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# Cellular Responses in Rous Sarcoma in Arkansas Rous Sarcoma Regressor and Progressor Chickens

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Cellular Responses to Rous Sarcoma in Arkansas Rous Sarcoma Regressor and Progressor  
Chickens

An Undergraduate Honors Thesis

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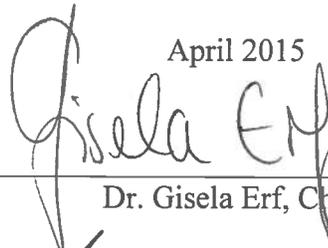
Dale Bumpers College of Agricultural, Food & Life Sciences

Submitted in partial fulfillment of the requirements for the  
University of Arkansas  
Dale Bumpers College of Agricultural, Food and Life Sciences  
Honors Program

by

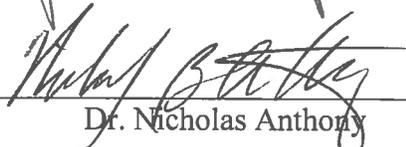
Kallie Alyce Sullivan

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Dr. Gisela Erf, Chair



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Dr. Nicholas Anthony



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Dr. Charles Rosenkrans

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## Abstract

The nature of the cellular immune response to tumors, such as sarcomas is not well understood. The Arkansas Progressor (AP) and Regressor (AR) chickens maintained at the University of Arkansas offer unique opportunity to examine anti-tumor immune responses. When injected into the wing web with Rous sarcoma virus (RSV), chickens from both lines develop tumors which continue to grow rapidly in AP chickens or regress in AR chickens to the point of elimination. Little is known concerning the nature of the cellular responses that allow the tumor to escape the immune system in AP chickens versus the effective anti-tumor response in AR chickens. Using the growing feather (GF) tissue as a test-site (Erf-US patent U.S. Patent No 8,216,551), the objective of this study was to monitor cellular immune response to tumor lysates in vivo in the AP and AR chicken model. Tumor lysates were prepared from a progressing AP and a regressing AR tumor. Lysates were injected into GF (18 GF/bird) of AP and AR chickens that were not injected with RSV (unsensitized groups), and in AP and AR chickens with actively progressing or regressing sarcomas (sensitized groups), respectively (6 birds/group). Injected GF were collected before (0), and 1, 2, 3, 5, and 7 d post GF-injection for phenotypic cell population analysis and relative quantitation of responding immune cells. For this, GF pulp cell suspensions were prepared and immunofluorescently stained using a panel of chicken leukocyte-specific fluorescently labeled monoclonal antibodies. The proportions of various leukocytes infiltrating the pulp were determined by flow cytometry. Within the AP and AR groups of chickens, few temporal, qualitative and quantitative differences in the cellular response were observed between unsensitized and sensitized groups. This would suggest a relatively weak protective memory response in both lines. However, side by side comparison of the cellular infiltration response in AP versus AR chickens points toward a fast and more sustained cell-mediated anti-tumor effector response in AR compared to AP chickens. The response appears to be ahead of the tumor in AR chickens and is lagging behind in the AP chickens, resulting in regression and continued growth of the tumors, respectively. Future studies on functional activities of the tumor infiltrating cells will provide important insight into effective and ineffective immune responses to tumors and how tumors evade the immune response.

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## **List of Abbreviations**

AP	Arkansas Progressor Chickens
AR	Arkansas Regressor Chickens
CTL	Cytotoxic Lymphocyte
FSC	Forward Scatter (Size)
GF	Growing Feather
MHC	Major Histocompatibility Complex
NK	Natural Killer Cell
PBS	Phosphate Buffered Saline
RSV	Rous Sarcoma Virus
S-AP	Sensitized Arkansas Progressor
S-AR	Sensitized Arkansas Regressor
SSC	Side Scatter (Complexity)
TCR	T Cell Receptor
U-AP	Unsensitized Arkansas Progressor
U-AR	Unsensitized Arkansas Regressor
v-src	Viral-src

## **Introduction**

Most vertebrate animals are equipped with two types of immune systems that defend against harmful microbes: innate and adaptive immunity. Innate immunity is the body's first and earliest line of defense against infections. Adaptive immunity is activated later and acts against pathogens that resist innate immunity. T and B lymphocytes are the cells of adaptive immunity which are able to focus on small molecular features of antigens using highly specific antigen receptors. Adaptive immunity takes 7-14 days to develop during a first encounter with a pathogen (antigen), but the responses are highly specialized to most effectively eliminate the antigen. Once an adaptive immune response developed, adaptive immunity has the ability to respond to a previously encountered antigen and react faster, more strongly, more effectively and with more specificity than the response following initial exposure. This feature of adaptive immunity is called memory (Abbas et al. 2015). Adaptive immunity includes two types: humoral immunity mediated by B-lymphocytes, and cell-mediated immunity mediated by T-lymphocytes. Both innate and adaptive immunity involve a complex system of numerous cells and cellular responses to pathogens and cellular abnormalities, such as cancer cells.

Cancer is a highly prevalent problem worldwide and presents a significant challenge to the immune system of all mammals and vertebrates. Malignant tumors are especially lethal, with an uncontrolled growth that metastasizes throughout the body and challenges the host. Malignant phenotypes of cancers have the ability to evade and challenge cellular mechanisms such as regulation of cell proliferation, resistance of tumor cells to apoptotic death, ability of tumor cells to invade host tissues and metastasize to different sites, and evade the host immune defense

mechanisms (Abbas et al., 2015). A significant portion of research in the immunology field is dedicated to tumor immunology and to understanding the complexity of the pathways of its development and complete invasion of a host's system.

The tumor cells are capable of activating immune responses, specifically within in the adaptive immunity (Abbas et al., 2015). Histopathology studies give insight to the specific cellular responses to tumor cells, showing that tumors are surrounded by T lymphocytes (T cells), natural killer (NK) cells, and macrophages. T cells are the key players in cell-mediated immunity as they act against viruses and bacteria that are generated inside cells or have been taken up by cells through phagocytosis and therefore are inaccessible to antibodies. Antibodies are produced by B-lymphocytes (B cells) and are able to help in the elimination of extra-cellular antigens during humoral responses. Natural killer cells are specialized innate immunity cells that act in antiviral defense. Macrophages and neutrophils (called heterophils in birds) are involved in phagocytizing microbes and killing them by the activation of phagolysosomes.

Unfortunately, immune responses often fail to prevent the growth and spread of tumors throughout the system, which maintains the urgency for continued research in tumor immunology. Immune responses often fail due to the weak immunogenicity of tumor antigens and the immunosuppressive effects tumor cells tend to have on the immune system (Abbas et al., 2015).

The Arkansas Rous sarcoma Regressor (AR) and Progressor (AP) lines of chickens are an excellent animal model system for the study of natural regression and progression of tumors, respectively. Additionally, in the chicken model we can examine cellular immune responses to tumor-associated antigens *in vivo*, using the dermis of growing feathers as a tissue test-site. My research is a novel study examining the types, relative amounts, and time-course of immune cells

responding to tumor lysates *in vivo*. Conducting these studies in the AR and AP animal model will generate important new insight into cellular response in the natural regression and progression of tumors.

## Literature Review

### Characteristics of Arkansas Progressor and Regressor Chicken Lines

The Arkansas Rous Sarcoma Regressor (AR) and Progressor (AP) lines of chickens serve as a useful animal model for the study of tumor regression and progression. These lines of chickens were developed by Dr. N. R. Gyles of the University of Arkansas and have been selected over multiple generations based on their ability to regress and progress Rous Sarcoma Virus (RSV)-induced tumors, respectively (Gyles et al., 1977). The AR line of chicken was introduced in 1965 by crossing Giant Jungle Fowl males with White Leghorn females of the original progressor line that has been maintained at the University of Arkansas for many years (Gyles et al., 1977). The Giant Jungle Fowl males were obtained from a closed line of Giant Jungle Fowl that had been reproduced from two males and ten females acquired from Dr. J.N. Thompson of Pottsville, AR in 1963. These chickens were originally received from the descendants of ten hatching eggs imported from a backyard flock in Southeast Asia during World War II (Gyles et al., 1977).

When AP and AR chickens are injected with RSV into the wing-web, birds from both lines of chickens will develop a tumor. Gyles et al. (1967) and Gyles and Brown (1971) determined that the AP and AR lines give different responses to the growth of Rous sarcomas. The AP line permits approximately 90 percent of the sarcomas in the wing-web to grow and kill the chickens, while in the AR line 70 percent of the sarcomas regress (Gyles et al., 1976). These significant differences were determined by Gyles to be under genetic influence (Gyles et al., 1976). The AP and AR lines of chickens are still maintained at the Arkansas Experiment Station's Poultry Farm in Fayetteville, AR, under the care of Poultry Geneticist, Dr. Nicholas B.

Anthony, professor in the Department of Poultry Science. Dr. Anthony further selected the AP and AR lines for high incidence of progression and regression of RSV-induced tumors, respectively, and for uniformity of tissue-type (MHC genes) within each line, which allows for tissue-transfer between AP and AP chickens and AR and AR chickens.

The idea that viruses could be involved in tumor development came about in the early 1900s when it was shown that the RSV caused tumors in chickens (Fields and Knipe, 1990). As a member of the family *Retroviridae*, RSV is an acute transforming retrovirus containing *v-src* oncogene (Fields and Knipe, 1990). The mechanisms by which viruses can transform cells involves various proto-oncogenes (cellular in origin) that play no role in the virus life cycle (Fields and Knipe, 1990).

Rous sarcoma virus is an RNA virus that affects birds, particularly poultry. RSV is named after Peyton Rous who discovered in 1911 that cancer could be induced in healthy chickens by injecting them with cell-free extract of the tumor of a RSV infected chicken (described in Becsei, 2010). Rous sarcoma virus is related to the avian leukosis virus, and is oncogenic due to the *v-src* oncogene (Spanakos 2007). Rous sarcoma virus infects cells through cell surface receptors that are specific for each viral subgroup. The virus uses host cells for proliferation until many cells are infected with the viral RNA. Following infection of target cells, the infected cell will be transformed into a cancer cell by the *v-src* oncogene at the site of inoculation.

Rous sarcoma can also be induced in chickens using just the *v-src* DNA. According to previous studies, chickens that had never been inoculated with the *v-src* gene had a greater increase in sarcoma size coupled with a slower regression of sarcoma compared to chickens with a previous exposure to the *v-src* DNA (Wisner et al., 1991). This indicates that a primary and

secondary exposure to tumor antigens resulted in the development of anti-tumor responses, with characteristics of a memory response (Wisner et al., 1991). How strong this memory response is and whether or not it can be maintained has not been determined.

T cell-mediated cellular immunity was shown to be most important mediator of immune responses to tumors (Fleischer and Bauer, 1981). Prat et al. (1987) showed that CD4+ T helper cells alone provided full protection against *v-src* induced sarcomas. CD4+ T helper cells are responsible for orchestrating adaptive immunity, and in the case of anti-tumor responses, the activity of these cells was found to be positively influenced by cytokines such as interferon-gamma (INF- $\gamma$ ). INF- $\gamma$  is a very potent activator of macrophages, boosts natural killer cell activity, and regulates proliferation and functional differentiation of cytotoxic (CD8+) lymphocytes (Prat et al., 1987). INF- $\gamma$  also plays an important role in increasing the expression of MHC class I molecules which present tumor antigens to cytotoxic T lymphocytes (CTLs) (Prat et al., 1987). Therefore, INF- $\gamma$  plays a critical role in making target cells more susceptible to lysis by tumor-antigen specific CTLs.

Research findings in the response of Japanese quail to RSV induced tumors indicated that the quail progressor line experienced immune response suppression by malignant cells (Janes et al., 1994). In this experiment, the progressor line maintained an elevated leukocyte population in the blood throughout the experiment. In AP and AR lines of chickens that had received a primary challenge with RSV, Gyles et al. (1967) observed a more rapid recruitment of blood leukocytes following a secondary RSV exposure in AR compared to AP chickens (Gyles et al., 1967). However, both lines showed a similar immune response after 15 days, indicating the lines may eventually maintain a similar response after a longer period of time.

## Immune System Cells and Responses

As discussed previously, the immune system separates into innate and adaptive immunity, which can be described as the native and acquired immunity. There are several types of leukocytes, including lymphocytes, granulocytes (heterophils, eosinophils, basophils, mast cells), monocytes, and macrophages. Lymphocyte and monocytes, or macrophages are also referred to as mononuclear cells. Innate immunity provides the earliest line of defense against microbes, and includes cells such as heterophils, macrophages, monocytes, eosinophil, mast cells and basophils. Heterophils are the avian equivalent to neutrophils in mammals, and mediate the earliest phase of inflammation. Monocytes, once recruited from the blood to the site of infection, will differentiate into macrophages. Macrophages are also involved in inflammation, but at a later phase than heterophils and also remain in the inflamed tissue longer. Heterophils and macrophages are phagocytes, removing extracellular microbes by taking them inside the cells where they are killed in phagolysosomes. While heterophils are phagocytosing microbes early in the infection they die very soon and are not as effective as the long-lived macrophages. Macrophages are specialized phagocytes and with T cell help (IFN- $\gamma$ ) become highly activated and able to kill microbes that survive inside the phagolysosome (intracellular bacteria). Additionally, macrophages can present antigens to T helper cells.

Adaptive immunity, which is acquired after an exposure to antigens, involves B and T-lymphocytes. B-lymphocytes produce antibodies and mediate humoral immunity. T-lymphocytes coordinate cell-mediated immunity and include many different subsets such as CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic lymphocytes (CTL). Subsets of T cells are also determined by the type of antigen-receptor, or T cell receptor (TCR), they express on their surface. Gamma-delta T cells represent a smaller subset of T cells that possess a TCR consisting of two glycoprotein chains

called gamma and delta TCR chains on their surface. These T cells act more like cells of innate immunity in that the specificity of their TCR is limited to frequently encountered microbes. Additionally, these cells do not require antigen processing into peptides and presentation of antigen with MHC molecules by antigen-presenting cells. Major histocompatibility complex (MHC) molecules are specialized proteins that display host cell-associated antigens for recognition by CD4<sup>+</sup> and CD8<sup>+</sup> alpha-beta T cells. Alpha-beta T cells have a TCR composed of two glycoprotein chains called alpha and beta TCR chains. Alpha-beta T cells are the more sophisticated type of T cell of adaptive immunity with a large repertoire of antigen-specificities in their TCR. They are highly restricted in antigen-recognition, only being activated if their TCR can recognize antigen-peptide-MHC complex on antigen presenting cells. Subsets of alpha-beta T cells include the CD4<sup>+</sup> T cells that are restricted to antigen-recognition in association with MHC class II molecules. Functionally, CD4<sup>+</sup> T cells are the T helper cells that orchestrate adaptive immunity and help other cells of the immune system to become activated. T helper cells can do this through direct cell-cell contact and/or secretion of cytokines, such as IFN- $\gamma$ . IFN- $\gamma$  is produced in cell-mediated immune responses where it has several functions, including activation of macrophages with intracellular bacteria and activation of antigen-presenting cells where it plays an important role of increasing expression of MHC antigens. Another subset of T cells is the CD8<sup>+</sup> T cells that function as the cytotoxic (or killer) cells of adaptive immunity. These cells are very important in eliminating viral infection and tumor cells – cells that generate antigen in their cytosol. They typically are alpha-beta T cells that need to recognize antigen-peptide in association with MHC class I. To become activated they need to recognize their antigen-peptide MHC class I complex on an antigen presenting cells and receive additional signals from T helper

cells. Once the CD8<sup>+</sup> T cells have differentiated into a killer cells (effector cells; CTL), it can go to the site of infection or tumor and kill the virus infected /tumor target cells.

The other subset of adaptive immune cells, or B cells, are responsible for producing antibodies that bind to extracellular microbes and cell surface antigens, block their ability to infect host cells, and promote microbe/antigen ingestion. B cells mediate humoral immunity and differentiate into antibody-secreting plasma cells.

## **Tumor Immunology**

The immune system does have the ability to be activated to effectively kill and eradicate tumors, despite the complexity and sometimes lethality of their nature. A new goal of treatment of tumors is in fact the use of tumor immunotherapy in which augmentation of the host anti-tumor response is the goal (Abbas et al., 2015). The very presence of this host anti-tumor response indicates tumors express antigens that can be recognized by the host as something foreign. The classifications for these tumor antigens are based on their patterns of expression, such as tumor-specific antigens and tumor-associated antigens (Abbas et al., 2015). Tumor-specific antigens are antigens expressed on tumor cells but not on normal cells while tumor-associated antigens are antigens that are expressed on tumor cells and normal cells (Abbas et al., 2015).

Tumors express genes whose products are essential for malignant transformation and for the maintenance of the malignant phenotype (Abbas et al., 2015). These genes are maintained by point mutations, deletions, chromosomal translocations, or viral gene insertions affecting cellular proto-oncogenes or tumor suppressor genes (Abbas et al., 2015). The products of these genes can

be presented on MHC class I molecules by tumor cells to CD8<sup>+</sup> CTL which will then kill the tumor cells by induction of apoptosis.

There is evidence that adaptive immunity does have the ability to eradicate virus-induced tumors by preventing the growth of tumors. Certain cancers are more likely to arise in individuals who are already immunosuppressed, such as in human papilloma virus-associated (HPV) cervical cancers or AIDS patients. Healthy patients or animals are less likely to experience tumor development because of their healthy adaptive immune system that is not currently challenged. This discovery of the ability of the adaptive immune system to combat tumors is the reason certain vaccines have been developed such as vaccines against HPV for women and men.

## **Objectives**

The purpose of this study was to monitor *in vivo* the type, amount and time-course of leukocytes responding to syngeneic tumor lysates injected into the dermis of growing feathers in unsensitized (no RSV) and sensitized (tumor bearing) AP and AR chickens. By evaluating the cellular infiltration profiles in unsensitized chickens, we can gain insight into the innate response to the tumor lysate in each line; whereas monitoring these aspects in sensitized chickens will provide insight into adaptive immune activity. Side by side comparison of the leukocyte infiltration profiles in unsensitized and sensitized AP vs AR chickens may reveal differences that allow one line to regress the tumor whereas in the other line the tumor continues to progress.

## **Hypothesis**

We hypothesize that within each line of chickens there will be differences between the leukocyte infiltration response in unsensitized and sensitized chickens with respect to the time-course, amount and type of cell responding to the lysate injection. Additionally, side-by-side comparison of the data may reveal differences in the time-course, amount and type of the responding leukocytes between AP and AR chickens.

## Materials and Methods

### A. Experimental Animals

For this study, Arkansas Rous Sarcoma Progressor chickens (AP) and Arkansas Rous Sarcoma Regressor chickens (AR) were randomly selected from populations maintained by Dr. Nicholas B. Anthony, Poultry Geneticist, Department of Poultry Science, University of Arkansas, Division of Agriculture, Fayetteville, AR. Newly hatched, wing-banded chicks were placed into a floor pen with wood shaving litter at the Arkansas Experiment Station Poultry Farm in Fayetteville, AR. Chicks were reared following standard vaccination (Marek's disease), temperature, lighting, and diet protocols and food and water were available *ad libitum*. The wellbeing of the chicks was checked daily. All protocols involving animals were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC Protocol #11019).

### B. Experimental Design

A total of twenty AR chicks and twenty AP chicks were used. Ten chickens of both groups were injected intradermal (i. d.) with 100  $\mu$ L of 700 pfu RSV/mL in PBS at seven weeks of age in the right wing web (sensitized). Another group of age-matched AR and AP chickens were injected similarly with the same volume of PBS alone and served as the non-RSV control (unsensitized). All chickens were monitored for tumor development three times per week. To determine tumor development, growth, and regression or progression, chicks were examined visually by the physical appearance of tumors. For each chick, the wing band number was checked and the tumor was measured (width and length) using a digital caliper (accurate to 0.1

mm) (Table 1). Measurements were recorded and later given a tumor score based on the criteria in Table 1. If a tumor had grown visibly large enough to reduce quality of life of a bird, the bird was euthanized by inhalation of carbon dioxide gas (if less than 6 weeks of age) or injected with pentobarbital, (65 mg/mL, 1 mL/kg of body weight) and the tumors were removed. Felicity Johnson, who withdrew from the Honors Program in 2014, carried out the Progressor chicken work except for the phenotypic analyses of the cells responding to the tumor lysate injected into the pulp of GF.

### **Tumor Lysate**

To prepare tumor lysates, a progressing RSV-induced tumor and a regressing RSV-induced tumor were collected from one AP and AR chicken, respectively. Under aseptic conditions, Mr. Robert Dienglewicz removed the capsule and skin of the tumor. The tumor was cut through the midline and the non-necrotic or “good” tumor tissue was removed based on its pink color and texture which differs from necrotic tissue. The tumor tissue was cut into small pieces. Using sterile forceps the pieces were placed in a 20 mL sterile glass homogenizer, PBS was added and the tissue homogenized on ice. To make the lysate, the homogenized tissue was then subjected to five freezing/thawing steps in liquid nitrogen and hot water, respectively. This process was to break the cells into a usable lysate. The protein concentration of the lysates was then determined using the Bradford protein assay and the concentration of the lysates adjusted to 2.44 mg/mL.

**Table 1.** Criteria for determining tumor scores based on measurable size of tumor in wing web. Scores were determined based on size in both Progressor and Regressor chickens.

Score	Criteria
0	No palpable tumor
1	Up to 50 mm <sup>2</sup>
2	Between 50 and 100 mm <sup>2</sup>
3	Between 100 and 200 mm <sup>2</sup>
4	Between 200 and 300 mm <sup>2</sup>
5	Between 300 and 400 mm <sup>2</sup>
6	Between 400 and 500 mm <sup>2</sup>
7	Between 500 and 600 mm <sup>2</sup>
8	Between 600 and 700 mm <sup>2</sup>
9	Death (euthanasia) during study

### **Injection of Growing Feathers**

To monitor leukocyte infiltration in response to tumor lysate, 6 unsensitized AR and AP chickens and 6 sensitized AR and AP chickens with actively regressing or progressing Rous sarcoma for at least two weeks were selected for feather injections.

For each chicken, nine 2- to 3-week-old growing feathers (GF) of each breast tract were selected and marked for injection by cutting off the emerging barbs just above the epidermal cap surrounding the pulp (dermis). The prepared GF of the unsensitized (U) and sensitized (S) AP chickens were injected into the pulp with syngeneic AP tumor lysate (2.44 mg protein/mL; 10

$\mu\text{L}/\text{GF}$ ; 18 GF/chicken). Similarly, GF of U-AR and S-AR chickens were injected into the pulp with syngeneic AR tumor lysate (2.44 mg protein/mL; 10  $\mu\text{L}/\text{GF}$ ; 18 GF/chicken). For all groups of chickens, GF were collected before (0 d) and 1, 2, 3, 5, and 7 d post-injection. Pulp cell suspensions were prepared and immunofluorescently stained.

### **Preparation of Pulp Cell Suspensions and Immunofluorescent Staining**

To prepare single cell suspensions from the pulp of injected GF, GF were sliced longitudinally along the shaft and forceps were used to extract the pulp. A few drops of PBS were put onto a nylon mesh covering a beaker and then the pulp was placed onto the nylon mesh. The pulp was pushed through the mesh with a blunt end of a syringe stopper while cold phosphate-buffered saline (PBS, isotonic solution) was added to cover the pulp. The nylon mesh has a defined pore size of 60  $\mu\text{m}$  diameter that allows single cell suspensions to be obtained when pushing tissue through the screen. The contents of the beaker were then added to 130 X 10 mm tubes. The cells prepared from each pulp were then spun at 250 x g (1200 rpm) at 4°C for 10 minutes.

When the cells were washed, the supernatant fluid was discarded. After the supernatant fluid was discarded, a second wash was conducted. The supernatant fluid was then discarded and the cells resuspended in 0.4 mL of PBS+. PBS+ consists of 0.1M phosphate buffered saline (pH 7.4) with 0.1% sodium azide (to prevent cells from internalizing the markers and labels) and 1% bovine serum albumin (to prevent non-specific binding of specific antibodies). Fifty  $\mu\text{L}$  of each cell suspensions were added to 6 wells in a row of a 96-well round-bottom plate. The cells were then stained using a panel of mouse anti-chicken (m-a-c) leukocyte-specific monoclonal antibodies (mAb) following one- or two-color direct immunofluorescent staining procedure. For

this, 50  $\mu$ L of fluorescently labeled (FITC and/or PE) mAbs were added to the cells in the following combinations: PE&FITC labeled isotype control (mouse IgG1 with irrelevant specificity), m-a-c CD45-FITC (single stain); CD4-FITC & CD8-PE (dual stain);  $\alpha\beta$ TCR-FITC & MHC class II-PE;  $\gamma\delta$ TCR-FITC & CD8-PE; Bu-1-FITC and Macrophage-PE. All mAb were purchased from Southern Biotech in Birmingham, Alabama. The isotype control mAbs were used to detect non-specific binding of fluorescent labeled m-a-c leukocyte specific mAbs and to decide the cut-off between fluorescence positive and fluorescence negative populations.

### **Cell Population Analysis by Flow Cytometry**

Phenotypic analysis of cells was conducted using a FACSort Becton Dickinson flow cytometer equipped with an argon laser at 488 nm with detectors for fluorescein isothiocyanate (FITC), phycoerythrin (PE), and quantum red. For each sample, data from  $10^4$  cells were acquired and analyzed using the CellQuest<sup>TM</sup> software. For analysis, a dot plot showing forward scatter (FSC; size) vs. side scatter (SSC; internal complexity) was generated. A region was drawn around the live cell populations (live cell gate). Another dot plot with FITC fluorescence intensity on the x-axis and PE fluorescence intensity on the y-axis was drawn to determine the percentage of each fluorescence-defined leukocyte-subpopulation. Data were expressed as the percentage of each cell-type in the total pulp cell suspension.

### **Statistical Analysis**

For each line of chicken, Systat computer software was used to conduct two-way analysis of variance (ANOVA) to determine differences in the effects of time (days post-injections) and treatment (U vs S) on the qualitative and quantitative nature of the pulp infiltration by leukocytes

in response to tumor antigens. Tukey's Honest multiple mean comparisons were conducted to determine treatment differences at each time-point. Data were expressed as mean  $\pm$  SEM and differences with P-values  $\leq 0.05$  were considered significant.

## Results

### Leukocyte Infiltration:

For AP chickens, 2-way ANOVA revealed treatment by day interactions for leukocyte infiltration (Table 2). Individual mean comparison at each time point revealed higher leukocyte levels (% pulp cells) on Day 1 and lower levels on Day 7 in sensitized (S) compared to unsensitized (U) AP chickens (Figure 1a). In both U-AP and S-AP chickens, leukocyte infiltration peaked at 1-day post-GF injection and returned to pre-injection levels Day 2-5, but increased again in U-AP chickens on Day 7.

In AR chickens, there were no differences in leukocyte infiltration overall and at individual time points between U-AR and S-AR chickens (Table 2, Figure 1b). Leukocyte infiltration levels were elevated on Day 1 and 2 for both AR groups.

Side-by-side comparison of leukocyte infiltration in AR and AP chickens suggests higher infiltration levels in AP groups on day 1 than in AR groups (Figure 1)

### Mononuclear Leukocyte Infiltration:

For AP chickens, 2-way ANOVA revealed treatment by day interactions for mononuclear cell infiltration (lymphocytes and macrophages) (Table 2). Individual mean comparison at each time point revealed higher leukocyte levels on Day 1 and lower levels on Day 7 in S-AP compared to U-AP chickens (Figure 2a). In both AP groups, mononuclear infiltration peaked at 1-day post-GF injection and returned to pre-injection levels Day 3-5, but increased again in U-AP chickens on Day 7.

In AR chickens, 2-way ANOVA revealed treatment by day interactions for mononuclear cell infiltration (Table 2). Individual mean comparison at each time point revealed higher

mononuclear cell levels on Day 1 in both S-AR and U-AR but returned to pre-injection levels at day 3 (Figure 2b).

Side-by-side comparison of mononuclear cell infiltration in AR and AP chickens suggests higher infiltration levels in AP groups on day 1 than in AR groups.

#### Heterophil Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions for heterophil infiltration and there was no main effect difference between treatments (Table 2). Individual mean comparison of the two treatment groups at each time point revealed higher heterophil infiltration on Day 7 in U-AP than S-AP chickens (Figure 3a). In both AP groups, heterophil infiltration peaked at 1-day post-GF injection and returned to pre-injection levels Day 5, but increased again on Day 7.

In AR chickens, 2-way ANOVA revealed treatment by day interactions for heterophil infiltration (Table 2). Individual mean comparison of the two treatment groups at each time point revealed higher heterophil infiltration levels on Day 2 in S-AR compared to U-AR chickens. In S-AR chickens, heterophil infiltration peaked on Day 2 post-GF injection and then decreased at Day 3. From Day 3-7 the heterophil infiltration remained elevated above pre-injection levels (Figure 3b). In U-AR chickens, heterophil infiltration was elevated on Day 1, returned to near pre-injection levels on Day 2, peaked on Day 3 and remained elevated on Day 5-7 (Figure 3b).

Side-by-side comparison of heterophil infiltration in AR and AP chickens suggests higher infiltration levels on Day 1 in AP chickens for both groups while the S-AR chickens had higher infiltration levels on Day 2.

### Macrophage Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions for macrophage infiltration and no overall differences (main effect) between the S-AP and U-AP chickens (Table 2). Individual mean comparison at each time point revealed higher macrophage levels on Day 1 in S-AP compared to U-AP chickens (Figure 4a). In both AP groups, macrophage infiltration peaked at 1-day post-GF injection and returned to pre-injection levels for Day 3-7.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions for macrophage infiltration and no effect of treatment was observed between the two groups (Table 2). In the U-AR chickens, macrophage infiltration peaked on Day 1 post-GF injection and was below pre-injection levels from Day 3-7 (Figure 4b).

Side-by-side comparison of macrophage infiltration in AR and AP chickens suggests higher infiltration levels in S-AP chickens on Day 1 post injection.

### MHC Class II+ Cell Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions for MHC class II+ cell infiltration and no effect of treatment (main effect)(Table 2). Individual mean comparison at each time point also revealed no differences in percentage of MHC class II+ cells in S-AP compared to U-AP chickens (Figure 5a). In both AP groups, MHCII+ cells gradually increased, reaching the highest level by Day 7.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions for MHC class II+ cell infiltration and no differences (main effect) in % MHC class II+ cells infiltrating the pulp between S-AR and U-AR chickens (Table 2).For both S-AR and U-AR chickens, infiltration

levels of MHC class II+ cells peaked on Day 1 and remained at pre-injection levels on Day 3-7 (Table 2, Figure 5b).

Side-by-side comparison of MHC class II+ cell infiltration profiles in AP and AR chickens suggests lower infiltration levels in AP chickens on Day 1 but higher levels on Day 3-7 compared to AR groups (Figure 5).

### B Cell Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions for B cell infiltration and no overall treatment differences (main effect)(Table 2). Individual mean comparison at each time point revealed lower levels on Day 3 and higher levels Day 5-7 in S-AP compared to U-AP chickens (Figure 6a). In both AP groups, B cell levels were highest on Day 1 and Day 2 post-GF injection. In U-AP chickens, B cell levels gradually decreased from Day 1-7 to pre-injection levels, whereas in S-AP chickens levels dropped substantially from Day 2 to 3 and remained near pre-injection levels between Day 3 to 7.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions for B cell infiltration but an overall (main effect) higher B cell infiltration in U-AR compared to S-AR chickens (Table 2). Individual mean comparison at each time point revealed higher levels in U-AR compared to S-AR chickens on Day 2. In both groups of AR chickens B cell infiltration levels were above pre-injection levels on Day 2-5 (Table 2, Figure 6b).

Side-by-side comparison of B cell infiltration suggests that B cell levels increased earlier (Day 1 vs. Day 2) and proportionally less (2 fold vs. 6 fold) in AP chickens compared to AR chickens, respectively. Moreover, the elevated levels of B cells were sustained from Day 2 to 5 in AR chickens (Figure 6).

### T Cell Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions for T cell infiltration and no overall treatment effects (main effect)(Table 2). Individual mean comparison at each time point revealed higher T cell levels in U-AP compared to S-AP chickens on Day 7 (Figure 7a). In both AP groups, T cell infiltration peaked at 1-Day post-GF injection and then decreased to pre-injection levels between Day 2-5 in U-AP chickens and Day 2-7 in S-AP chickens.

In AR chickens, 2-way ANOVA revealed no differences in T cell infiltration between the two groups of AR chickens and no main effect of treatment (Table 2). In both AR groups, T cell levels were elevated on Day 1 and Day 2 (Table 2, Figure 7b).

Side-by-side comparison of T cell infiltration revealed that AR chickens had higher and more sustained initial infiltration (Day 1-2) while AP chickens had higher T cell infiltration on Day 7 (Figure 7).

### Alpha-beta TCR+ T Cell Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions for alpha-beta TCR+ T cell infiltration and no overall treatment effects (main effect)(Table 2). Individual mean comparison at each time point revealed higher alpha-beta TCR+ T cell levels on Day 7 in U-AP compared to S-AP chickens (Figure 8a). In both groups, alpha-beta TCR+ T cell levels were elevated on Day 1, and returned near to pre-injection levels from Day 2-5 in U-AP and Day 2-7 in S-AP chickens.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions for alpha-beta TCR+ T cells and no overall treatment effects (main effect)(Table 2). In both groups, alpha-beta

TCR+ T cell levels peaked on Day 1-2 and returned near pre-injection levels from Day 3-7 (Table 2, Figure 8b).

Side-by-side comparison of alpha-beta TCR+ T cell levels suggests more a higher and more sustained increase of alpha-beta TCR+ T cells in AR compared to AP chickens (Day 1-2 vs. Day 1).

#### Gamma-delta T Cell Infiltration:

For AP chickens, 2-way ANOVA revealed no treatment by day interactions and no overall treatment differences for gamma delta T cell infiltration (main effect)(Table 2) (Figure 9a). In both AP groups, gamma delta T cell infiltration peaked on Day 1 and then returned to pre-injection levels Day 2-5 and then increased again on Day 7.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions and no overall treatment differences for gamma delta T cells (main effect)(Table 2). Individual mean comparison at each time point revealed greatly elevated gamma delta T cell levels in U-AR compared to S-AR chickens on Day 2. In both AR groups, gamma delta T cell levels were elevated on Day 1 and Day 2 (Table 2, Figure 9b).

Side-by-side comparison of gamma delta T cell infiltration suggests greater infiltration of gamma delta T cells on Day 2 in U-AR compared to AP chickens (Figure 9)

#### CD4+ T Cell Infiltration:

For AP chickens, 2-way ANOVA revealed treatment by day interactions for CD4+ T cell infiltration (Table 2). Individual mean comparison at each time point revealed higher CD4+ T cell levels on Day 7 in U-AP compared to S-AP chickens (Figure 10a). In both AP groups, levels

of CD4+T cell infiltration decreased from Day 0-2 and then returned to near pre-injection levels for the remainder of the study.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions and no overall treatment differences (main effect) for CD4+ T cells (Table 2, Figure 10b). In both AR groups levels of CD4+ T cells peaked on Day 2 and returned to pre-injection levels thereafter.

Side-by-side comparison suggests a diverging response in terms of T helper cell infiltration (CD4+) in AP compared to AR chickens with levels decreasing in AP, and increasing in AR chickens on Day 1 and Day 2 (Figure 10).

#### CD8+ T Cell Infiltration:

For AP chickens, 2-way ANOVA revealed treatment by day interactions for CD8+ T cell infiltration (Table 2). Individual mean comparison at each time point revealed higher levels of CD8+ T cell levels on Day 7 in U-AP compared to S-AP chickens (Figure 11a). In both AP groups, infiltration of CD8+T cells peaked on Day 1, remained elevated on Day 2, and dropped to pre-injection levels on Day 3-5 in U-AP and Day 3-7 in S-AP chickens.

In AR chickens, 2-way ANOVA revealed no treatment by day interactions and no overall treatment differences for CD8+ T cells (main effect)(Table 2). Individual mean comparison at each time point revealed higher CD8+ T cell levels on Day 2 in U-AR compared to S-AR chickens (Table 2, Figure 11b). In S-AR and U-AR chickens, CD8+ T cell levels peaked on Day 1 and Day 2, respectively, and returned to pre-injection levels thereafter.

Side-by-side comparison of CD8+ T cell infiltration revealed that AP chickens had higher CD8+ T cell infiltration at Day 1 and Day 2 than the AR chickens (Figure 11)

### CD4:CD8 Cell Ratio:

For AP chickens, 2-way ANOVA revealed no time by day interactions and no overall treatment differences for CD4:CD8 ratios (main effect)(Table 2). In both groups, the CD4:CD8 ratio dropped substantially on Day 1 and Day 2 and returned to pre-injection levels thereafter (Figure 12a).

In AR chickens, 2-way ANOVA revealed no time by day interactions and no overall treatment differences for CD4:CD8 ratios (main effect)(Table 2). The CD4:CD8 ratios were slightly elevated on Day 2 and Day 3 in U-AR chickens and gradually increase in S-AR chickens from Day 1-7 (Table 2, Figure 12b).

Side-by-side comparison suggests a diverging response with CD4:CD8 ratios dropping substantially on Day 1 and Day 2 and never returning above pre-injection levels in AP chickens, whereas in AR chickens the ratio increased and remained above pre-injection levels on Day 2-7 (Figure 12).

### T Cell:B Cell Ratios

For AP chickens, 2-way ANOVA revealed treatment by day interactions for T cell:B cell ratios (Table 2). Individual mean comparison at each time point revealed a higher T cell:B cell ratio levels on Day 7 in U-AP compared to S-AP chickens (Figure 13a). In both AP groups, T cell:B cell ratios remained near pre-injection levels Day 1-5 in U-AP and Day 1-7 in S-AP chickens.

In AR chickens, 2-way ANOVA revealed treatment by day interactions for T cell:B cell ratio (Table 2). Individual mean comparison at each time point revealed higher levels in S-AR compared to U-AR on Day 7. In both groups, T cell:B cell ratios peaked on Day 1 and then

dropped to below pre-injection levels on Day 2-5 and then started to increase again on Day 7  
(Table 2, Figure 13b)

Side-by-side comparison revealed that AP chickens had substantially lower T cell:B cell ratios on Day 0, 1, and 7 than AR chickens (Figure 13)

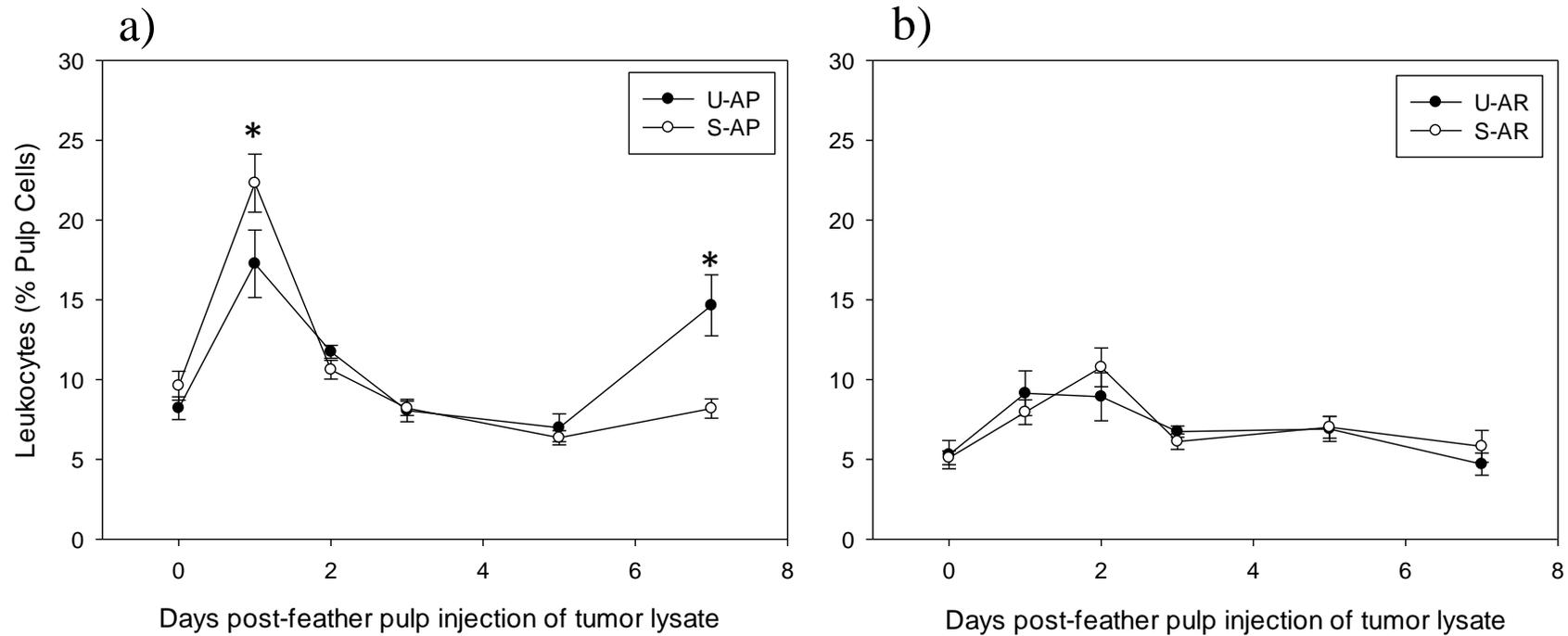
**Table 2.** Two way analysis of variance (ANOVA) determining the main effect of treatment (No RSV and Progressing/Regressing Rous sarcoma), the main effect of day post lysate injection into growing feathers, and treatment by day interactions<sup>1</sup>. Growing feathers were injected in the Arkansas Rous sarcoma progressing (AP) and regressing (AR) chickens.

	AP P-Value	AR P-Value
Leukocytes Treatment Day Treatment X Day	0.672 < 0.001 < 0.001	0.675 < 0.001 0.304
Mononuclear Cells Treatment Day Treatment X Day	0.505 < 0.001 0.003	0.250 0.010 < 0.001
Heterophils Treatment Day Treatment X Day	0.400 < 0.001 0.287	0.250 0.010 < 0.001
Macrophages Treatment Day Treatment X Day	0.110 < 0.001 0.116	0.916 < 0.001 0.596
MHCII Treatment Day Treatment X Day	0.808 < 0.001 0.094	0.059 < 0.001 0.650
T Cells Treatment Day Treatment X Day	0.292 0.003 0.117	0.083 < 0.001 0.134
Alpha-beta TCR+ T Cells Treatment Day	0.069 0.005	0.407 < 0.001

Treatment X Day	0.053	0.906
gamma delta T Cells		
Treatment	0.254	0.081
Day	< 0.001	< 0.001
Treatment X Day	0.734	0.273
CD4+ Cells		
Treatment	0.180	0.070
Day	< 0.001	< 0.001
Treatment X Day	0.013	0.130
CD8+ Cells		
Treatment	0.445	0.955
Day	< 0.001	0.115
Treatment X Day	0.070	0.416
CD4:CD8 Ratio		
Treatment	0.989	0.293
Day	< 0.001	< 0.001
Treatment X Day	0.870	0.788
T Cell: B Cell Ratio		
Treatment	0.047	0.018
Day	< 0.001	< 0.001
Treatment X Day	< 0.001	0.009

<sup>1</sup>Treatment: Tumor lysates were prepared from progressing tumors from a progressor (AP) and regressing tumors from a regressor chicken (AR) (2.44 mg protein/mL). Treatments included injections of respective tumor lysate (AP lysate into AP chickens; AR lysate into AR chickens)(10 µL/GF) into growing feathers (12 GF/chicken) of unsensitized AP and AR chickens (no-RSV) and AP and AR chickens with progressing/regressing Rous sarcoma.

Day: Growing feathers were collected before (0) and 1, 2, 3, 5, and 7 d post injection for determination of leukocyte infiltration profiles in response to the tumor lysates.

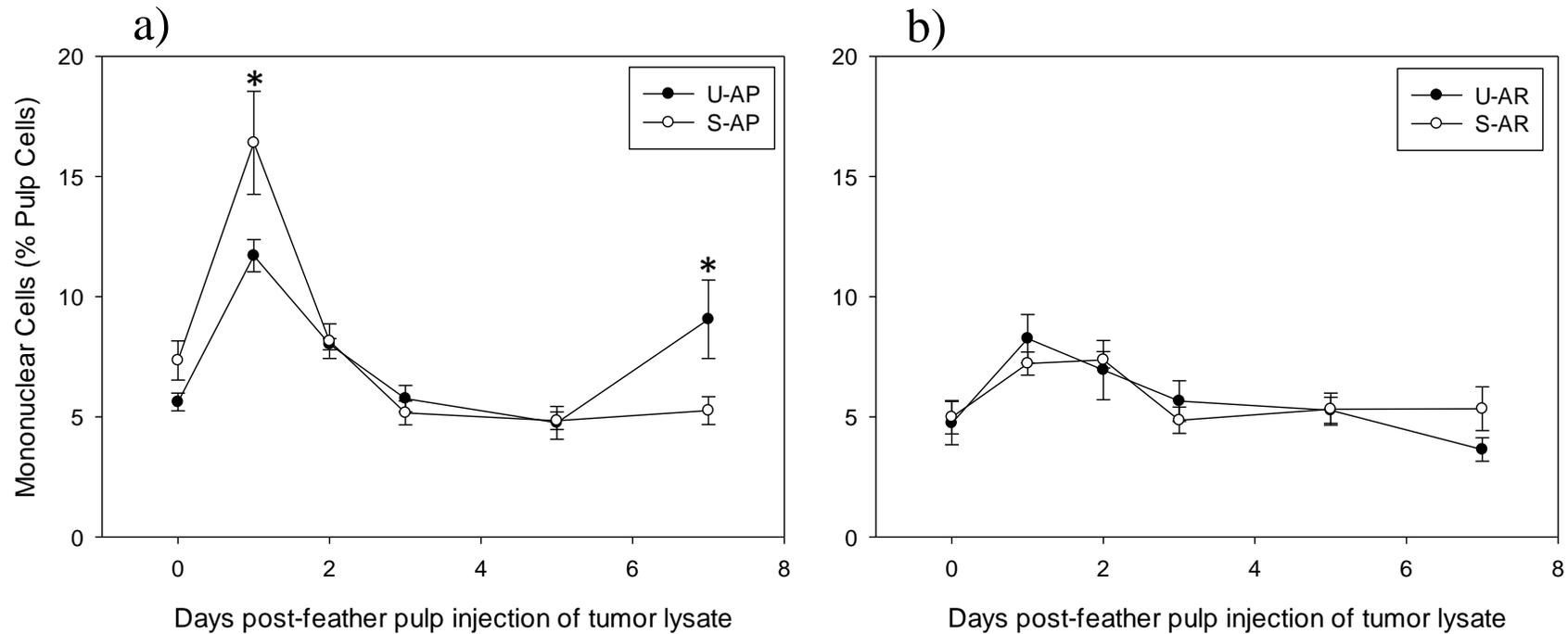


**Figure 1.** Proportions of leukocytes in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (sensitized; S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and leukocytes identified by immunofluorescent staining using a fluorescently labeled chicken leukocyte-specific mouse monoclonal antibody (CD45). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

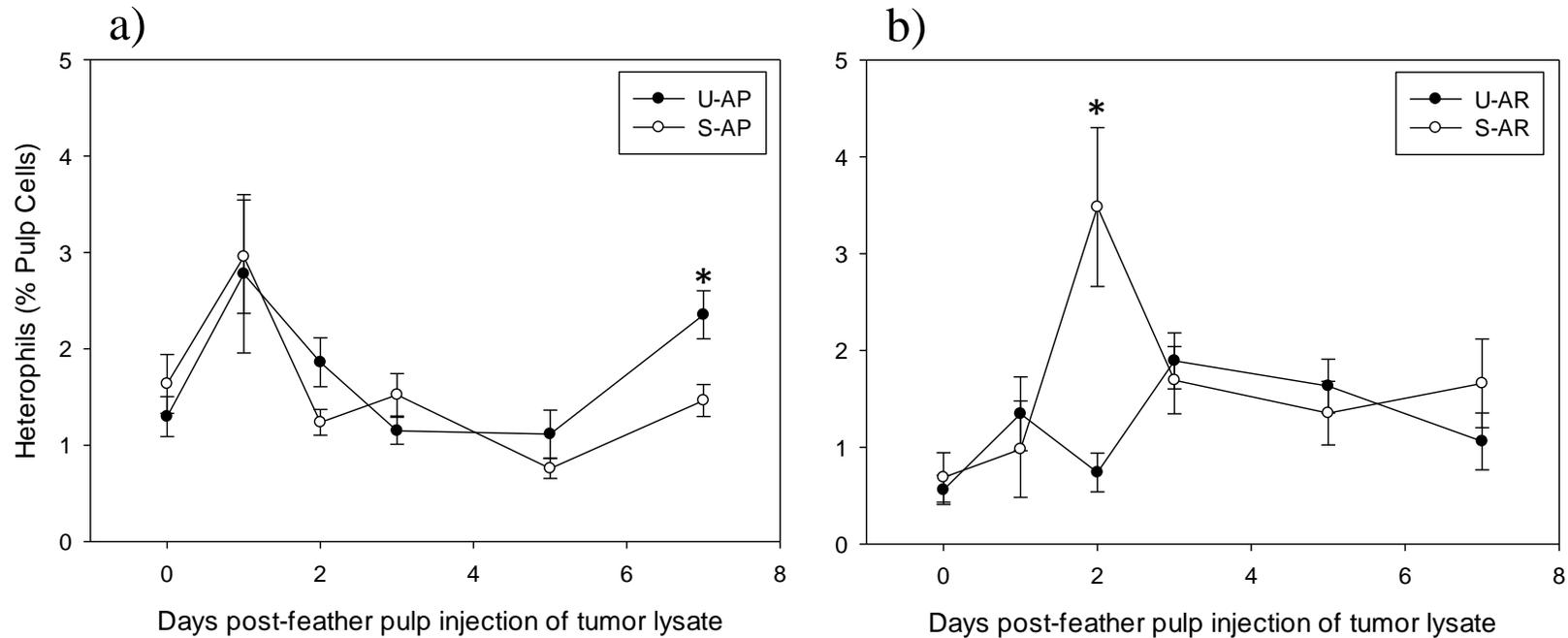


**Figure 2.** Proportions of mononuclear cells (total lymphocytes and macrophages) in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and lymphocytes and macrophages identified by immunofluorescent staining using a fluorescently labeled chicken-specific mouse monoclonal antibodies. Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

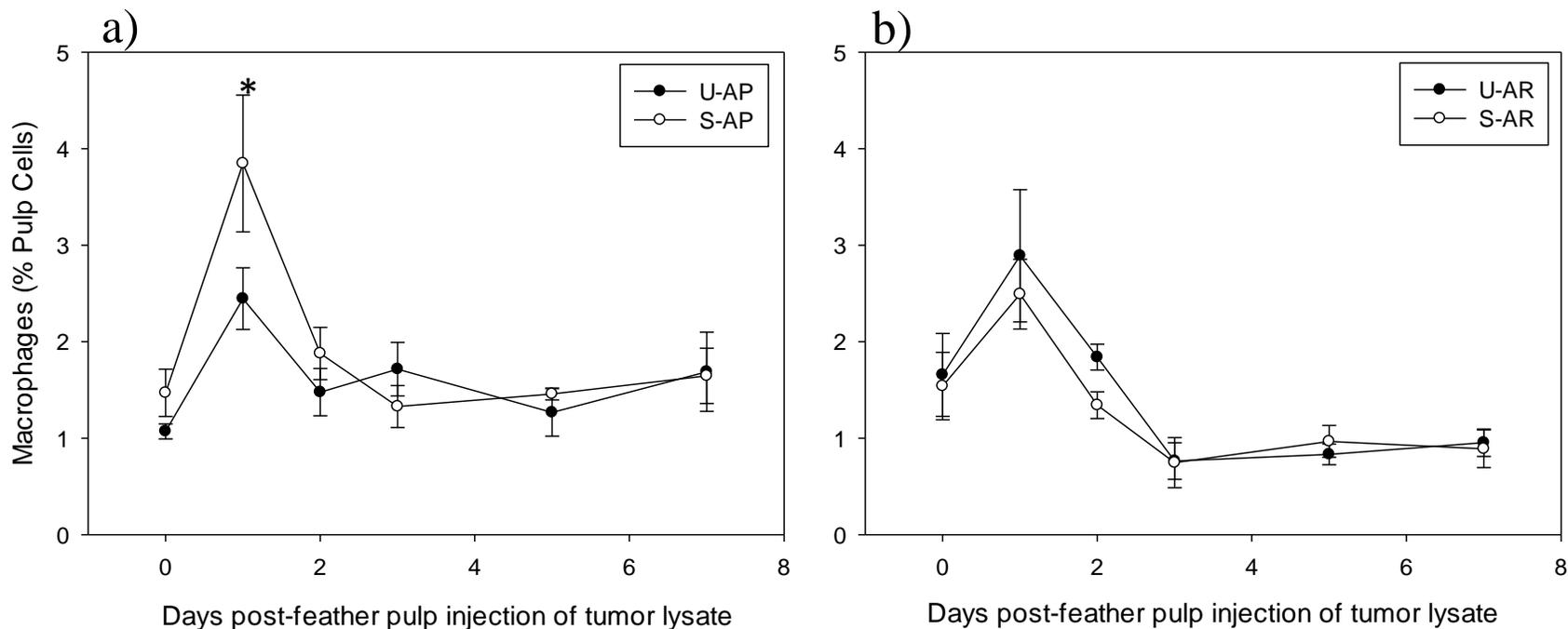


**Figure 3.** Proportions of heterophils in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared. The percentage of heterophils in the pulp cell suspension was determined based on forward and side scatter characteristics using a flow cytometer. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

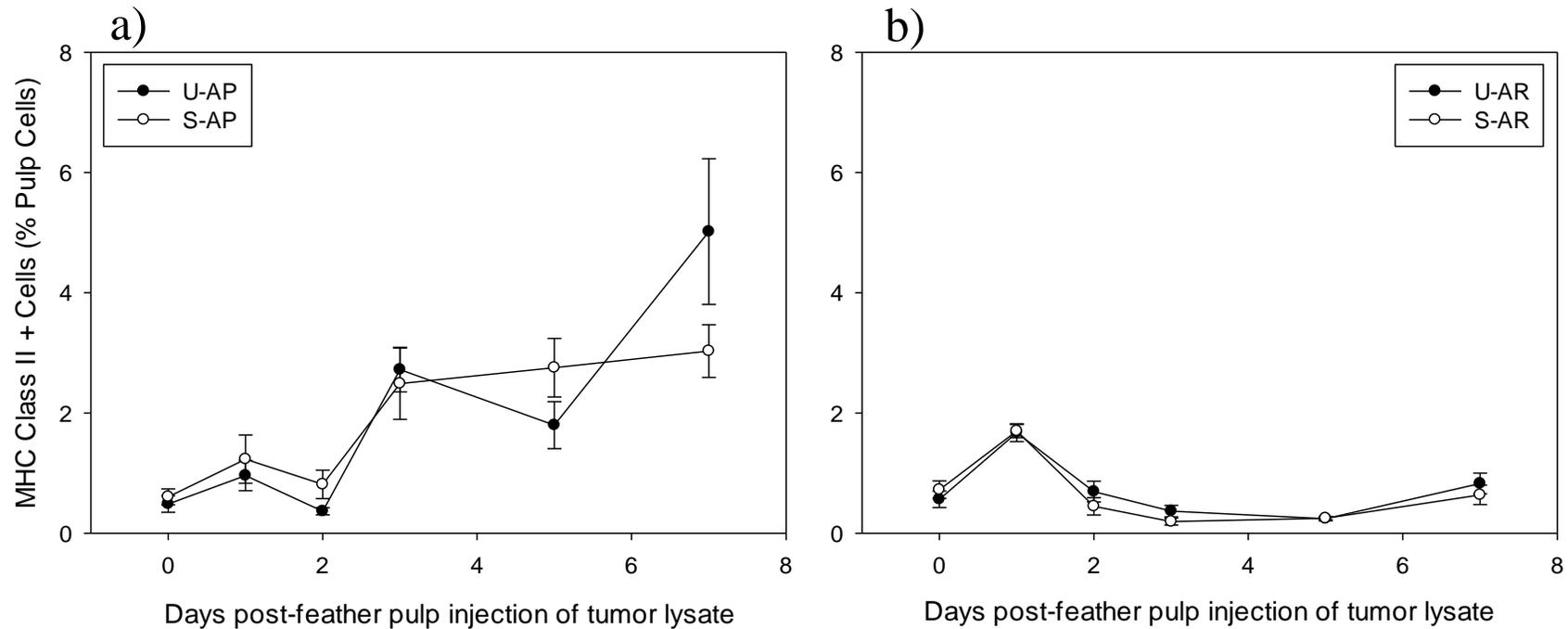


**Figure 4.** Proportions of macrophages in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and macrophages identified by immunofluorescent staining using a fluorescently labeled chicken macrophage-specific mouse monoclonal antibody (KUL01). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

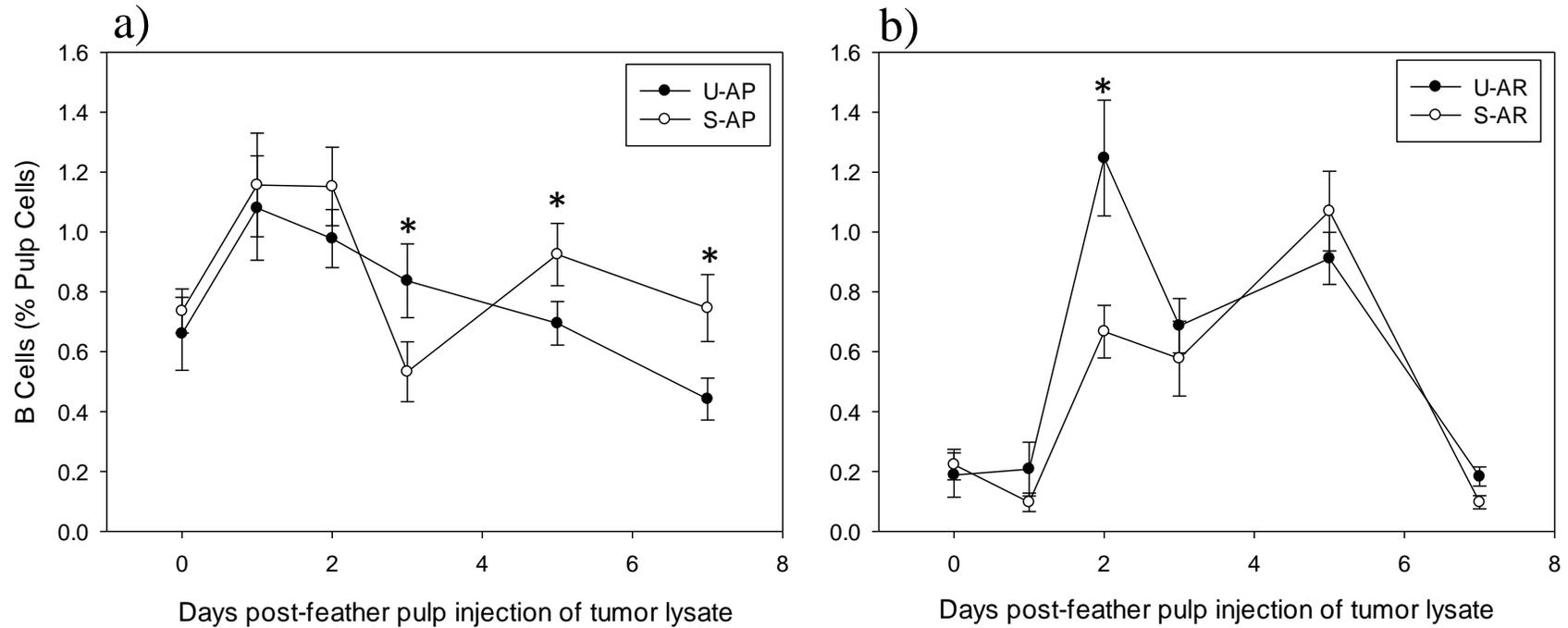


**Figure 5.** Proportions of MHCII+ cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and MHCII+ cells identified by immunofluorescent staining using a fluorescently labeled chicken MHCII-specific mouse monoclonal antibody (Ia). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

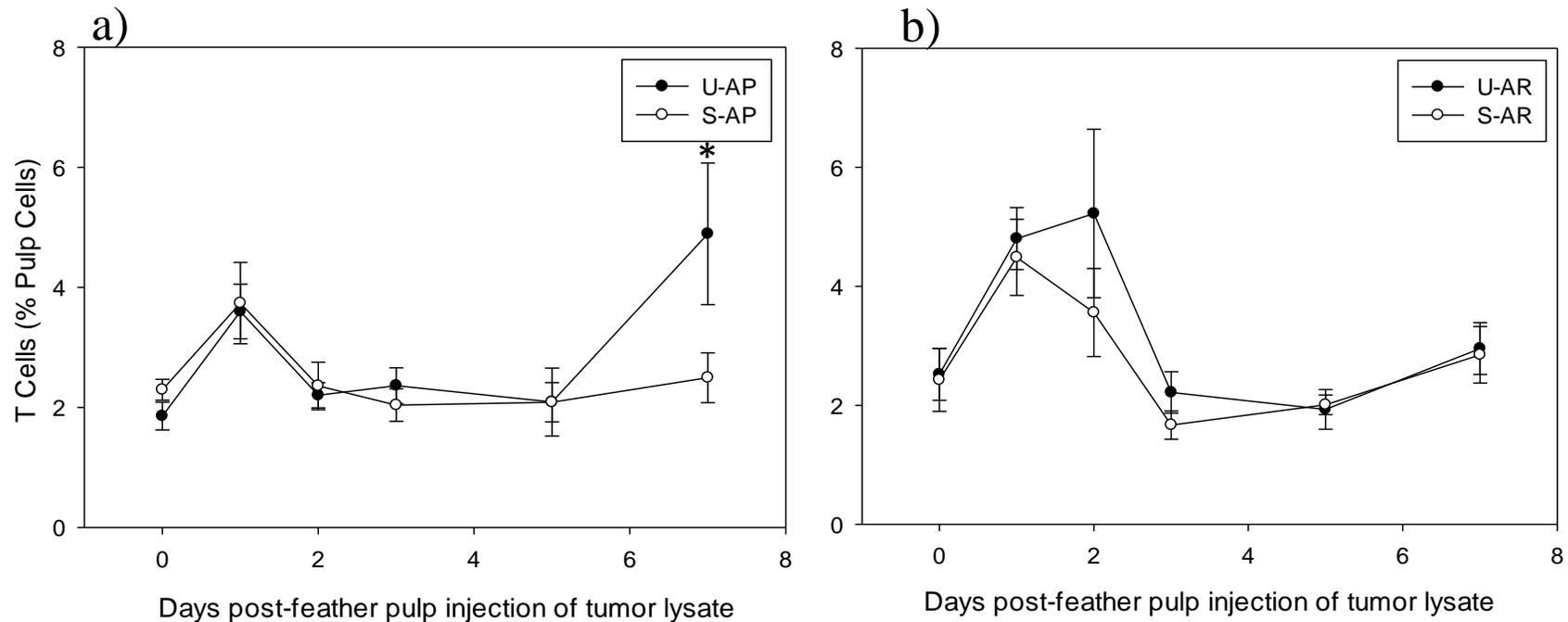


**Figure 6.** Proportions of B cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and B cells identified by immunofluorescent staining using a fluorescently labeled chicken B cell-specific mouse monoclonal antibody (Bu-1). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

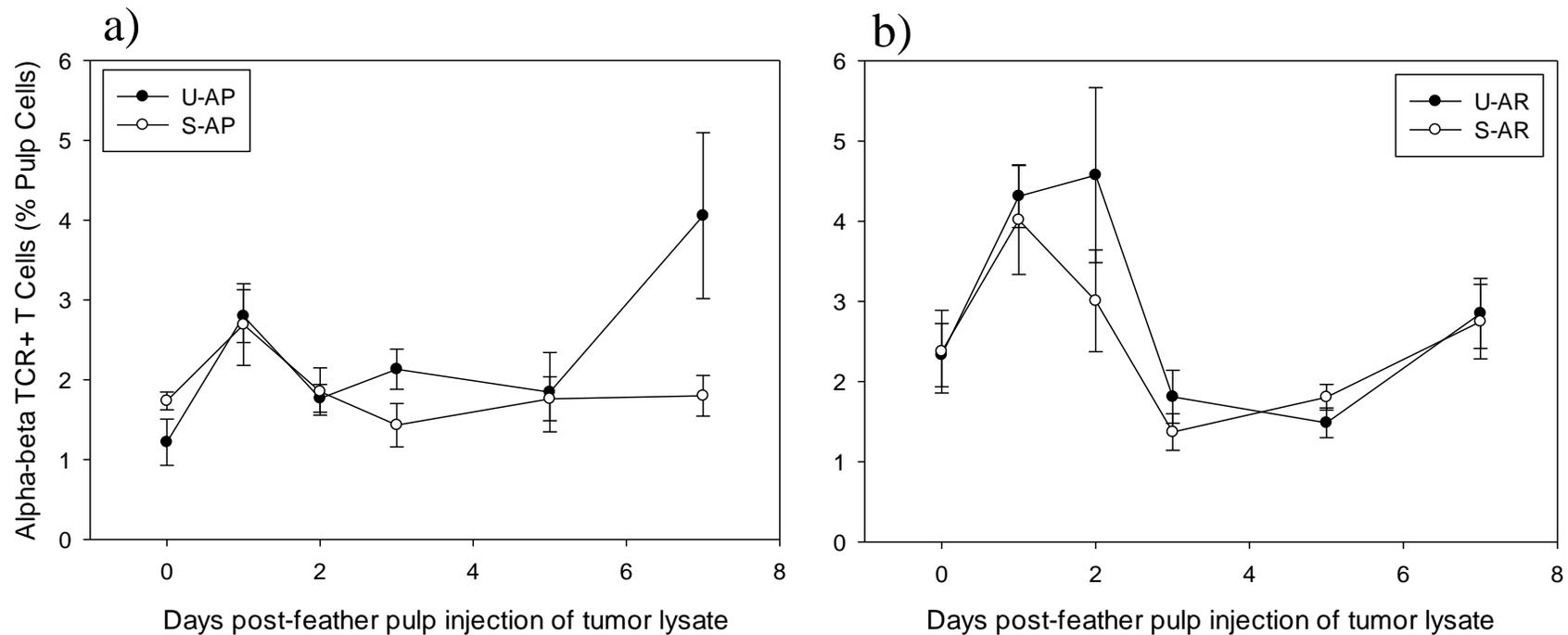


**Figure 7.** Proportions of T cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and T cells identified by immunofluorescent staining using a fluorescently labeled chicken T cell-specific mouse monoclonal antibody (CD3). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

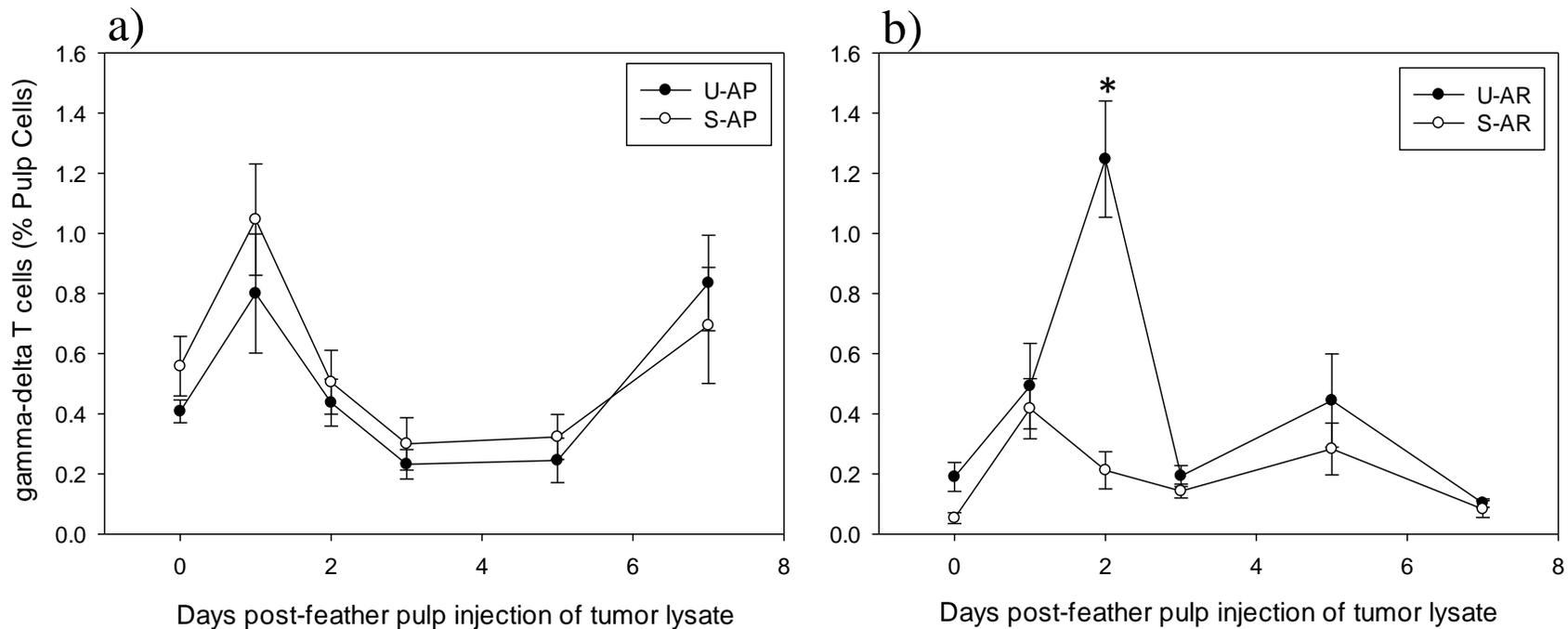


**Figure 8.** Proportions of alpha-beta TCR+ T cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and alpha-beta TCR+ T cells identified by immunofluorescent staining using a fluorescently labeled chicken alpha-beta TCR -specific mouse monoclonal antibody. Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

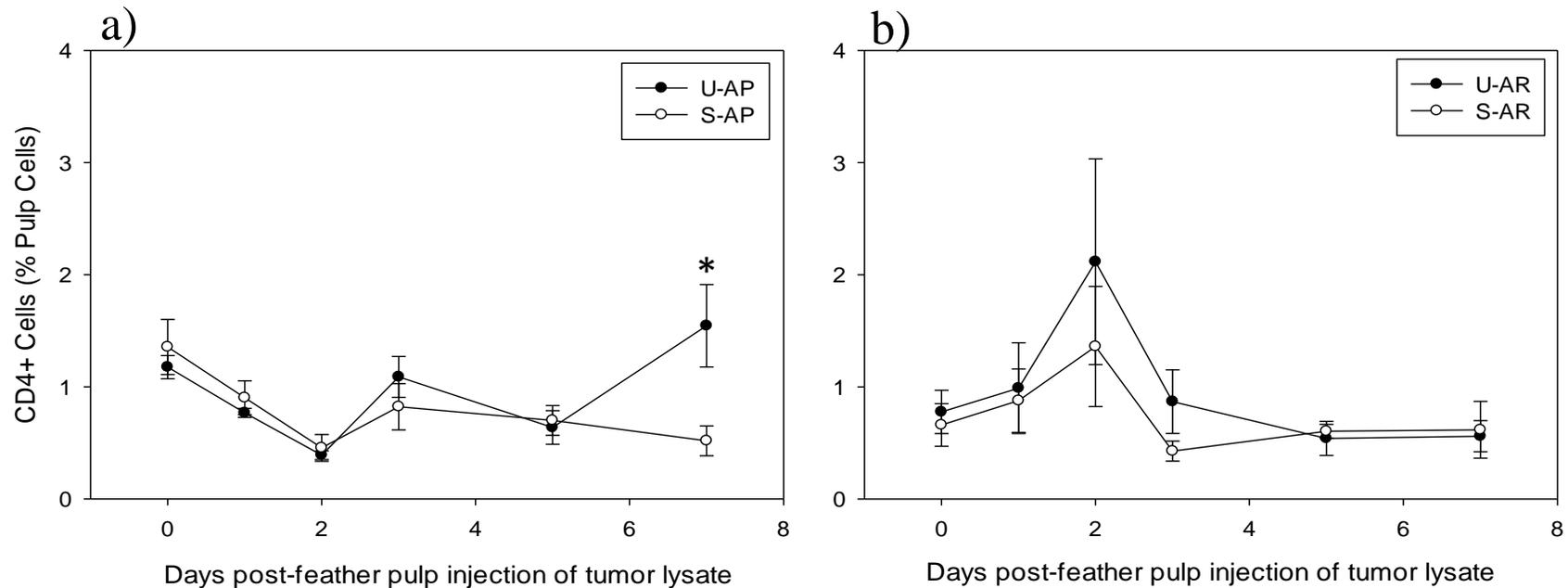


**Figure 9.** Proportions of gamma-delta T cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and CD8<sup>+</sup> gamma-delta T cells identified by immunofluorescent staining using a fluorescently labeled chicken gamma-delta T cell-specific mouse monoclonal antibody. Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

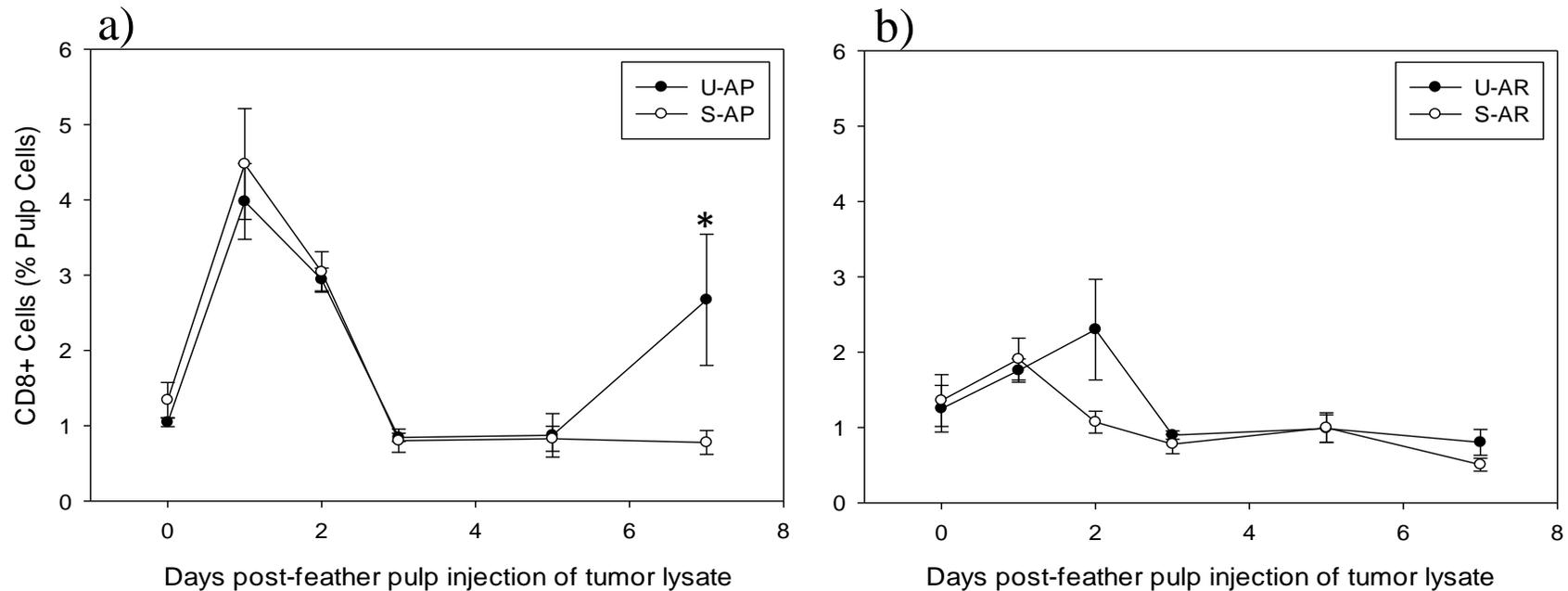


**Figure 10.** Proportions of CD4+ T cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and CD4+ identified by immunofluorescent staining using a fluorescently labeled chicken CD4-specific mouse monoclonal antibody (T4). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

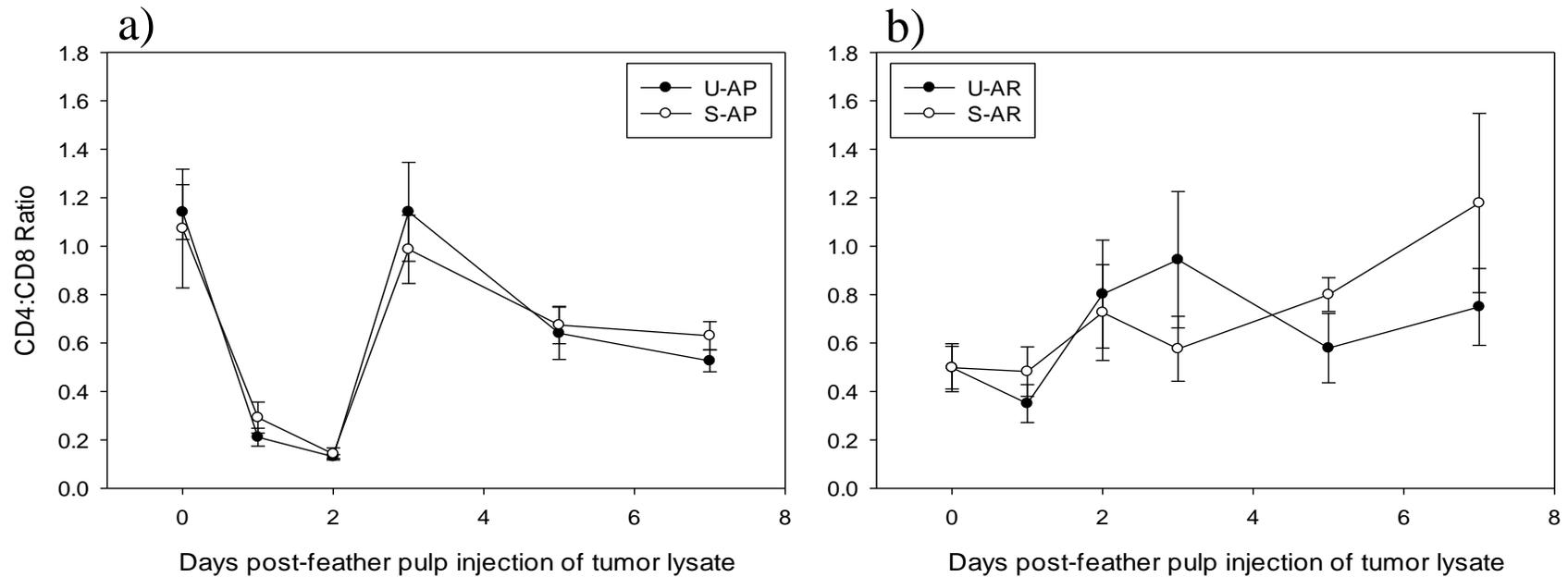


**Figure 11.** Proportions of CD8+ cells in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and CD8+ identified by immunofluorescent staining using a fluorescently labeled chicken CD8-specific mouse monoclonal antibody (T8). Cell population analysis was carried out by flow cytometry. Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

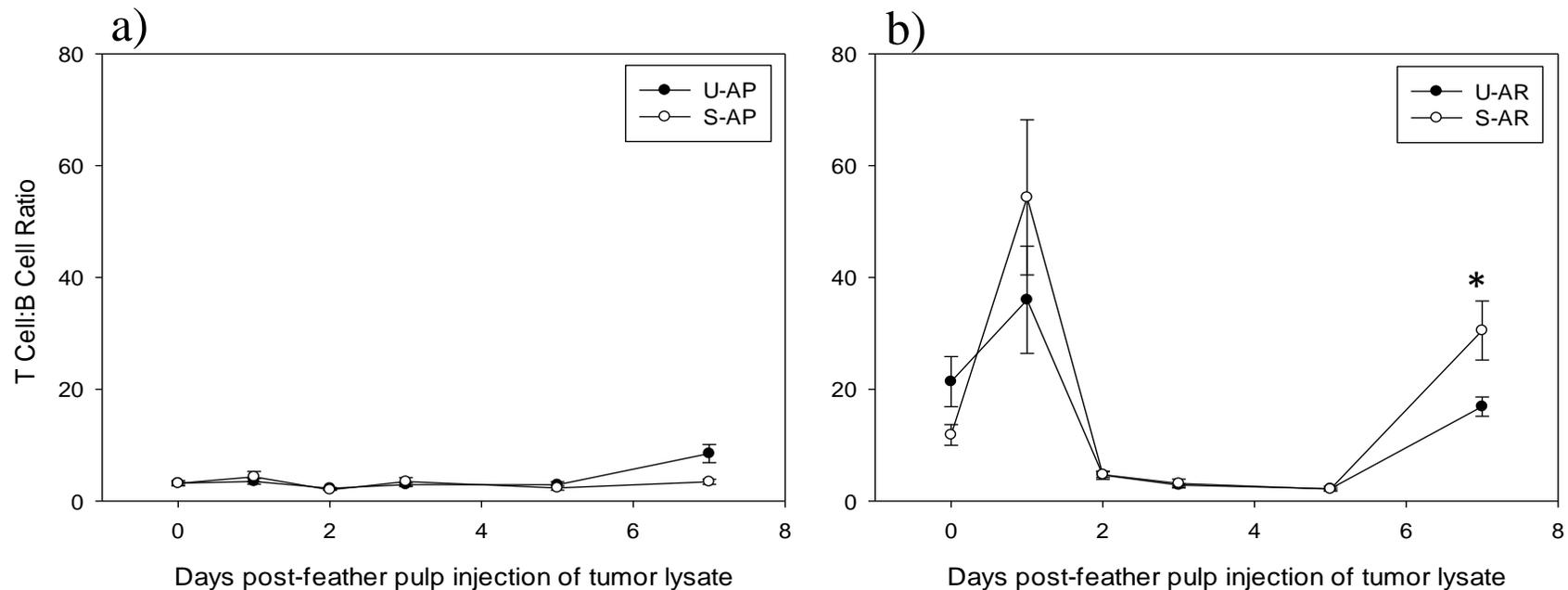


**Figure 12.** Proportions of CD4 to CD8 ratios in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and CD4 to CD8 ratios calculated by dividing % CD4 by %CD8 (see figure 10 and 11 for CD4 and CD8 profiles). Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.



**Figure 13.** Proportions of T cell to B cell ratios in the pulp of growing feathers (GF) injected with tumor lysate in Arkansas Rous sarcoma Progressor (AP) and Regressor (AR) chickens.

(a) the pulp of GF of unsensitized AP chickens (U-AP; n=6) and AP chickens bearing progressing Rous sarcoma (sensitized; S-AP; n=6) was injected with syngeneic tumor lysate prepared from a progressing AP tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

(b) the pulp of GF of unsensitized AR chickens (U-AR; n=6) and AR chickens bearing regressing Rous sarcoma (S-AR; n=6) were injected into the pulp with syngeneic tumor lysate prepared from a regressing AR tumor (2.44 mg protein/mL; 10  $\mu$ L/GF; 12 GF/chicken).

For all groups of chickens (a & b) GF were collected before (0) and 1, 2, 3, 5, and 7 d post injection. Pulp cell suspensions were prepared and T cell to B cell ratios calculated by dividing % T cell by % B cell (see figure 6 and 7 for B and T cell profiles). Data shown are mean  $\pm$  SEM; \*means within a time point are different ( $P \leq 0.05$ ) as determined by Tukey's multiple means comparison tests.

## Discussion

The Arkansas Rous sarcoma Regressor (AR) and Progressor (AP) chickens develop tumors following intra-dermal injection of Rous sarcoma virus (RSV) into the wing web. The most distinguishing feature between the AR and AP lines of chickens is the ability of the AR chickens to then completely regress and eliminate the tumor whereas the tumors in the AP chickens will continue to progress. These tumor regression/progression characteristics in AR/AP chicken make these lines an excellent animal model to examine effective versus ineffective immune activities in tumor immunology. The few studies carried out on the immune response in AR and AP chickens suggest a faster and more protective (adaptive memory phenotype) cell-mediated anti-tumor response in AR chickens following a second exposure to tumor antigens (Gyles et al., 1976; Erf et al., 1998, 1999). However, there is no information on the local *in vivo* cellular and tissue responses to tumor antigens in AR and AP chickens.

Our lab recently developed an *in vivo* method that enables monitoring of cellular responses to antigens in tissues (U.S. Patent No 8,216,551). This method uses growing feathers (GF) as a dermal test-site to monitor tissue/cellular responses to test-compounds injected into the feather pulp (dermis). This method involves injection of several GFs at the same time and then sampling the GFs various times post-injection (minutes, hours, days). Collection of injected GF is a minimally invasive procedure (GF are not firmly attached to the follicles and can easily be removed), the GF provide sufficient tissue to carry out several *ex vivo* analyses, and for the first time provide insight into the types and relative amounts of immune cells responding to test-material, as well as, the time course of the response. Using this *in vivo* test system, we now have opportunity to examine the response of unsensitized (U) and tumor-sensitized (S) AP and AR

chickens to a first (innate response) and second exposure (adaptive/memory response) to tumor/RSV antigens. Specifically, my thesis project focused on examining the type, relative amount, and time-course of various types of leukocytes infiltrating the GF pulp in response to injection of syngeneic tumor lysates in U-AP compared to S-AP chickens and U-AR compared to S-AP chickens.

For these types of studies, injection of a tissue-matched (syngeneic, same MHC-type) lysate is important to avoid generating a tissue-rejection response that would mask the tumor lysate specific innate and adaptive immune response (Abbas et al, 2015). The MHC type of the AP chickens is  $B^{13/13}$  while the AR chickens is MHC type  $B^{21/21}$  (Sponakas, 2007). Because of the different MHC-types in the AR compared to the AP line of chickens, tumor lysate prepared from an AP progressing tumor was injected into GF of AP chickens, and the lysate prepared from an AR regressing tumor was injected into GF of AR chickens. With the tissue compatibility in individuals within a line, the responses observed to the lysate can be interpreted to be triggered by tumor antigens.

To examine leukocyte infiltration into the pulp of tumor lysate injected GF, pulp cells suspension were prepared and immunofluorescently stained to identify various types of leukocyte populations. Cell population analysis was then carried out by flow cytometry to determine the relative amount (% pulp cell suspension) of the various immune cell populations infiltrating/present in the pulp at various times post-lysate injection throughout the course of one week. We hypothesized that within a line of chickens (AP, AR) the types, amount and time-course of the responding leukocytes would differ between the innate/primary immune response in U-chickens and adaptive effector/memory response in S chickens.

For both the AP and AR lines of chickens, however, only few differences were observed in the leukocyte infiltration profiles between U and S chickens; although two way analysis of variance revealed treatment (U vs S) by time (days post-lysate injection) interactions indicating that the two treatment groups responded differently over the time course of the study (Table 2). For AP chickens, the few quantitative differences in leukocytes infiltration in U-AP compared to S-AP chickens included lower total leukocyte-, total mononuclear cell- and macrophage-infiltration levels on Day 1 (Figure 1, 2 and 4), and higher total leukocyte-, total mononuclear cell-, heterophil-, total T cell-, CD4+ T helper cell-, and CD8+ cytotoxic cell-infiltration levels on Day 7 (Figures 1-3, 8, 10, and 11). The early (Day 1) heightened response of the S-AP compared and U-AP suggests development of cell-mediated immunity in S-AP chickens to the tumor lysate. The later (Day 7) heightened infiltration of various T cell subpopulations and higher T to B cell ratio (Figure 13) in U-AP compared to S-AP suggests development of a primary cell-mediated immune response in the U-AP chickens and recruitment of effector cells to the site of lysate injection. Further studies on the functional state of these infiltrating cells are needed to confirm development of an anti-tumor cell-mediated response in AP chickens and assess the functional abilities of the responding cells.

For AR chickens, the few quantitative differences in leukocytes infiltration in U-AP compared to S-AP chickens included lower heterophil-infiltration on Day 2 (Figure 3) and higher B cell-, gamma-delta T cell-, and CD8+ cytotoxic T cell-infiltration on Day 2 (Figure 6, 9 and 11). Additionally, the T to B cell ratio was elevated in both U-AR and S-AR chickens on Day 7, with higher levels in S-AR chickens (Figure 13). Together, these observations suggest an early recruitment of lymphocyte population in U-AR chickens, such as B cells, gamma-delta T and CD8+ lymphocytes that may be part of “innate” immune activities setting the stage for

appropriate anti-tumor adaptive immune response. Gamma-delta T cells are often considered to be part of innate immunity and further analysis may reveal the participating B cells to be IgM+ B1 B cell and the CD8+ lymphocytes to be natural killer cells, which would also categorize these lymphocytes as part of innate immunity (Abbas et al., 2015). Together, these findings do however suggest a cell-mediated immune activities in AR chickens. Development of cell-mediated immunity is further supported by the high T to B cell ratio observed on Day 7 in both U-AR and S-AR chickens, like representing T effector/memory cell arrival in the target tissue.

Although statistical comparison between AP and AR chickens is not possible, because the studies were conducted at different times by different individuals, side by side comparisons between the profiles of responding leukocyte does reveal differences that need further investigation. Some apparent differences between AP and AR leukocyte infiltration profiles were observed on Day 1 post-lysate injection when AP chickens had higher levels of total leukocyte-, total mononuclear cell-, heterophil-, macrophage-, B cell-, and gamma-delta T cell-infiltration than AR chickens (Figure 1-4, 6 and 9). Other striking differences between AP and AR infiltration responses to tumor lysates were observed regarding recruitment of T cells. Total T cell-, alpha-beta TCR+ T cell-, and CD4+ T cell-infiltration was lower in AP then AR chickens on Day 1 and 2 (Figure 7, 8 and 10), as was the CD4 to CD8 cell ratio on Day 1&2 (Figure 12) and the T to B cell ratio on Day 1 and 7 post-lysate injection (Figure 13). Together, the early higher and more sustained infiltration of T cells and the participation of the more “sophisticated” alpha-beta TCR+ T cells in AR compared to AP chickens supports the concept of an earlier and qualitatively better cell-mediated anti-tumor response in AR chickens. The most-striking differences between AP and AR chickens that emerged through side-by-side comparison were the infiltration profiles of CD8+ cells and CD4+ cells. In AP chicken, relative levels of CD4+ T

cells dropped substantially below pre-injection levels between Day 0 and 2 (Figure 10 ), whereas in AR chickens, CD4 + T cell levels increased between Day 0 and 2, reaching peak infiltration levels on Day 2. Moreover, in AP chickens, levels of CD8+ cell infiltration on Day 1 and 2 (Figure 11) were much higher than those observed in AR chickens. These different infiltration profiles of CD4+ and CD8+ cells in AP compared to AR chickens are also clearly reflected in the CD4 to CD8 cell ratio, which was substantially lower in AP chickens than AR chickens on Day 1 and 2 (Figure 12). Considering the important role of CD4+ T helper cell in orchestrating and activating an effective cell-mediated immune response, this early divergence in the CD4+ and CD8+ lymphocyte infiltration profiles in AP and AR chickens may well be responsible for the ineffective anti-tumor response of AP chickens.

Taken together, this descriptive study of the types, amounts and time-course of leukocytes responding to tumor lysate injected into GF of unsensitized and sensitized AP and AR chickens, revealed qualitative, quantitative and time-course differences that provide important new knowledge needed to further examine an effective versus ineffective tumor-specific immune response in this unique chicken model.

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