS	+ LPS	- LPS	+ LPS	SEM	ARG	LPS	ARG X LPS
IL-1β											
	2	0.68	4.28	1.00	9.53	0.34	10.27	2.88	0.5302	<0.01	0.5262
	24	0.96	1.71	0.92	1.20	1.06	1.72	0.51	0.7636	0.1676	0.8861
IL-6											
	2	0.55	15.91	1.00	56.49	0.40	73.20	22.86	0.4445	<0.05	0.4517
	24	1.04	1.24	0.94	0.53	0.96	1.16	0.57	0.7398	0.9905	0.8266
IL-18											
	2	1.68	15.81	1.00	18.18	1.18	21.12	3.26	0.7450	<0.01	0.6770
	24	1.29	3.24	2.56	4.07	2.09	2.70	0.74	0.3016	0.0317	0.6574
IFN-γ											
	2	1.49	35.74	1.00	49.28	9.73	102.69	21.05	0.1895	<0.01	0.3670
	24	0.69	2.02	0.94	2.06	1.80	1.43	0.63	0.9172	0.1957	0.3592
TGF-β2											
	2	0.77	0.69	1.00	0.82	0.81	0.89	0.26	0.7702	0.7525	0.8846
	24	0.89	1.75	0.87	0.68	1.53	1.01	0.53	0.5346	0.9036	0.4152
TGF-β4											
	2	0.94	2.17	1.00	6.95	1.40	2.39	1.17	0.1480	<0.05	0.0848
	24	1.36	1.05	1.08	1.25	1.40	0.71	0.34	0.9014	0.3263	0.4551

[1] Values represent means ± pooled SEM (n=4). For each gene, data are presented as the normalized fold-change in mRNA abundance relative to 1.30% ARG at 2 h post-injection from un-injected chicks.

[2] Abbreviations: ARG, arginine; h, hour; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; TGF, transforming growth factor.

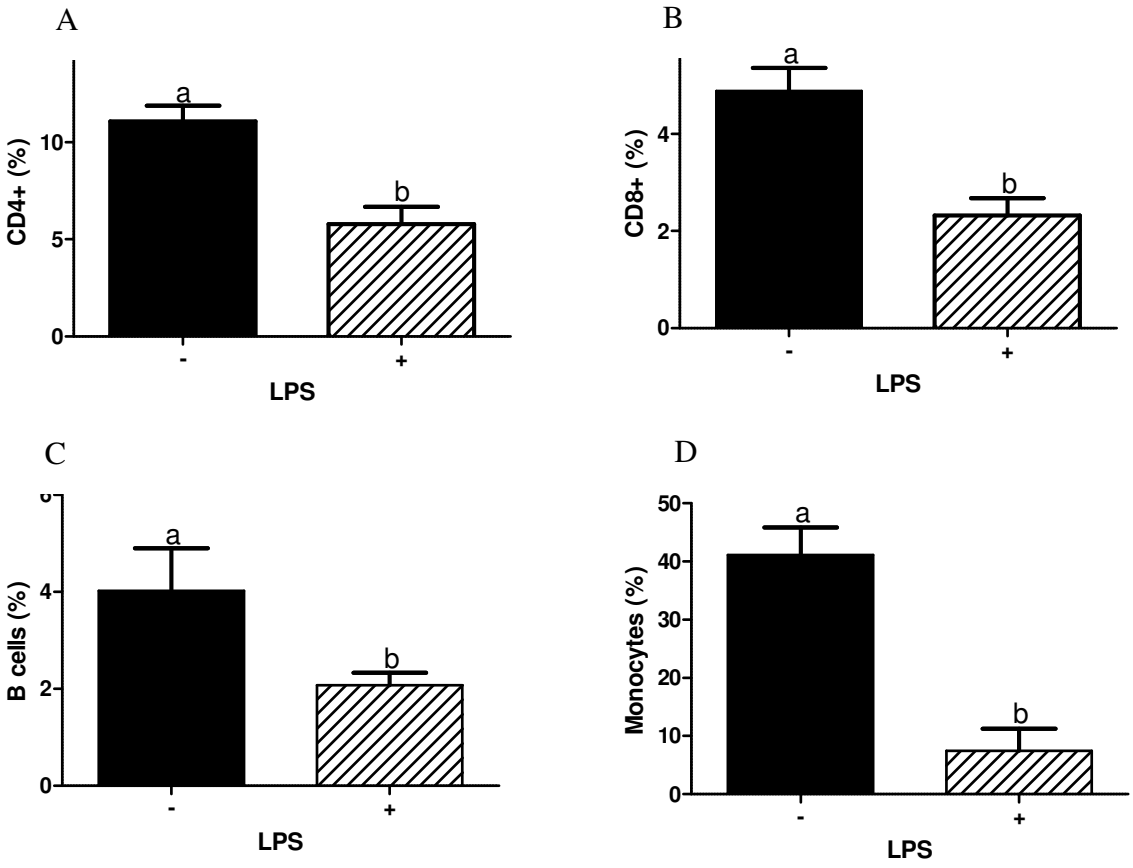
A
CD4+ (%)
10
5
0
a
b
-
+
LPS
B
CD8+ (%)
4
2
0
a
b
-
+
LPS
C
B cells (%)
4
2
0
a
b
-
+
LPS
D
Monocytes (%)
50
40
30
20
10
0
a
b
-
+
LPS

Figure 1.

Figure 1. PBMC phenotyping of A) $CD4^+$ T cells, B) $CD8^+$ T cells, C) B cells, and D) monocytes isolated from broiler chicks fed 1.1%, 1.3%, or 1.5% dietary ARG at 2 h post LPS administration. PBMC were isolated, enumerated, and phenotyped as described in sections 3.3 and 3.4. Values represent means ± SEM (n=4). Means not sharing a common superscript differ ($P<0.05$). Abbreviations: ARG, arginine; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell.

CHAPTER 4

Conclusion

4.1. Summary

In the present studies, broiler chicks served as an animal model not confounded by *de novo* ARG synthesis. These studies suggest that the optimal level of dietary ARG supplementation for utilization by genes coordinating ARG uptake and release in leukocytes is higher than what is sufficient for growth and is both age and leukocyte dependent. These studies also suggest that the level of dietary ARG supplementation to manipulate leukocyte phenotype in peripheral blood is also higher than what is sufficient for growth. Additionally, ARG transporter mRNA abundance indicates that the immune system has redundant pathways for ARG acquisition and retention in both quiescent (PBMC) and proliferating (thymocytes) cells. In PBMC, ARG importer (CAT-1) mRNA abundance on d7 posthatch was increased with 1.35% dietary ARG compared to 1.20% dietary ARG. In thymocytes, ARG exporter (y^+LAT-2) mRNA abundance was decreased with 1.35% dietary ARG compared to 1.20% dietary ARG. When considering leukocyte phenotype, the percentage of B cells and monocytes in peripheral blood were higher from chicks fed 1.5% ARG than from those fed 1.1% and 1.3% ARG on d17 and d14 posthatch, respectively. Also, the present studies have shown that dietary ARG can be utilized to manipulate the severity of the APR. Adequate dietary ARG levels tended to alleviate negative feed conversion and increase the splenic mRNA abundance of the anti-inflammatory cytokine, TGF-β4.

4.2. Future Studies

Many additional studies should be performed to further explore the impact of dietary ARG supplementation on ARG utilization for immunity. First, the functional response of PBMC to dietary ARG levels should be evaluated. For instance, monocyte/macrophage phagocytosis and/or NO production in cells isolated from peripheral blood of chicks fed different dietary ARG levels will demonstrate if dietary ARG impacts their function and taken together with the changes in ARG transporter mRNA abundance in the present studies it will help further our knowledge concerning ARG utilization by leukocytes.

Second, to compliment evaluation of ARG's impact on NO production, experiments that examine the ARG metabolizing enzymes, iNOS and arginase, in leukocytes should be carried out to further understand how dietary ARG level impacts ARG utilization in immune cells. The mRNA abundance or the protein abundance of these enzymes in PBMC and thymocytes could be determined by quantitative real-time PCR or Western blot, respectively. However, to examine the respective proteins by western blot, antibodies for these enzymes in broiler chicks would need to be manufactured. Alternatively, iNOS and arginase activities could be examined with commercially available kits.

Third, to extend the thymocyte ARG transporter and proliferation data obtained in these studies, an additional experiment examining recent thymic emigrants in peripheral blood of chicks fed different dietary ARG levels could be performed. This would indicate if ARG's impact on developing cells from the thymus has implications for T cell

populations in peripheral blood and could be easily performed via flow cytometry by labeling peripheral blood T cells with a commercially available antibody for ChT1 which is present on recent thymic emigrants (1).

Fourth, an experiment that would be quite interesting would be to attempt to determine dietary ARG's impact on the T cell receptor (TCR) complex in aves in comparison to findings in mammalian T cells in which ARG impacts expression of the primary signal transduction element (CD3ζ chain) of the TCR complex (2). This would provide information pertaining to ARG's impact on T cell signaling and cell-mediated immunity.

Finally, additional studies concerning dietary ARG's impact on ARG utilization for immunity under periods of infection should be considered. Dietary ARG's impact on the severity of the APR should be considered in a much larger study that characterizes cytokine profiles in additional cells and tissues.

4.3. Implications

The present studies have indicated that dietary ARG levels for growth are not necessarily appropriate for ARG utilization by leukocytes. This indicates that understanding nutrient supplementation for immunocompetence in addition to nutrient supplementation for growth has important implications for animal nutrition and dietary recommendations. Additionally, since ARG is an immunomodulator as a substrate utilized by leukocytes, real-time adjustments of dietary ARG levels may eventually be used to manipulate an immune response in a particular situation.

References

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