THE EFFECT OF THE AQUEOUS EXTRACT OF *LONICERA JAPONICA* ON

ANTIGEN-STIMULATED T CELL FUNCTION

A THESIS

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF SCIENCE

BY

AUSTIN D. BROOKS

ADVISOR: HEATHER BRUNS

BALL STATE UNIVERSITY

MUNCIE, INDIANA

MAY 2013

THE EFFECT OF THE AQUEOUS EXTRACT OF *LONICERA JAPONICA* ON

ANTIGEN-STIMULATED T CELL FUNCTION

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL

FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

BY

AUSTIN BROOKS

ADVISOR: HEATHER BRUNS

Committee Approval:

_____   _____
Committee Chairperson   Date

_____   _____
Committee Member   Date

_____   _____
Committee Member   Date

Departmental Approval:

_____   _____
Departmental Chairperson   Date

_____   _____
Dean of Graduate School   Date

BALL STATE UNIVERSITY
MUNCIE, INDIANA
MAY 2013

TABLE OF CONTENTS

LIST OF FIGURES

Figure   Page

CHAPTER 1

APPENDICES

ABSTRACT

THESIS: The effects of the aqueous extract of *Lonicera japonica* on antigen-

stimulated T cell function

STUDENT: Austin Brooks

DEGREE: Master of Science

COLLEGE: Science and Humanities

DATE: May 2013

PAGES: 40

Lonicera japonica is a honeysuckle species commonly used in traditional Chinese medicine to treat diverse ailments such as headache, fever, cough and sore throat. The aqueous fraction of *L. japonica* has been shown to have a variety of anti-inflammatory properties in addition to *L. japonica* being hailed as an immune enhancer. Previous studies examining flavonoids, common constituents of *L. japonica* extracts, have demonstrated that flavonoids have varying abilities to inhibit lymphocyte functions. The goal of these studies was to examine the effect of the aqueous fraction of *L. japonica* on T cell functions. Purified murine T cells were assessed for proliferation using an MTT assay following stimulation with plate bound anti-CD3 and soluble anti-CD28 antibodies and/or treatment with *L. japonica*. The expression of CD25, CD95, CD152, and CD178 as well as cell death using propidium iodide staining were analyzed on CD4+ T cells using flow cytometry. An ELISA was used for assessing IL-2 production. Neutralization assays were performed using αCD178 or αCD152 antibodies in determining alterations in T cell death or proliferation. *L. japonica* inhibited T cell proliferation and reduced CD25 expression on activated T cells, while having no effect on IL-2 production. In naïve and

activated CD4+ T cells, *L. japonica* increased the expression of CD95, CD152, and CD178 in addition to cell death. Neutralization of CD178 or CD152 did not abrogate increased cell death or reduced proliferation resulting from *L. japonica* treatment. Our results showed that the aqueous extract of *L. japonica* is inhibitory to T lymphocyte proliferation and potentially occurs through decreased expression of CD25. In addition, *L. japonica* increased cell death which was independent of upregulated CD95 and CD178 expression. Findings from this work provide insights into the immunomodulatory properties of *L. japonica* and subsequent effects on T cell responses.

ACKNOWLEDGEMENTS

CHAPTER 1: The effects of the aqueous extract of *Lonicera japonica* on antigen-stimulated T cell function

INTRODUCTION

During the course of any infection, an important component in generating an effective immune response against the invading pathogen is the activation of T lymphocytes. T cell activation occurs mechanistically as an interaction between a naïve T lymphocyte and an antigen presenting cell (APC) (Davis et al. 2003; Smith-Garvin et al. 2009; Fooksman et al. 2010). This interaction requires engagement of the T cell receptor (TCR), costimulation and adhesion by other surface receptors followed by intracellular signaling launched by phosphorylation of tyrosine residues. TCR engagement is initiated through the peptide display by the major histocompatability complex (MHC) of the APC which becomes bound by the TCR complex consisting of a heterodimer αβ chain and several variant chains of CD3 (Smith-Garvin et al. 2009). Antigen presentation between peptide MHC and TCR is stabilized by CD4 or CD8 associating with the MHC. Antigen presentation alone will produce non-responsive T lymphocytes to stimulation; therefore, T lymphocytes require costimulation for enhanced signaling by a cell surface receptor, mainly CD28, binding with CD80 or CD86 expressed by the APC (Davis et al. 2003; Smith-Garvin et al. 2009). Given that TCR engagement and costimulation occurs over a rate of several hours, activation must be maintained and stabilized by a peripheral ring of

integrins like leukocyte function-associated antigen-1 (LFA-1) and CD2 adhering with their ligands, intracellular adhesion molecule-1 (ICAM-1) and CD58 respectively, on the APC (Bromley et al. 2001; Davis et al. 2003; Smith-Garvin et al. 2009). With sustained TCR stimulation, signaling pathways are activated by the cytoplasmic tails of the TCR and CD28 (Smith-Garvin et al. 2009). Signaling via the TCR occurs by phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) allowing the assembly of multiple adaptor proteins and initiating signaling cascades through phospholipase Cγ, DAG, and calcium-mediated pathways for T cell proliferation and function. Additionally, proline motifs in the cytoplasmic tails of CD28 attract subunits of PI3K for supporting cell proliferation. In summary, T cell activation requires sustained engagement between the TCR and peptide MHC as well as ligation of costimulatory molecules for effective downstream signaling pathways that initiate proliferation and effector functions.

Resulting from downstream signaling following T cell activation, several genes that support T cell proliferation are expressed. One important change is the expression of interleukin-2 (IL-2) and the IL-2 receptor (IL-2R), which is increased following TCR and CD28 engagement (Malek 2008). The IL-2R is expressed as a low or high affinity receptor but only the latter increases T cell activation upon ligand binding of IL-2Rα (CD25) (Malek 2008). The low affinity receptor consists of IL-2Rβ (CD122) and the common gamma chain, while the high affinity IL-2R has CD25, CD122, and the gamma chain (Malek 2008; Boyman et al. 2012). IL-2 is rapidly produced following T cell activation, and binds IL-2R through CD25 on activated T cells to enhance proliferation (Boyman et al. 2012). Although TCR stimulation occurs for hours (Davis et al. 2003), IL-2 and IL-2R components are expressed briefly then the quaternary complex is

internalized and CD25 is recycled back to the cell surface while the remaining components undergo proteosomal degradation (Malek 2008). Recycling maintains a supply of CD25 for re-stimulation of this key signaling pathway in the activation of T cells and makes CD25 a good indicator of T cell activation.

While changes in the expression of the IL-2R components serve to increase T cell proliferation, changes in the expression of other genes occur to downregulate the activated T cell response. T cell activation is mainly terminated through the expression of cytotoxic T lymphocyte associated antigen-4 (CTLA-4 or CD152) after 24-48 hours when CD152 expression is maximized (Smith-Garvin et al. 2009). During maximized expression, CD152 becomes competitively bound to CD80 and CD86 with a greater affinity and avidity than CD28 thereby inhibiting CD28 function and T cell activation (Teft et al. 2006). While ligation of CD152 inhibits T cell proliferation and cytokine production, it also functions to prevent T cells from undergoing activation induced cell death (Teft et al. 2006; Wing et al. 2011). In addition to CD152, Fas (CD95) and FasL (CD178) are also expressed on T cells following activation (Strasser et al. 2009). Ligation of CD95 with CD178 induces apoptosis, which is important for maintaining the homeostasis of T lymphocyte populations generated in response to antigen. The complex activities of T cell activation, expansion, down-regulation, and homeostasis are necessary for the appropriate immune defense and clearance of pathogens.

Medicines are frequently used to aid in immune defense, and herbs are often a major component of medicinal compounds in traditional Chinese medicine. *Lonicera japonica*, commonly known as Japanese Honeysuckle, is one such herb that is commonly used (Schierenbeck 2004). It originates from regions of Asia and is referenced in

traditional Chinese medicinal texts as being beneficial in treating edema, fever and dysentery (Schierenbeck 2004; Shang et al. 2011). Historically, *L. japonica* has been administered alone or mixed with other herb and plant extracts as a tea. Within various brewed concoctions, *L. japonica* has been successful in the prevention and treatment of many infections and ailments including laryngitis, congestion, ulcers, constipation, and inflammation (Shang et al. 2011). The cumulative result of many investigations over several years has been the isolation and identification 147 different compounds from varying components of the plant (i.e. flower, leaf, root, etc) that have diverse functions (Shang et al. 2011), owing to its many uses within traditional Chinese medicine.

The history of *L. japonica* within traditional Chinese medicine has led many researchers to identify its effects on the biological processes that may contribute to the medicinal properties of the herb. Research into the effects of *L. japonica* extracts on immune cells has demonstrated its capabilities as an anti-inflammatory (Shang et al. 2011). In these studies, the aqueous and methanol extracts of *L. japonica* decreased the inflammatory activities of macrophages such as nitric oxide and TNF-alpha production (Kang et al. 2004; Park et al. 2005) as well as inhibited inflammatory enzymatic functions required to produce leukotrienes and prostaglandins for initiating inflammation (Tae et al. 2003; Xu et al. 2007; Ryu et al. 2010).

Brewed tea concoctions of *L. japonica* such as Yin Zhi Huang have been investigated for its influence on immune cell function. Yin Zhi Huang inhibited the proliferation of antigen-stimulated splenocytes, which was partially recovered by the addition of IL-2 (Chen et al. 2004). Similarly, *L. japonica* is a source of numerous flavonoids which have individually demonstrated immunomodulatory effects on

lymphocytes (Shang et al. 2011). The flavone and organic acids, luteolin, chlorogenic acid and caffeic acid, inhibit antigen-stimulated splenocytes (Chen et al. 2004). Other flavones including chrysin, apigenin, luteolin(e), kaempferol, and quercetin diminished the proliferation of Concanavalin-A activated splenocytes (Namgoong et al. 1994; Lee et al. 1995), and ochnoflavone demonstrated the ability to irreversibly inhibit splenocyte proliferation (Namgoong et al. 1994). Although Concanavalin-A is an antigen capable of inducing T cell proliferation through the TCR, this lectin elicits a non-specific response from activated T cells (Dwyer et al. 1981). Additional *in vitro* studies examining the effects of quercetin and apigenin on cytotoxic T lymphocyte function demonstrated the ability of those compounds to inhibit proliferation and target cell killing by cytotoxic T lymphocytes (Schwartz et al. 1984). Together, these studies demonstrate the potential ability of varying preparations and compounds of *L. japonica* to influence the function of immune cells.

L. japonica, in varying forms, is becoming increasingly common in immune boosting supplements, such as Airborne, Cold Snap and Nutriferon that are reported to aid in immune responses against viral infections leading to colds or influenza. The current literature demonstrates its ability to serve as an anti-inflammatory, but studies investigating the effects of a variety of *L. japonica* preparations and individual compounds on adaptive immune cell function suggest that *L. japonica* may inhibit cellular functions that would be counter-productive to the generation of an effective immune response to pathogen invasion. Thus, to more clearly identify the effect of *L. japonica* on a specific population of activated lymphocytes, this study investigated the

ability of the aqueous fraction of *L. japonica* to alter T lymphocyte proliferation, survival, and protein expression.

MATERIALS AND METHODS

Mice: Adult male and female C57BL/6J mice between the ages of 8-12 weeks, bred from mating pairs purchased from The Jackson Laboratory (Bar Harbor, ME), were used for each study. Methods involving mice were approved by the Ball State University Animal Care and Use Committee (IACUC).

Preparation of L. japonica: The extract was generated by dissolving 1 g of Honeysuckle flower 5:1 single herb extract powder (MayWay, Oakland, CA) into 10 mL of RPMI-1640, generating a 100 mg/mL solution. The solution was then filtered through a series of membranes with increasingly smaller pores until a final filter of 0.45 μm was used.

Lymphocyte isolation: Lymphocytes were harvested from the spleen by maceration in complete RPMI-1640 culture medium supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), penicillin-streptomycin, sodium pyruvate, non-essential amino acids, $_L$-glutamine, HEPES, and $5x10^{-5}$ M 2-mercaptoethanol (all from Sigma Chemical, St. Louis, MO). Lymphocyte suspensions were treated with RBC lysis buffer with 0.83% NH_4Cl (MP Biomedicals, Santa Ana, CA), 0.01 M Tris (Sigma), pH 7.4, then washed and resuspended in complete RPMI-1640. Numbers of lymphocytes were determined by trypan blue exclusion.

In vitro stimulation: Total splenocytes ($2x10^6$ cells/mL) were divided into 4 treatment groups and plated in triplicate. Total splenocytes were treated with plate bound 0.5 μg/mL anti-CD3 (eBioscience, San Diego, CA) and 1.0 μg/mL anti-CD28 (eBioscience)

or neither in the presence or absence of 1000 µg/mL *L. japonica* (MayWay). Plates were coated with 0.5 µg/mL anti-CD3 for 24 hours before T lymphocyte stimulation. Splenocytes were incubated at 37ºC and 5% CO_2 for 6, 12, 24, 48 or 96 hours as indicated in the results.

Cell surface marker analysis: Following incubation, cells were resuspended in FACS buffer (1X PBS with 2% BSA, 0.1% NaN_3) and incubated (10 min, 4ºC) with fluorochrome-conjugated antibodies: anti-mouse CD4, CD95, CD178, CD25, and CD152 (eBioscience). Experiments were performed with 3 replicates for each treatment condition. Cells were analyzed by an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The mean fluorescence intensity (+/- SEM) of CD95, CD178, CD25, and CD152 on CD4-gated lymphocytes per time point was normalized to unstained controls. One way ANOVA with a SNK (Student-Newman-Keuls) multiple comparison test was used for statistical analysis of mean fluorescence intensity of treatment groups within time points using SigmaPlot 12.0 software.

Analysis of Cell Death: Following incubation, lymphocytes were stained with propidium iodide (Sigma Chemicals) and FITC-conjugated anti-mouse CD4 (eBioscience). Experiments were performed with 3 replicates for each treatment condition. Percentages of propidium iodide stained CD4+ T cells were determined using an Accuri C6 flow cytometer (BD Biosciences). One way ANOVA with a SNK multiple comparison test was used for the statistical analysis of the percentage of PI+ CD4 cells for treatment groups within time points using SigmaPlot 12.0 software.

Lymphocyte purification: The MidiMACS cell separator kit was used to purify T lymphocyte populations from the spleen according to the manufacture's guidelines (Miltenyi Biotech, Cambridge, MA). Briefly, cell suspensions were suspended in MACS buffer (1X phosphate buffer solution, 0.5% bovine serum albumin, 2mM EDTA, and pH 7.2). Beads conjugated to anti-CD90 beads isolated T lymphocytes using the Miltenyi LS column. Following isolation of the desired cell population, a cell count via trypan blue exclusion was performed and isolated lymphocytes were used in the proliferation assay.

Proliferation Assay: Purified T cells ($1x10^6$ cells/mL) were added to a 96 well plate (100,000 cells/well) and stimulated as described above according to the 4 treatment groups. T cells were incubated at 37ºC (5% CO_2) for 24, 48, and 96 hours. Following incubation, proliferation was assessed using the MTT Cell Proliferation Assay kit (ATCC, Manassas) according to the manufacturer's guidelines. Absorbance was determined using a BIO-RAD Model 680 microplate reader at 570 nm. Experiments were performed with 3 replicates for each treatment condition. The absorbance (+/- SEM) was normalized to unstained controls. Kruskal-Wallis ANOVA with a SNK multiple comparison test was used for statistical analysis of treatment groups within time points using SigmaPlot 12.0 software.

Enzyme-linked Immunosorbant Assay (ELISA): Supernatants from T cells stimulated for 24 or 96 hours were analyzed via ELISA for levels of IL-2 (quantification kit from Leinco Technologies, St. Louis, MO) according to the manufacturer's guidelines. Briefly, plates were coated with capture antibody (anti-mouse IL-2). After overnight incubation

(4ºC), plates were washed and a blocking solution was added. After 30 minutes, samples and standards were added for a 2 hour incubation at room temperature, which was followed by a wash and then the addition of a detection antibody (anti-mouse IL-2 conjugated to HRP). Following incubation with the detection antibody, the plates were washed and substrate was added. Plates were incubated with substrate for 15-20 minutes followed by the addition of stop solution (2N H_2SO_4). Experiments were performed with 3 replicates for each treatment condition. Absorbance at 450nm was determined using a BIO-Rad model 680 plate reader. One-way ANOVA was used for statistical analysis of treatment groups within time points using SigmaPlot 12.0 software.

Fas Ligand neutralization: Splenocytes ($2x10^6$ cells/mL) were divided into 4 treatment groups as described above and 15 µg/mL anti-Fas Ligand neutralizing antibody (Leinco) was added to cells in each treatment group. Splenocytes were incubated at 37ºC and 5% CO_2 for 24 and 96 hours. Following the appropriate incubations, the splenocytes were resuspended in FACS buffer and incubated (10 min, 4ºC) with FITC-conjugated anti-mouse CD4 (eBioscience) and propidium iodide (Sigma Chemicals). Experiments were performed with 3 replicates for each treatment condition. Cells were analyzed by an Accuri C6 flow cytometer (BD Biosciences). The mean fluorescence intensity (+/- SEM) per time point was normalized to unstained controls. One way ANOVA with a SNK multiple comparison test was used for statistical analysis of mean fluorescence intensity of treatment groups within time points using SigmaPlot 12.0 software.

CTLA-4 neutralization: Purified T cells were divided into 4 treatment groups as described above, and 20 μg/mL anti-mouse CD152 antibody (Leinco) was added to cells in each treatment group. T cells were incubated at 37ºC (5% CO_2) for 24, 48, and 96 hours. Following incubation, proliferation was assessed using the MTT Cell Proliferation Assay kit (ATCC) according to the manufacturer's guidelines. Absorbance was determined using a BIO-RAD Model 680 microplate reader at 570 nm. Experiments were performed with 3 replicates for each treatment condition. The absorbance (+/- SEM) was normalized to background controls. One way ANOVA with a SNK multiple comparison test was used for statistical analysis of treatment groups within time points using SigmaPlot 12.0 software.

RESULTS

        To increase the knowledge about the effect of the herbal supplement, *L. japonica*, on immune function, the influence of the aqueous fraction of *L. japonica* on T lymphocyte functions was examined. Critical to the initiation of any effective immune response is the activation and subsequent proliferation of T lymphocytes. Previous research involving compounds present in *L. japonica* have demonstrated that biflavonoids can inhibit the proliferation of splenocytes (Namgoong et al. 1994; Lee et al. 1995). To examine the effects of *L. japonica*, on a purified population of antigen-stimulated T lymphocytes specifically, T cells were isolated from the spleens of C57BL6/J mice by magnetic sorting and stimulated with anti-CD3 and anti-CD28 in the presence or absence of *L. japonica*. Relative levels of proliferation between treatment groups were determined using an MTT assay. Similar to previous studies, treatment of anti-CD3-stimluated T cells with *L. japonica* inhibited proliferation compared to controls (Fig 1). These results demonstrate that proliferation of activated T lymphocytes is reduced in the presence of *L. japonica.*

        The proliferation of T lymphocytes involves a complex mechanism requiring the secretion of Interleukin-2 (IL-2) for clonal expansion and development (Malek 2008). A potential explanation for the decreased proliferation is a lack of IL-2 production from T lymphocytes. To investigate the effect of *L. japonica* on IL-2 production, lymphocytes isolated from the spleens of C57BL/6J mice were stimulated with anti-CD3 and anti-CD28 in the presence or absence of *L. japonica*. After 24 and 96 hours, supernatants were collected and examined for levels of IL-2 by ELISA. *L. japonica* did not significantly alter IL-2 production by stimulated T cells at 24 or 96 hours (Fig 2 A, B). Although our
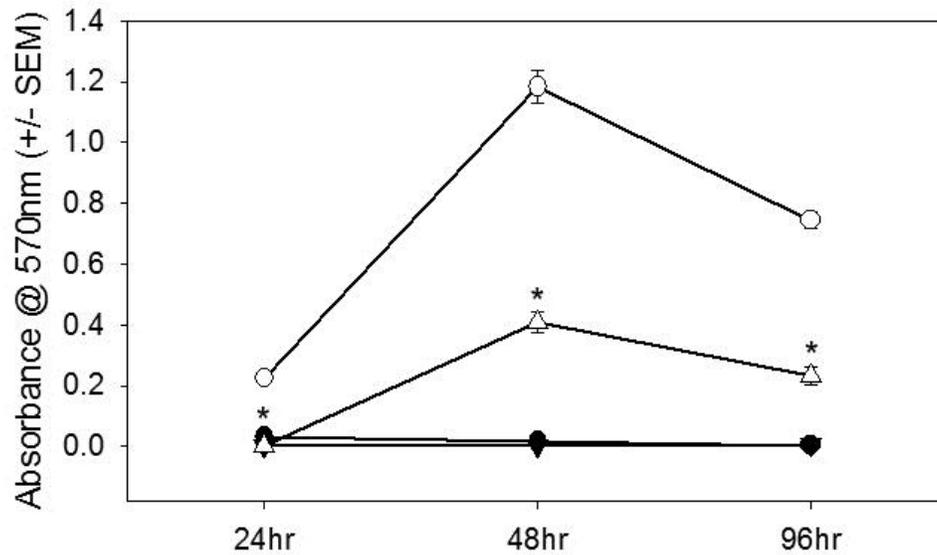
Figure 1: *L. japonica* **inhibits the proliferation of stimulated T lymphocytes.** Isolated T cells were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*. Amount of proliferation was determined at indicated time points using the MTT assay. Experiments were performed in triplicate. Statistically significant differences were determined by Kruskal-Wallis ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * p ≤ 0.05 αCD3 vs αCD3/LJ. Treatment groups are designated as Control (filled circle), αCD3 (open circle), *L. japonica* (filled triangle), and αCD3+*L. japonica* (open triangle).

A.

B.
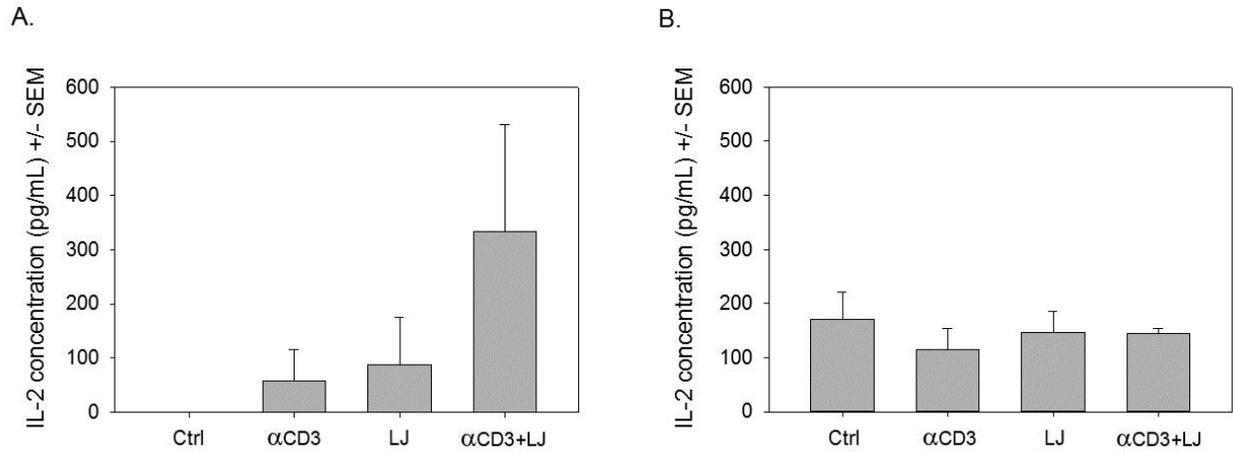
**Figure 2:** *L. japonica* **does not alter IL-2 production at 24 (A) or 96 hours (B).** Total splenocytes were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*. At 24 and 96 hours, supernatants were harvested and IL-2 concentration was examined using a mouse IL-2 ELISA. IL-2 concentrations were determined based on a recombinant IL-2 standard along with supernatants using a Bio-Rad Model 680 Microplate Reader. IL-2 concentration was compared among treatment groups and statistically analyzed by One-Way ANOVA using SigmaPlot software.
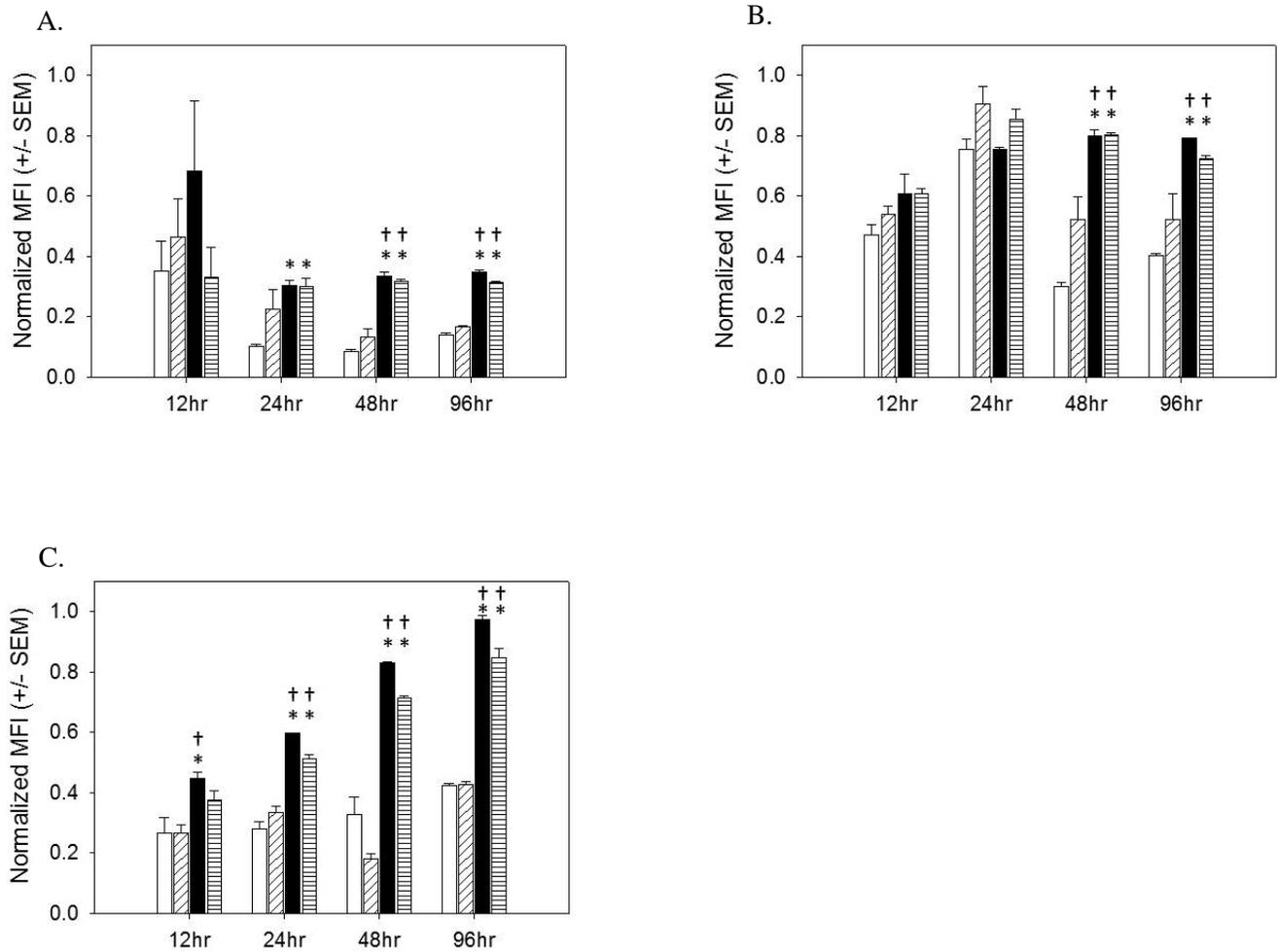
findings did not demonstrate an effect of *L. japonica* treatment on IL-2 production by activated T cells, *L. japonica* could alter the sensitization of activated T cells to IL-2 by altering the expression of the IL-2R. In the high affinity IL-2R, the presence of CD25 increases IL-2 binding and subsequent signaling during T cell activation, enhancing proliferation (Boyman et al. 2012). To determine the effect of *L. japonica* treatment on CD25 expression, total splenocytes were isolated and stimulated with anti-CD3 and anti-CD28 in the presence or absence of *L. japonica*. *L. japonica* treatment reduced anti-CD3-stimulated-CD25 expression (Fig 3), suggesting that *L. japonica* suppresses the proliferation of activated T cells by decreasing the expression of CD25.

In addition to analyzing CD25 expression, the effect of *L. japonica* treatment on the expression of other surface markers indicative of T cell activation was examined. *L. japonica* increased the expression of CD95 and CD178 on anti-CD3-stimulated and non-stimulated CD4+ T cells (Fig 4A, B). Likewise, CD152 expression on both anti-CD3-stimulated and non-stimulated CD4+ T cells was enhanced by *L. japonica* treatment alone (Fig 4C). These results demonstrate the ability of *L. japonica* to alter the expression of proteins indicative of activation and suggest that the observed decrease in proliferation may result not solely from decreased CD25 expression but potentially through the increased expression of CD152 (downregulating T cell activation) and CD95 and CD178 (inducing cell death).

To assess the effect of *L. japonica* on cell death, lymphocytes were isolated from the spleen and stimulated with anti-CD3 and anti-CD28 in the presence or absence of *L. japonica*. After each time point, CD4+ T lymphocytes were analyzed by propidium iodide (PI) staining to assess cell death. After 24 hours, *L. japonica* treatment alone or in
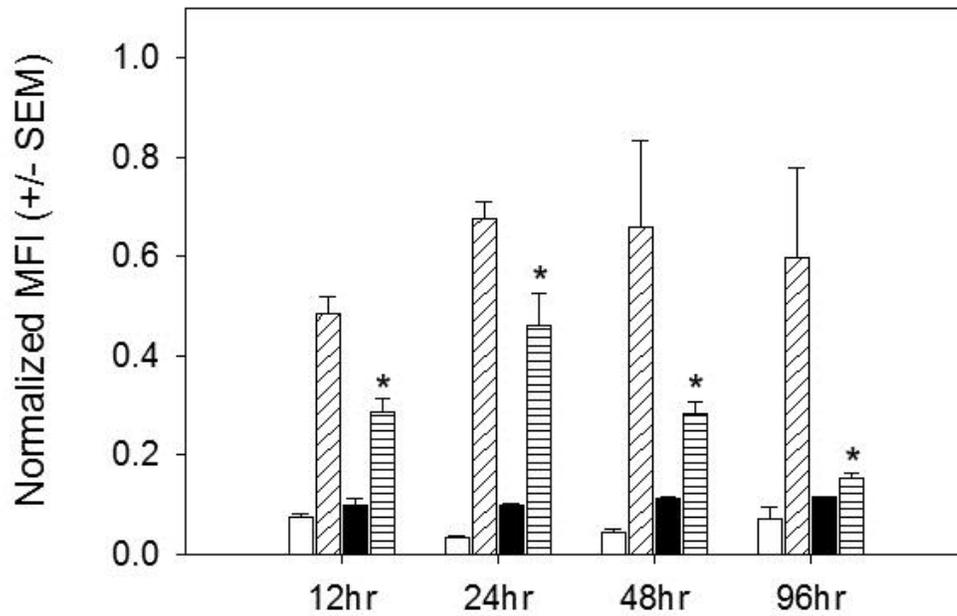
Figure 3: CD25 (IL2Rα) is suppressed by *L. japonica* in stimulated T lymphocytes. Total splenocytes were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*. At each time point, cells were harvested and stained with anti-mouse antibodies for CD4 and CD25. Relative levels of αCD3-stimulated CD25 expression on CD4+ T cells were determined using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Mean fluorescence intensity values were normalized to negative controls and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * p≤ 0.05 αCD3 vs αCD3+LJ. Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), and αCD3+*L. japonica* (horizontal hash).

A.

B.

C.


Figure 4: *L. japonica* **enhances the expression of CD95 (A), CD178 (B), and CD152 (C) in unstimulated and αCD3-stimulated T lymphocytes over time.** Total splenocytes were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*. At each time point, cells were harvested and stained with antibodies to CD4, CD95, CD178, and CD152. Relative levels of anti-CD3-stimulated CD95 (A), CD178 (B), or CD152 (C) expression on CD4+ T cells were determined using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Mean fluorescence intensity values were normalized to negative controls and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * $p \leq 0.05$ vs ctrl, † $p \leq 0.05$ vs αCD3. Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), and αCD3+*L. japonica* (horizontal hash).

conjunction with anti-CD3 stimulation increased cell death, as demonstrated by an increased percentage of PI+ cells compared to the negative control and anti-CD3 stimulation control (Fig 5). These results demonstrate that treatment with *L. japonica* increases cell death in activated T cell populations and suggests another mechanism contributing to the diminished proliferation of T lymphocytes.

Programmed cell death is activated through the receptor-ligand binding of CD95 and CD178 (Strasser et al. 2009). Given the increased death of activated T cells and the expression of CD95 and CD178 on T cells treated with *L. japonica*, the survival of T cells was assessed in the presence of a neutralizing antibody to CD178. Total splenocytes were isolated and stimulated with anti-CD3 and anti-CD28 with or without *L. japonica* in the presence or absence of a CD178 neutralizing antibody. After 24 and 96 hours, cells were analyzed using PI staining to determine cell death. While the CD178 neutralizing antibody decreased cell death in the control groups at 96 hours, the increase in cell death stimulated by *L. japonica* was not dependent upon CD178, as demonstrated by similar percentages of PI+ cells in the *L. japonica* treated cells in the presence and absence of CD178 neutralizing antibody (Fig 6). This result suggests the death of CD4 T cells occurs independent of the increased expression of CD95 and CD178.

Proliferation in T cells may be minimized through mechanisms other than cell death. After a T cell response, activated T cells are downregulated by increased expression of CD152 at the activation complex, and proliferation of activated T cells is reduced through maintained CD152 expression (Smith-Garvin et al. 2009). To determine if decreased proliferation in CD4+ T cells from *L. japonica* treatment was induced by the upregulation of CD152, T lymphocytes were purified and stimulated with anti-CD3 and
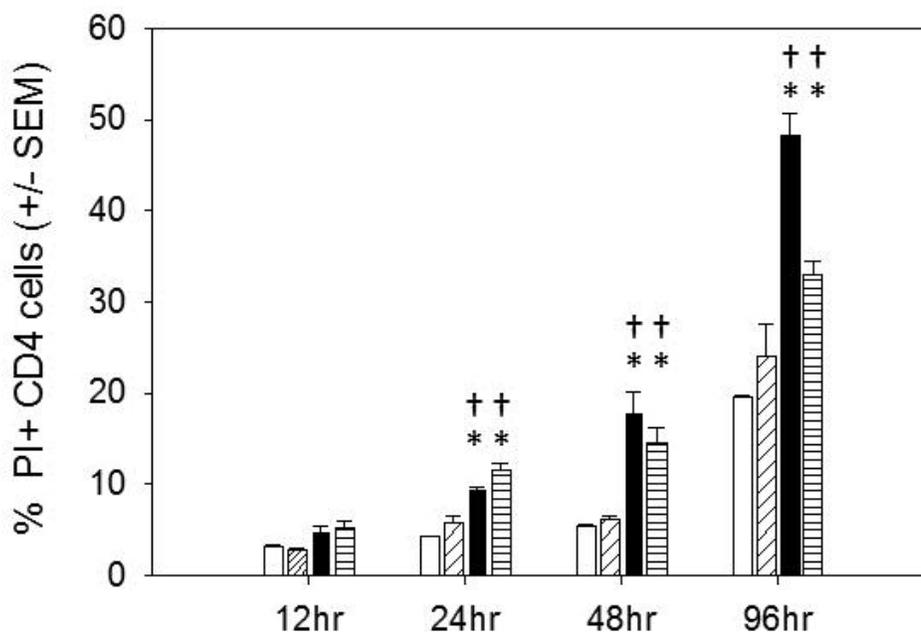
Figure 5: ***L. japonica* enhances cell death in CD4+ T lymphocytes.** Total splenocytes were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*. At each time point, cells were harvested and stained with propidium iodide (PI). Percentage of PI+ cells was determined for treatment groups using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Percentages of PI+ cells were determined from CD4+ cells and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * $p \leq 0.05$ vs ctrl, † $p \leq 0.05$ vs αCD3. Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), and αCD3+*L. japonica* (horizontal hash).
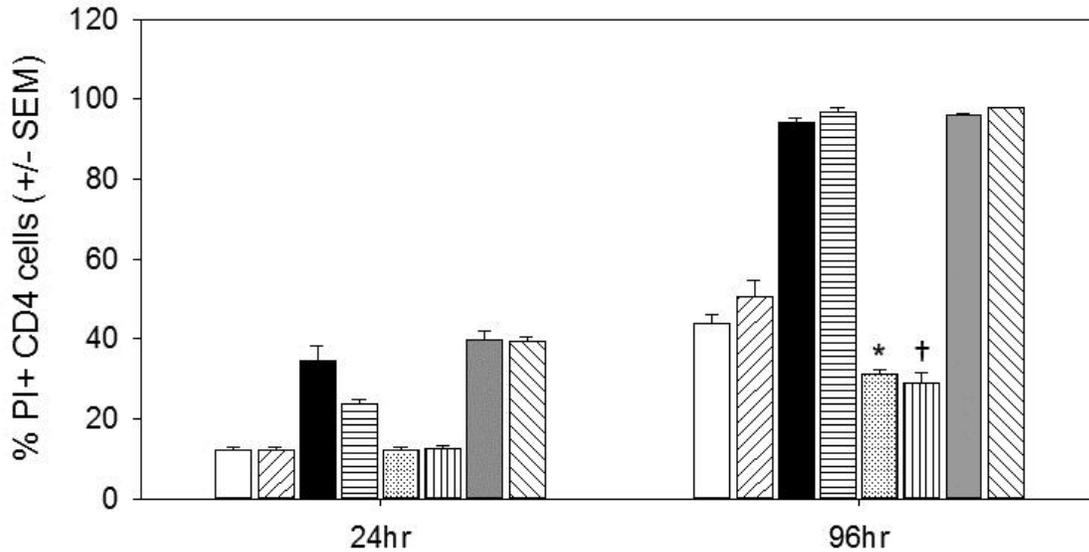
Figure 6: *L. japonica* **boosts CD4+ T cell death in a CD178-independent manner.** Total splenocytes were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*, with or without 15 μg/mL αCD178 antibody. At each time point, cells were harvested and stained with propidium iodide. Percentage of PI+ cells was determined for treatment groups using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Percentages of PI + cells were determined from CD4+ cells and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * $p \leq 0.05$ vs ctrl, † $p \leq 0.05$ vs αCD3. Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), αCD3+*L. japonica* (horizontal hash), αCD178 Control (dots), αCD178 αCD3 (vertical hash), αCD178 *L. japonica* (grey), and αCD178 αCD3+*L. japonica* (left angle hash).

23

anti-CD28 with or without *L. japonica* in the presence or absence of a neutralizing antibody to CD152. After 24, 48, and 96 hours, cells were analyzed using the MTT assay to determine proliferation in the presence of anti-CD152. Although treatment with anti-CD152 increased proliferation in anti-CD3 stimulated T cells at 48 and 96 hours, this result was not observed in the anti-CD3 stimulated T cells treated with *L. japonica* (Fig 7), suggesting that increased expression of CD152 on activated T cells following treatment with *L. japonica* did not contribute to the reduced proliferation of those T cells.

These data demonstrate that *L. japonica* decreases the proliferation of activated T cells potentially through its decreased expression of CD25 on activated T cells, and through a mechanism that is independent of enhanced CD152 expression. Additionally, activated CD4+ T cells treated with *L. japonica* have increased cell death concomitant with enhanced CD95 and CD178 expression. However, neutralization of CD178 did not result in decreased cell death. Taken together these data demonstrate that the aqueous fraction of *L. japonica* has dramatic effects on T cell proliferation and survival.
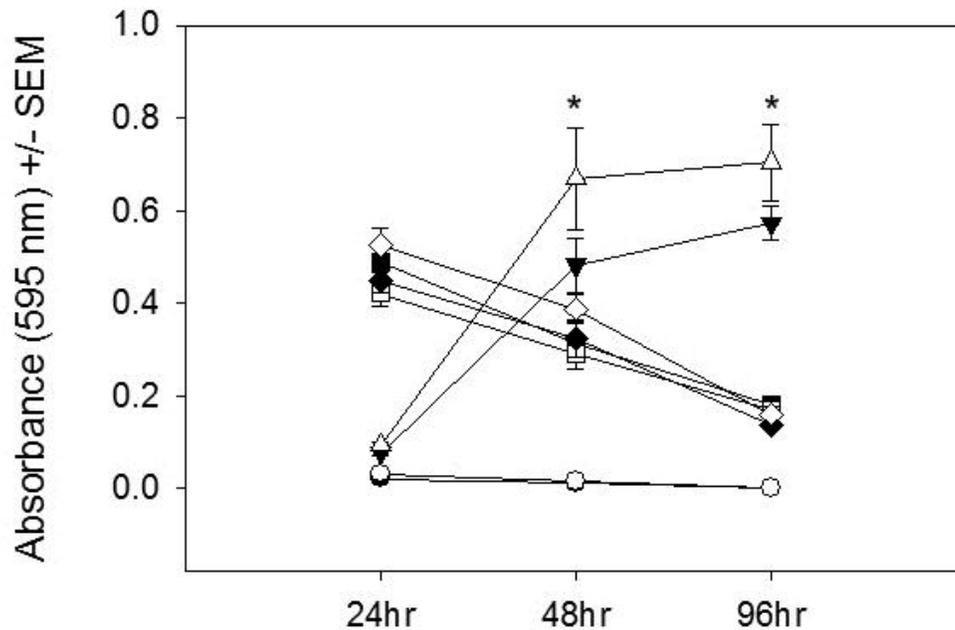
Figure 7: *L. japonica* diminishes T cell proliferation independent of CD152. Isolated T cells were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica* with or without 10 or 20 µg/mL αCD152 antibody. Amount of proliferation was determined at indicated time points using the MTT assay. Experiments were performed in triplicate. Statistically significant differences were determined by ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * $p \leq 0.05$ vs αCD3. Treatment groups are designated as Control (filled circle), αCD3 (filled triangle), *L. japonica* (filled square), and αCD3+*L. japonica* (filled diamond), αCD152 Control (open circle), αCD152 αCD3 (open triangle), αCD152 *L. japonica* (open square), and αCD152 αCD3+*L. japonica* (open diamond).

DISCUSSION

In understanding the immune properties of *L. japonica*, various preparations, decoctions and individual compounds of *L. japonica* have been examined for their varying cellular effects on both innate and adaptive immune responses. To the best of our knowledge, the findings from this study are novel due to the type of *L. japonica* preparation investigated and the first to demonstrate the suppression of function and cytotoxic effect of the aqueous extract of *L. japonica* alone on T lymphocytes. The aqueous fraction of *L. japonica* inhibited the proliferation of antigen-stimulated CD4+ T lymphocytes, concomitantly reducing CD25 and increasing CD152 expression. Furthermore, treatment of CD4+ T cells with *L. japonica* increased cell death which paralleled but was independent of increased CD95 and CD178 expression. These data suggest that the use of this aqueous extract of *L. japonica* is potentially detrimental to the generation of an effective T cell response and therefore may not have the desired medicinal benefits that are historically known of *L. japonica* in traditional Chinese medicine.

Flavonoids have been investigated for their immunomodulatory properties on T lymphocytes, and *L. japonica* has been documented to have a high content of flavonoids (Shang et al. 2011). More specifically, these studies investigated the effects of various flavonoids on stimulated murine T lymphocytes. First, Schwartz et al. (1984) showed the flavonoids apigenin, quercetin, and rutin suppressed cytotoxic T lymphocyte proliferation stimulated in a mixed leukocyte reaction (Schwartz et al. 1984). Likewise, several flavonoids including apigenin, chrysin, kampferol, luteolin(e), and quercetin inhibited splenocyte proliferation stimulated by Concanavalin-A or mixed leukocyte reactions

(Namgoong et al. 1994). Additionally, the specific biflavonoids apigenin and ochnoflavone suppressed Concanavalin-A stimulated splenocyte proliferation (Lee et al. 1995). Based on these studies, several flavonoids that are present in *L. japonica* (Shang et al. 2011) inhibit T lymphocyte proliferation independent of the mechanism of activation. Importantly, these studies did not show cytotoxicity of these compounds on T lymphocytes. While our study supports the prior investigations demonstrating the inhibitory effect of flavonoids on T cell function, it also demonstrates a potential harmful combination of compounds extracted from the flowers of *L. japonica* that elicit cytotoxic and not just suppressive characteristics.

A prior investigation into the immunomodulatory properties of decoctions containing multiple Chinese herbal medicines demonstrated the ability of Yin Zhi Huang (YZH) to inhibit antigen-stimulated T cell proliferation (Chen et al. 2004). A major component of YZH is the extract from the flower buds of *L. japonica*. Similar to our work, this study not only demonstrated a suppression of T cell proliferation but also decreased expression of CD25 on activated CD4+ T cells. Contrastingly, our study investigated the ability of the *L. japonica* aqueous extract alone to affect CD4+ T cell function. Our findings support those of Chen et al. (2004) demonstrating that the *L. japonica* extract alone or in combination with other herbal extracts can suppress T cell function. However, the study involving YZH states (the data was not shown) that there was no cytotoxic effect of YZH on the T lymphocytes as assessed by trypan blue exclusion. Our data demonstrated that the aqueous extract of *L. japonica* increases cell death of both resting and stimulated CD4+ T cells (Figure 5). A novelty of our findings was that increased cell death of CD4+ T cells occurred independently of increased

expression of the T cell activation inhibitor CD152 (Figure 6) or the apoptotic receptor/ ligand CD95 and CD178 (Figure 7). Taken together these results suggest that the aqueous extract of *L. japonica* alone may be more cytotoxic to T lymphocytes than when used in combination with other herbal extracts as a concoction.

While many investigations have been done *in vitro*, a few *in vivo* investigations have demonstrated varying properties of *L. japonica* concoctions to reduce lymphocyte presence during infection. For example, the herbal concoction Jin Ying Tang consists of nine different plant extracts including *L. japonica* that individually are known to have anti-bacterial, anti-inflammatory, and other immunomodulatory properties (Wang et al. 2012). Wang et al. (2012) demonstrated the effect of Jin Ying Tang treatment to reduce mastitis in rabbits infected with *Staphylococcus aureus*. In this study, pretreatment before or treatment after *S. aureus* infection with Jin Ying Tang reduced leukocyte aggregation and lymphocyte counts in infected mammary tissue. In addition to the loss of lymphocytes, Jin Ying Tang also decreased the degeneration of the infected epithelial mammary tissue during inflammation. The results of Wang et al. (2011) showed Jin Ying Tang decreased lymphocyte presence during *S. aureus* infection and contributed to reducing the occurrence of mastitis in rabbits, highlighting *in vivo* the benefit of downregulating T cell function due to *L. japonica* concoctions demonstrated by previous *in vitro* investigations. Our study revealed that the aqueous extract of *L. japonica* alone decreased proliferation but also increased cell death in CD4+ T lymphocytes, suggesting that individual *L. japonica* treatment may reduce an effective T cell response and thus not be immunologically advantageous. Furthermore, these are initial findings that support

further investigation into the effect of the aqueous extract of *L. japonica* on immune function *in vivo*.

In conclusion, the novelty of this study was demonstrated by the inhibitory and cytotoxic effect of the preparation of this aqueous extract of *L. japonica* on antigen-stimulated T lymphocytes. These findings highlight important differences in the preparations of *L. japonica* and its individual constituents on immune function and provide a basis for future investigations into the immune effects from varied extracts of *L. japonica* both *in vitro* and *in vivo*.
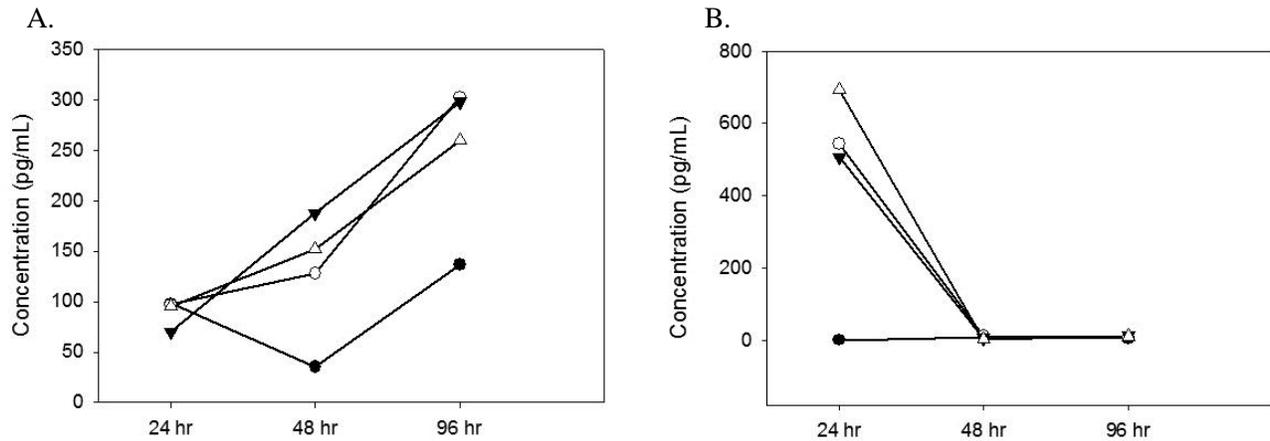
REFERENCES

Boyman, O. and J. Sprent (2012). "The role of interleukin-2 during homeostasis and activation of the immune system." Nat Rev Immunol **12**(3): 180-190.

Bromley, S. K., W. R. Burack, K. G. Johnson, K. Somersalo, T. N. Sims, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen and M. L. Dustin (2001). "The immunological synapse." Annu Rev Immunol **19**: 375-396.

Chen, X., T. Krakauer, J. J. Oppenheim and O. M. Z. Howard (2004). "Yin Zi Huang, an injectable multicomponent Chinese herbal medicine, is a potent inhibitor of T-cell activation." Journal of Alternative and Complementary Medicine **10**(3): 519-526.

Davis, M. M., M. Krogsgaard, J. B. Huppa, C. Sumen, M. A. Purbhoo, D. J. Irvine, L. C. Wu and L. Ehrlich (2003). "Dynamics of cell surface molecules during T cell recognition." Annu Rev Biochem **72**: 717-742.

Dwyer, J. M. and C. Johnson (1981). "The use of concanavalin A to study the immunoregulation of human T cells." Clin Exp Immunol **46**(2): 237-249.

Fooksman, D. R., S. Vardhana, G. Vasiliver-Shamis, J. Liese, D. A. Blair, J. Waite, C. Sacristan, G. D. Victora, A. Zanin-Zhorov and M. L. Dustin (2010). "Functional anatomy of T cell activation and synapse formation." Annu Rev Immunol **28**: 79-105.

Kang, O. H., Y. A. Choi, H. J. Park, J. Y. Lee, D. K. Kim, S. C. Choi, T. H. Kim, Y. H. Nah, K. J. Yun, S. J. Choi, Y. H. Kim, K. H. Bae and Y. M. Lee (2004). "Inhibition of trypsin-induced mast cell activation by water fraction of Lonicera japonica." Arch Pharm Res **27**(11): 1141-1146.

Lee, E. J., J. S. Kim, H. P. Kim, J. H. Lee and S. S. Kang (2010). "Phenolic constituents from the flower buds of Lonicera japonica and their 5-lipoxygenase inhibitory activities." Food Chemistry **120**(1): 134-139.

Lee, S. J., J. H. Choi, K. H. Son, H. W. Chang, S. S. Kang and H. P. Kim (1995). "Suppression of mouse lymphocyte proliferation in vitro by naturally-occurring biflavonoids." Life Sci **57**(6): 551-558.

Malek, T. R. (2008). "The biology of interleukin-2." Annu Rev Immunol **26**: 453-479.

Namgoong, S. Y., K. H. Son, H. W. Chang, S. S. Kang and H. P. Kim (1994). "Effects of naturally occurring flavonoids on mitogen-induced lymphocyte proliferation and mixed lymphocyte culture." Life Sciences **54**(5): 313-320.

Park, E., S. Kum, C. Wang, S. Y. Park, B. S. Kim and G. Schuller-Levis (2005). "Anti-inflammatory activity of herbal medicines: inhibition of nitric oxide production and tumor necrosis factor-alpha secretion in an activated macrophage-like cell line." Am J Chin Med **33**(3): 415-424.

Ryu, K. H., H. I. Rhee, J. H. Kim, H. Yoo, B. Y. Lee, K. A. Um, K. Kim, J. Y. Noh, K. M. Lim and J. H. Chung (2010). "Anti-inflammatory and analgesic activities of SKLJI, a highly purified and injectable herbal extract of Lonicera japonica." Biosci Biotechnol Biochem **74**(10): 2022-2028.

Schierenbeck, K. A. (2004). "Japanese Honeysuckle (*Lonicera japonica*) as an Invasive Species: History, Ecology, and Context." Crit Rev Plant Sci **23**(5): 391-400.

Schwartz, A. and E. Middleton, Jr. (1984). "Comparison of the effects of quercetin with those of other flavonoids on the generation and effector function of cytotoxic T lymphocytes." Immunopharmacology **7**(2): 115-126.

Shang, X., H. Pan, M. Li, X. Miao and H. Ding (2011). "Lonicera japonica Thunb.: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine." J Ethnopharmacol **138**(1): 1-21.

Smith-Garvin, J. E., G. A. Koretzky and M. S. Jordan (2009). "T cell activation." Annu Rev Immunol **27**: 591-619.

Strasser, A., P. J. Jost and S. Nagata (2009). "The many roles of FAS receptor signaling in the immune system." Immunity **30**(2): 180-192.

Tae, J., S. W. Han, J. Y. Yoo, J. A. Kim, O. H. Kang, O. S. Baek, J. P. Lim, D. K. Kim, Y. H. Kim, K. H. Bae and Y. M. Lee (2003). "Anti-inflammatory effect of Lonicera japonica in proteinase-activated receptor 2-mediated paw edema." Clin Chim Acta **330**(1-2): 165-171.

Teft, W. A., M. G. Kirchhof and J. Madrenas (2006). "A molecular perspective of CTLA-4 function." Annu Rev Immunol **24**: 65-97.

Wang, L. U., C. L. He, B. K. He, Q. Guo, C. G. Xiao and Q. Yi (2012). "Effects of Jin-Ying-Tang on Staphylococcus aureus-induced mastitis in rabbit." Immunopharmacol Immunotoxicol **34**(5): 786-793.

Wing, K., T. Yamaguchi and S. Sakaguchi (2011). "Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation." Trends Immunol **32**(9): 428-433.

Xu, Y., B. G. Oliverson and D. L. Simmons (2007). "Trifunctional inhibition of COX-2 by extracts of Lonicera japonica: direct inhibition, transcriptional and post-transcriptional down regulation." J Ethnopharmacol **111**(3): 667-670.

APPENDICES

CYTOKINE ANALYSES

ELISA: Supernatants from T cells stimulated for 24, 48 or 96 hours were analyzed via ELISA for levels of IL-4 and IFNγ (quantification kits from eBioscience, San Diego, CA) according to the manufacturer's guidelines. Briefly, plates were coated with capture antibody (anti-mouse IL-4 or IFNγ). After overnight incubation (4ºC), plates were washed and a blocking solution was added. After 30 minutes, samples and standards were added for a 2 hour incubation at room temperature, which was followed by a wash and then the addition of a detection antibody (anti-mouse IL-4 or IFNγ conjugated to HRP). Following incubation with the detection antibody, the plates were washed and substrate was added. Plates were incubated with substrate for 15-20 minutes followed by the addition of stop solution (2N H_2SO_4). Experiments were performed with 3 replicates for each treatment condition. Absorbance at 450nm was determined using a BIO-Rad model 680 plate reader.

Figure 8: *L. japonica* **enhances IL-4 production in unstimulated T lymphocytes (A) and stimulates IFNγ production in anti-CD3 stimulated T lymphocytes (B).** Total splenocytes were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica*. At 24, 48 and 96 hours, supernatants were harvested and IL-4 and IFNγ concentration was examined using a mouse IL-4 and IFNγ ELISA. IL-4 and IFNγ concentrations were determined based on a recombinant IL-4 and IFNγ standard along with supernatants using a Bio-Rad Model 680 Microplate Reader. IL-4 and IFNγ concentration was compared among treatment groups. Treatment groups are designated as Control (filled circle), αCD3 (open circle), *L. japonica* (filled triangle), and αCD3+*L. japonica* (open triangle).

From our results, *L. japonica* alters the expression of some surface proteins (Figs 3 and 4) necessary for certain signaling pathways; therefore certain secreted proteins, cytokines, may have altered expression with *L. japonica* treatment. Cultured total splenocytes were treated and incubated for respective times. Following timed incubations, supernatants were removed from cultured cells and analyzed for IL-4 and IFNγ levels using sandwich ELISAs. *L. japonica* alone maintained the production of IL-4 in naïve T cells similar to anti-CD3 stimulated T cells (Figure 8a). In contrast, *L. japonica* enhanced production of IFNγ in anti-CD3 stimulated T cells before 48 hours

(Fig 8b). Although not statistically analyzed, these results suggest *L. japonica* maintains IL-4 in naïve T cells and boosts IFNγ production in activated T cells.

# B CELL SURFACE MARKER EXPRESSION ANALYSES

In vitro stimulation: Total splenocytes ($2x10^6$ cells/mL) were divided into 4 treatment groups and plated in triplicate. For analyzing B lymphocytes, total splenocytes were treated with 2.5 µg /mL LPS (Sigma, St. Louis, MO) in the presence or absence of 1000 µg /mL *L. japonica*. Splenocytes were incubated at 37ºC and 5% CO_2 for 12, 24, 48 or 96 hours as indicated in the results.

Cell surface marker analysis: Following incubation, cells were resuspended in FACS buffer (1X PBS with 2% BSA, 0.1% NaN_3) and incubated (10 min, 4ºC) with fluorochrome-conjugated antibodies: anti-mouse MHC II, CD40, CD80, and CD19 (eBioscience). Cells were analyzed by an Accuri C6 flow cytometer (BD Biosciences). The mean fluorescence intensity (+/- SEM) of MHC II, CD40, and CD80 on C19-gated lymphocytes per time point was normalized to unstained controls for each treatment group. One way ANOVA with SNK multiple comparison test was used for statistical analysis of mean fluorescence intensity of treatment groups within time points using SigmaPlot 12.0 software.
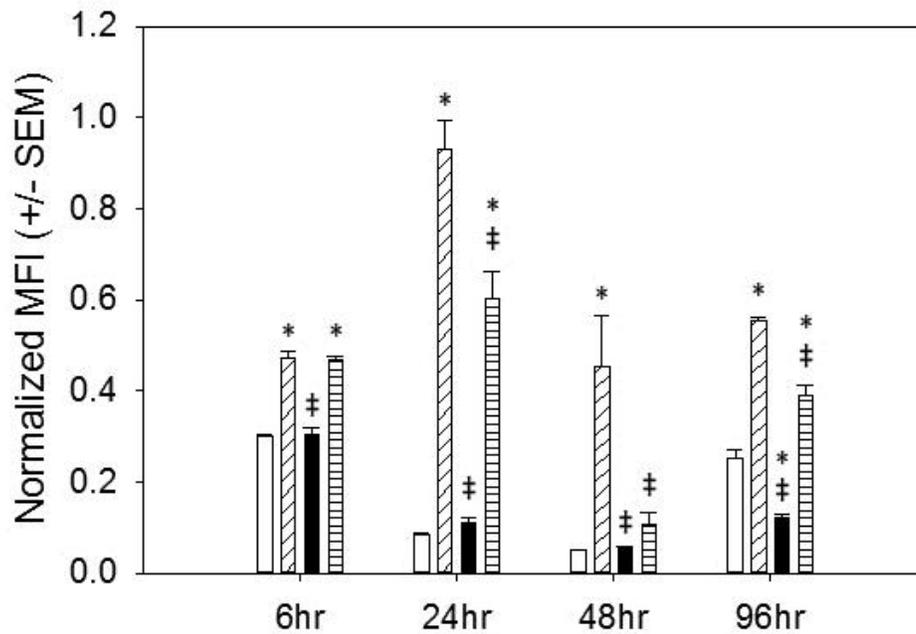
Figure 9: *L. japonica* **reduces MHC Class II expression on LPS-stimulated B lymphocytes after 24 hrs.** Total splenocytes were stimulated with 2.5 μg/mL LPS in the presence or absence of 1000 μg/mL *L. japonica*. At each time point, cells were harvested and stained with antibodies to CD19 and MHC II. Relative levels of LPS-stimulated MHC II expression on CD19+ B cells were determined using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Mean fluorescence intensity values were normalized to negative controls and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by $*$ $p \leq 0.05$ vs ctrl, ‡ $p \leq 0.05$ vs LPS. Treatment groups are designated as Control (white), LPS (right angle hash), *L. japonica* (black), and LPS+*L. japonica* (horizontal hash).
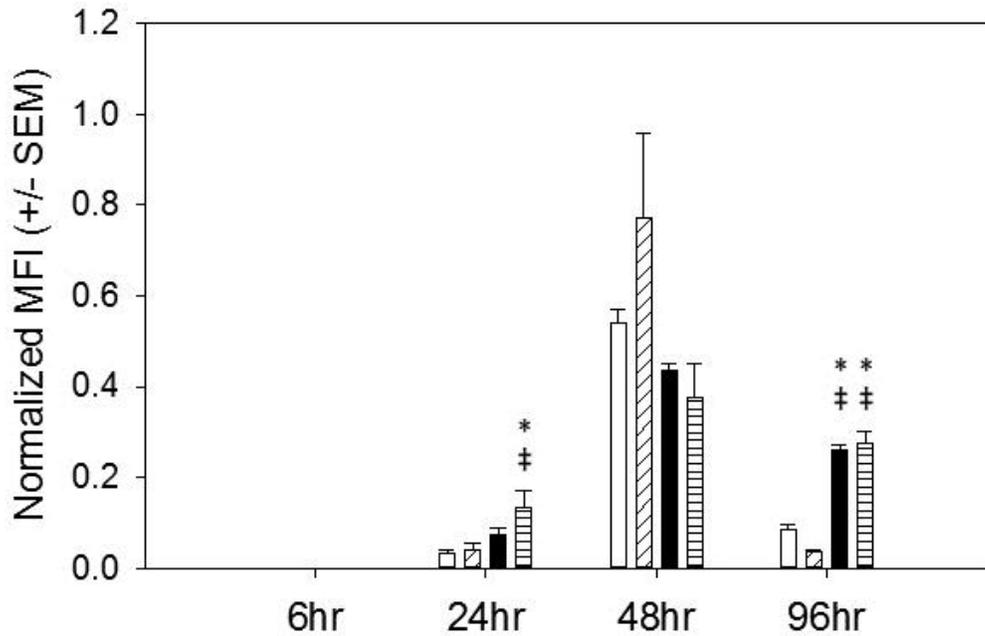
Figure 10: *L. japonica* **sustains CD40 expression on LPS-stimulated and non-stimulated B lymphocytes at 96 hrs.** Total splenocytes were stimulated with 2.5 μg/mL LPS in the presence or absence of 1000 μg/mL *L. japonica*. At each time point, cells were harvested and stained with antibodies to CD19 and CD40. Relative levels of LPS-stimulated CD40 expression on CD19+ B cells were determined using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Mean fluorescence intensity values were normalized to negative controls and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using SigmaPlot software. Statistically significant results signified by * $p \leq 0.05$ vs ctrl, ‡ $p \leq 0.05$ vs LPS. Treatment groups are designated as Control (white), LPS (right angle hash), *L. japonica* (black), and LPS+*L. japonica* (horizontal hash).
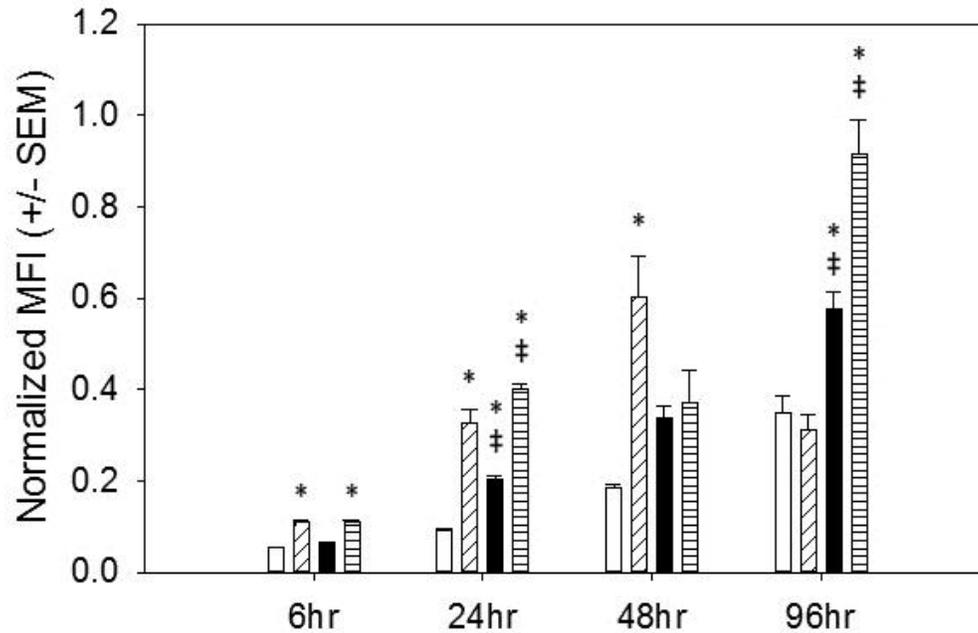
Figure 11: ***L. japonica* sustains CD80 expression in LPS-stimulated and non-stimulated B lymphocytes at 96hrs.** Total splenocytes were stimulated with 2.5 μg/mL LPS in the presence or absence of 1000 μg/mL *L. japonica*. At each time point, cells were harvested and stained with antibodies to CD19 and CD80. Relative levels of LPS-stimulated CD80 expression on CD19+ B cells were determined using an Accuri C6 flow cytometer. Experiments were performed in triplicate. Mean fluorescence intensity values were normalized to negative controls and statistically significant differences were determined by One-way ANOVA with a SNK multiple comparison test using Sigma Plot software. Statistically significant results signified by * $p \leq 0.05$ vs ctrl, ‡ $p \leq 0.05$ vs LPS. Treatment groups are designated as Control (white), LPS (right angle hash), *L. japonica* (black), and LPS+*L. japonica* (horizontal hash).

Our surface marker analysis of T lymphocyte suggested *L. japonica* altered the expression of surface proteins involved in the activation and regulation of T lymphocytes (Figs 3 and 4). Since B lymphocytes function in antigen presentation and therefore activation of T lymphocytes, *L. japonica* may alter the surface protein expression in B lymphocytes. To determine the effect of *L. japonica* treatment on B lymphocyte surface protein expression, total splenocytes were isolated and stimulated with LPS in the presence or absence of *L. japonica*. Following incubations, cells were harvested and

stained with antibodies for MHC II, CD19, CD40, and CD80. *L. japonica* treatment reduced LPS-stimulated-MHC Class II expression (Fig 9), while sustaining CD40 and CD80 expression in LPS-stimulated and non-stimulated B lymphocytes (Figs 10 and 11).