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Lonicera japonica alters antigen-stimulated T cell functions

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ABSTRACT
Lonicera japonica is a honeysuckle species commonly used in traditional Chinese medicine, having a variety of anti-inflammatory and immune-enhancing properties. Studies examining individual constituents of *L. japonica* as well as concoctions containing *L. japonica* have demonstrated that it inhibits the proliferation of stimulated T cells, potentially contradicting the immune-enhancing function of *L. japonica*. This study investigated the effect of *L. japonica* dried herb extract (1:5) on T cell function *in vitro* to elucidate the comprehensive effect of *L. japonica* on T cell-mediated immune function. Purified murine T cells were stimulated in the presence or absence of *L. japonica*, and alterations in protein expression, proliferation, and cell survival of T cells were assessed. *L. japonica* inhibited proliferation, altered protein expression, and increased the death of antigen-stimulated T lymphocytes, suggesting that *L. japonica* may serve a role in regulating T cell function, but may contribute to the generation of an ineffective immune response if used inappropriately.

Keywords: *Lonicera japonica*, T lymphocyte, herbal supplement, proliferation, protein expression.

1. Introduction
Lonicera japonica, also known as *Japanese Honeysuckle*, *Ren Dong*, and *Jin Yin Hua*, is an herb that is commonly used in traditional Chinese medicine (TCM) [1, 2] and whose nature in TCM is described as cold and sweet [3]. It is used to clear 'internal heat', a description applied to inflammatory and infectious conditions. It has direct anti-bacterial and anti-viral properties, and the diversity of chemical constituents found in *L. japonica* allows it to support general host resistance to pathogens. In general, it is used to treat fever, infections where there is pain and swelling, particularly sore throats and upper respiratory tract infections, bleeding ulcers, or other conditions resulting in blood in the sputum and stool, abscesses, and skin sores [3, 4]. Likely due to these properties, *L. japonica* is gaining popularity as an herbal supplement in the United States, particularly as a component of over-the-counter immune system supporter or immune enhancer products including Airborne ® and Nutriferon ®.

Supporting the use of *L. japonica* to treat fever and inflammation are several scientific studies examining the effect of *L. japonica* on a variety of activities that contribute to inflammation. 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 [5, 6] as well as inhibited inflammatory enzymatic functions required to produce leukotrienes and prostaglandins for initiating inflammation [7-10].

In contrast, there are few scientific studies examining the effect of *L. japonica* on adaptive immune functions. These studies are mainly limited to the examination of *L. japonica* in combination with other herbs or the examination of individual chemical constituents isolated from the flower buds of *L. japonica*. Yin Zhi Huang (YZH), an injectable herbal medicine used to treat liver disease in China, has four major chemical ingredients, one being the extract from the flower buds of *L. japonica*. Treatment of mouse splenocytes with YZH resulted in inhibited TCR-stimulated T cell proliferation and decreased expression of the activation proteins CD25 and CD69 and costimulatory proteins PD-1 and ICOS on CD4+ T cells [11]. Examinations of flavones, luteolin, ochnaflavone, and quercetin, isolated from the flower buds of *L. japonica* have demonstrated their varying abilities to inhibit mitogen-stimulated lymphocyte proliferation [12, 13].

As the orchestrators of immune responses, T cells secrete specific types of cytokines in large quantities that direct immune cell functions. In addition, T cell activation is necessary for the

generation of an effective and efficient immune response. Chemical constituents of *L. japonica* have been reported to suppress T cell proliferation and activation, which is in direct contrast to its purported immune enhancing properties. The goal of this study is to elucidate the influence of *L. japonica* on immune function by examining it's *in vitro* effects on antigen-stimulated T cell functions.

2. Materials and methods

2.1 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 (11/7/2012) by the Ball State University Animal Care and Use Committee (IACUC), protocol # 391567-1.

2.2 Preparation of Extract

An aqueous solution of *Lonicera japonica* was prepared using Plum Flower Honeysuckle flower 5:1 single herb extract powder (May Way, Oakland, CA). Powder from the flowers (sulfur-free and chlorine-free) of *L. japonica* [Caprifoliaceae] was generated using a low-temperature water extraction process that preserves essential oils and potency. The powder contains no additives and is tested for quality by thin layer chromatography. The *L. japonica* solution used in these experiments was generated by dissolving 1 g of the Honeysuckle flower 5:1 single herb extract powder in 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.

2.3 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.

2.4 *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 37 ºC and 5% CO_2 for 6, 12, 24, 48 or 96 hours as indicated in the results.

2.5 Cell surface marker analysis

Splenocytes ($2x10^6$cells/mL) were divided into 4 treatment groups as described above. Following stimulation/treatment, cells were harvested and resuspended in FACS buffer (1X PBS with 2% BSA, 0.1% NaN_3) and incubated (10 min, 4 ºC) with fluorochrome-conjugated antibodies: anti-mouse CD4, CD95, CD178, CD25, and CD152 (eBioscience). Each experiment was performed triplicate with 3 replicates for each treatment condition. Cells were analyzed on 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. For each experiment, samples were plated in triplicate. Data shown is representative of 3 experiments. One way ANOVA with a Holm-Sidak post-hoc analysis was used for statistical analysis of mean fluorescence intensity of treatment groups within time points using Sigma Plot 12.0 software.

2.6 Analysis of Cell Death

Splenocytes ($2x10^6$ cells/mL) were divided into 4 treatment groups as described above. Following stimulation/treatment, cells were harvested and resuspended in FACS buffer (1X PBS with 2% BSA, 0.1% NaN_3) and incubated (10 min, 4 ºC) with anti-mouse CD4 FITC (eBioscience). Following incubation, cells were washed and then incubated with 1 μg/mL propidium iodide (Sigma Chemicals) in FACS buffer (10 min, 4 ºC), washed and immediately analyzed. Each experiment was performed triplicate with 3 replicates for each treatment condition. Percentages of propidium iodide (PI) stained CD4+ T cells were determined using an Accuri C6 flow cytometer (BD Biosciences). For each experiment, samples were plated in triplicate. Data shown is representative of 3 experiments. One way ANOVA with a SNK (Student-Newman-Keuls) post-hoc analysis was used for statistical analysis of the percentage of PI+ cells in each treatment group at the indicated time points using Sigma Plot 12.0 software.

2.7 Fas Ligand (CD178) 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 37 ºC and 5% CO_2 for 24 and 96 hours. Following the appropriate incubations, cells were resuspended in FACS buffer and incubated (10 min, 4 ºC) 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. The mean fluorescence intensity (+/- SEM) per time point was normalized to unstained controls. One-way ANOVA with a Holm-Sidak post-hoc analysis was used for statistical analysis of mean fluorescence intensity of treatment groups within time points using SigmaPlot 12.0 software.

2.8 Intracellular Cytokine Staining

Splenocytes ($2x10^6$ cells/mL) were divided into 4 treatment groups as described above. After 2 hours of stimulation/treatment, 2 μM monensin (eBioscience) was added. Cells were harvested 4 hours after the addition of monensin and assessed for IL-2 production. Cells were incubated with anti-CD4 APC (Leinco Technologies, St. Louis, MO) in FACS buffer (10 min, 4 ºC). Following the incubation, cells were washed, fixed in FACS buffer with 2% formaldehyde, and permeabilized in FACS Buffer plus 0.1% saponin. Cells were then incubated with anti-IL-2 PE (Tonbo Biosciences, San Diego, CA) in permeabilization buffer for 30 minutes at 4 °C. Following the incubation, cells were washed and immediately analyzed on an Accuri C6 flow cytometer (BD Biosciences). For each experiment, samples were plated in triplicate. Data shown is representative of 3 experiments.

Significant differences in the mean fluorescence intensity (+/- SEM) of IL-2 on CD4-gated lymphocytes from each treatment group was determined by one-way ANOVA with a Holm-Sidak post-hoc analysis using SigmaPlot 12.0 software.

2.9 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.

2.10 Proliferation Assay

Purified T cells ($1x10^6$ cells/mL) were added to a 96 well plate (100,000 cells/well) and stimulated according to the 4 treatment groups as described above in the absence or presence (for assessment of CD152 neutralization) of 20 µg/mL anti-mouse CD152 antibody (Leinco) . T cells were incubated at 37 ºC (5% CO_2) for 24, 48, and 96 hours. Following incubation, proliferation was assessed using the MTT Cell Proliferation Assay kit (ATCC, Manassas, VA) according to the manufacturer's guidelines. Absorbance was determined using a BIO-RAD Model 680 microplate reader at 570 nm. For each experiment, samples were plated in triplicate. Data shown is representative of 3 experiments. The absorbance (+/- SEM) was normalized to unstained controls. Kruskal-Wallis One-Way Analysis of Variance on Ranks identified statistically significant differences between treatment groups at the indicated time points using SigmaPlot 12.0 software.

3. Results

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 [12, 13]. To examine the effect 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 (Figure 1).

IL-2 is rapidly produced following the activation of T cells [14] and is a necessary requirement for proliferation and clonal expansion [15]. Decreased IL-2 production by activated T cells treated with *L. japonica* may hinder proliferation. 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 6 hours, cells were harvested and levels of IL-2 produced by CD4+ T cells were analyzed by intracellular cytokine staining. Treatment with L. japonica did not alter IL-2 production by antigen-stimulated CD4+ T cells (Figure 2). Similarly, treatment with *L. japonica* did not alter levels of IL-2 in supernatants harvested from T cells stimulated for 24 hours as determined by ELISA (data not shown).

Sensitization of T cells to IL-2 and subsequent proliferation is enhanced following activation due to the increased but transient expression of the IL-2Rα chain (CD25) [16, 17]. Although IL-2 production by activated T cells was not altered by *L. japonica* treatment, reduced levels of CD25 on activated T cells may decrease an antigen-stimulated proliferative response. 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*. At all time points examined, treatment with *L. japonica* reduced anti-CD3-stimulated CD25 expression (Figure 3), suggesting that the decreased proliferative response of antigen-stimulated T cells in the presence of *L. japonica* may result from decreased sensitization to IL-2.

The ability of *L. japonica* to reduce CD25 expression may suggest it can influence the expression of other surface proteins expressed by T cells following activation. 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 (Figure 4A, B). Likewise, CD152 expression on both anti-CD3-stimulated and non-stimulated CD4+ T cells was enhanced by *L. japonica* treatment alone (Figure 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 CD95 and CD178 (inducing cell death) and CD152 (downregulating T cell activation).

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 conjunction with anti-CD3 stimulation increased cell death of CD4+ T cells, as demonstrated by an increased percentage of PI+ cells compared to controls (Figure 5). This finding suggests that the decreased proliferative response of activated T cells in the presence of *L. japonica* may not solely result from decreased CD25 expression but may also be a result of decreased numbers of T cells.

One pathway by which programmed cell death is activated is through the receptor-ligand binding of CD95 and CD178 [18, 19]. Given the increased death of activated T cells and the increased 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, CD4+ T 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 (Figure 6). Furthermore, caspase activity at 48 and 72 hours was increased in anti-CD3 stimulated T cells compared to anti-CD3-stimulated T cells treated with *L. japonica*. These results suggests that the *L. japonica*-stimulated death of CD4+ T cells occurs independent of the apoptotic mechanism of programmed cell death and increased expression of CD95 and CD178.

Proliferation of 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 [20-22]. To determine if decreased proliferation of CD4+ T cells from *L. japonica* treatment was induced by the upregulation of CD152, T lymphocytes were isolated by magnetic sorting and stimulated with anti-CD3 and 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 neutralization with anti-CD152 increased the proliferation of anti-CD3 stimulated T cells at 48 and 96 hours, proliferation was not recovered by CD152 neutralization in the anti-CD3- stimulated T cells treated with *L. japonica* (Figure 7), suggesting that increased expression of CD152 on activated T cells following treatment with *L. japonica* was not a major factor in reduced proliferation of activated T cells treated with *L. japonica*.

These data demonstrate that antigen-stimulated T cell proliferation is suppressed following treatment with *L. japonica*, and this may result from decreased CD25 expression but is independent of increased CD152 expression. Furthermore, treatment with *L. japonica* enhances cell death of activated CD4+ T cells, which is independent of Fas and FasL interactions, despite concomitant increases in CD95 and CD178. Taken together, these findings demonstrate the ability of *L. japonica* to profoundly influence antigen-stimulated T cell functions that may result in an inability to mount an effective T cell response.

3.1 Tables and Figures

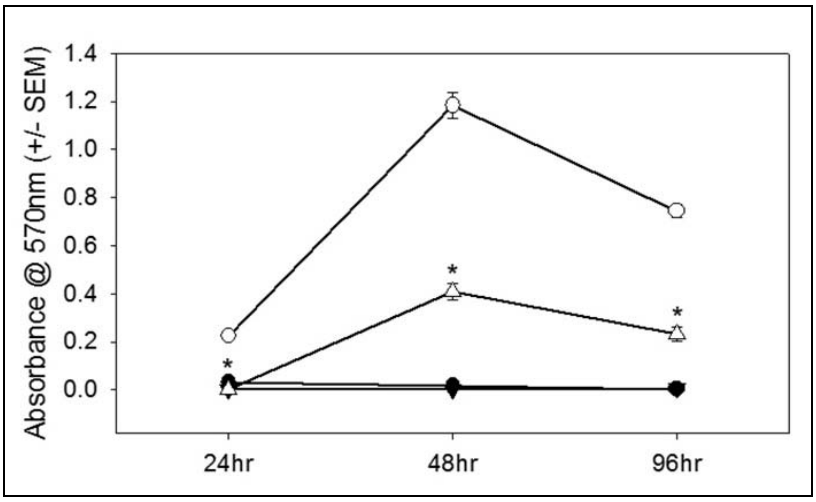

Fig 1: *L. japonica* inhibits the proliferation of antigen-stimulated T lymphocytes Isolated T cells were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica*. The amount of proliferation was determined at indicated time points using the MTT assay. *p ≤ 0.05 αCD3 vs αCD3/LJ as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks. Treatment groups are designated as Control (filled circle), αCD3 (open circle), *L. japonica* (filled triangle), and αCD3+*L. japonica* (open triangle).

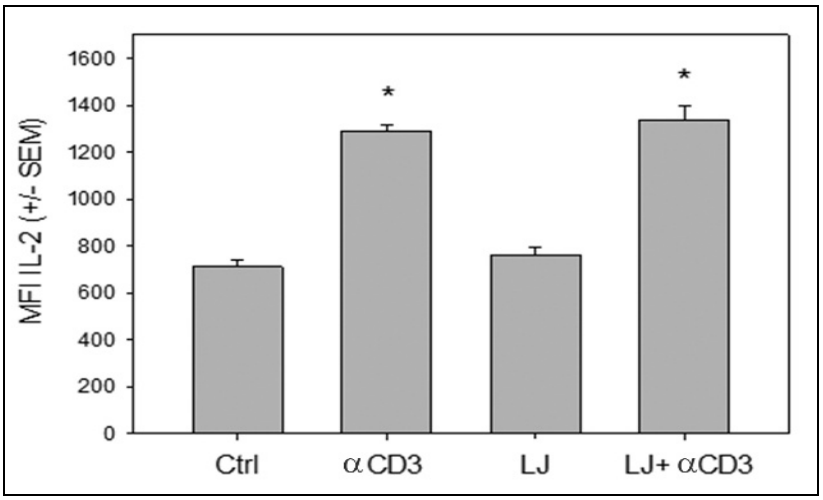

Fig 2: *L. japonica* does not alter IL-2 production. Total splenocytes were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica*. After 6 hours, cells were harvested and levels of IL-2 produced by CD4+ T cells were determined via intracellular cytokine staining. *Significant increase compared to Ctrl and LJ. *p ≤ 0.05 as determined by one-way ANOVA with a Holm-Sidak post-hoc analysis.

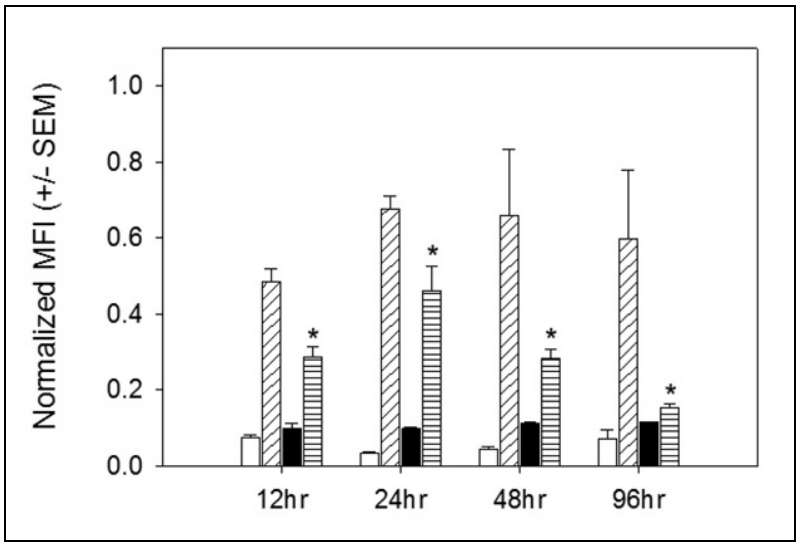

Fig 3: CD25 expression is suppressed by *L. japonica* in antigen-stimulated T lymphocytes. Total splenocytes were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica*. Relative levels of αCD3-stimulated CD25 expression on CD4+ T cells were determined at each time point. Mean fluorescence intensity (MFI) values were normalized to negative controls. * $p \leq 0.05$ αCD3 vs αCD3+LJ as determined by one-way ANOVA with Holm-Sidak post-hoc analysis**.** Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), and αCD3+*L. japonica* (horizontal hash).

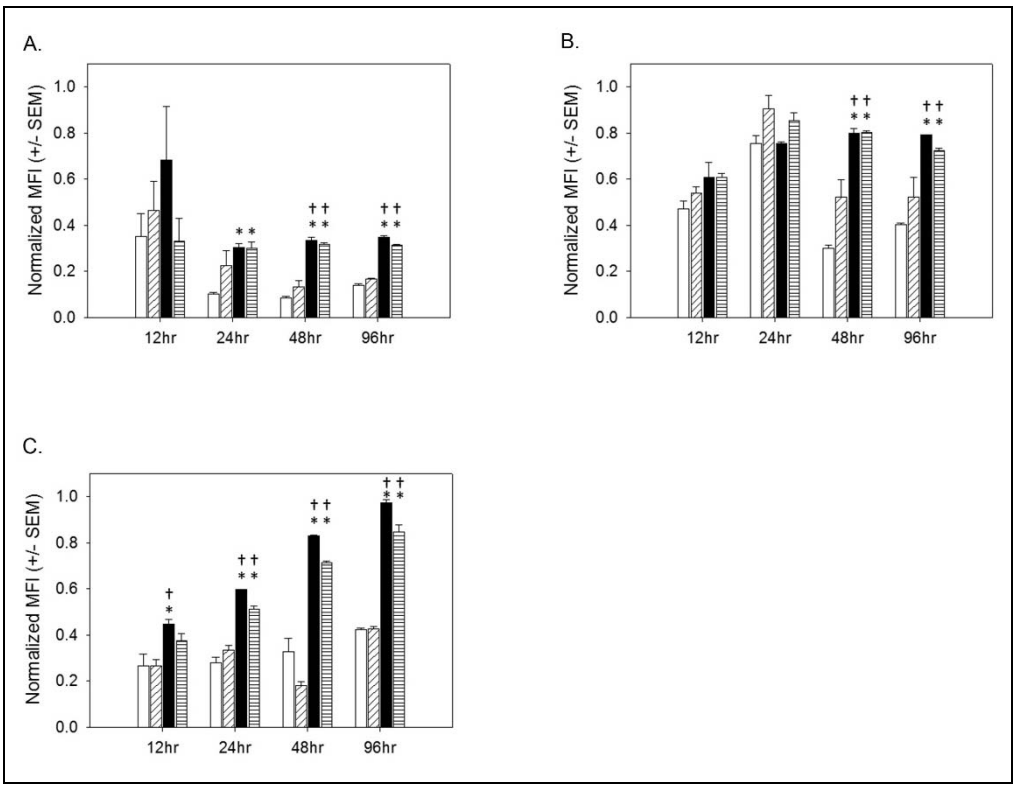

Fig 4: *L. japonica* enhances the expression of CD95, CD178, and CD152 in unstimulated and antigen-stimulated T lymphocytes. Total splenocytes were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica*. At each time point, relative levels of anti-CD3-stimulated CD95 (A), CD178 (B), or CD152 (C) expression on CD4+ T cells were determined. Mean fluorescence intensity values were normalized to negative controls. ***** $p \leq 0.05$ vs ctrl, † $p \leq 0.05$ vs αCD3 as determined by one-way ANOVA with Holm-Sidak post-hoc analysis. Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), and αCD3+*L. japonica* (horizontal hash).

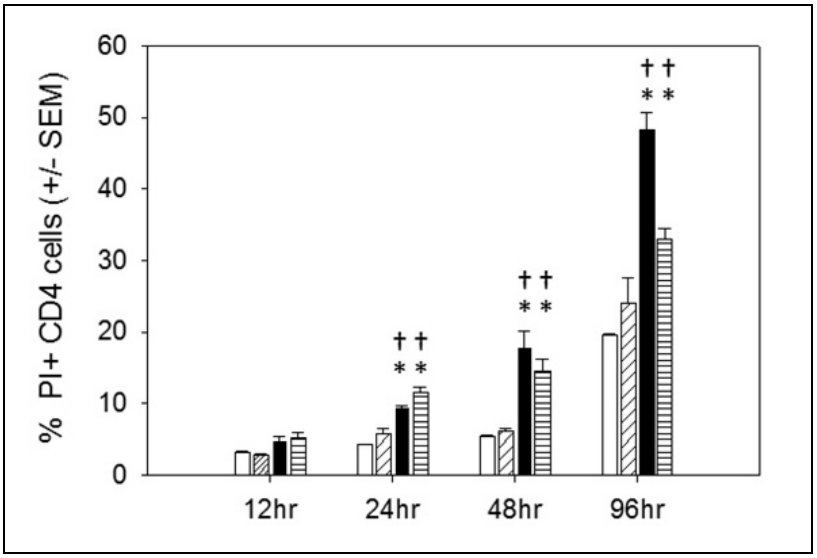

Fig 5: *L. japonica* enhances cell death in CD4+ T lymphocytes. Total splenocytes were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica*. At each time point, cells were harvested and stained with propidium iodide (PI). Percentages of CD4+, PI+ cells in each treatment group were determined to assess cell death. *****p ≤ 0.05 vs ctrl, † p ≤ 0.05 vs αCD3 as determined by one-way ANOVA with a Student-Newman-Keuls post-hoc analysis**.** Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), and αCD3+*L. japonica* (horizontal hash).

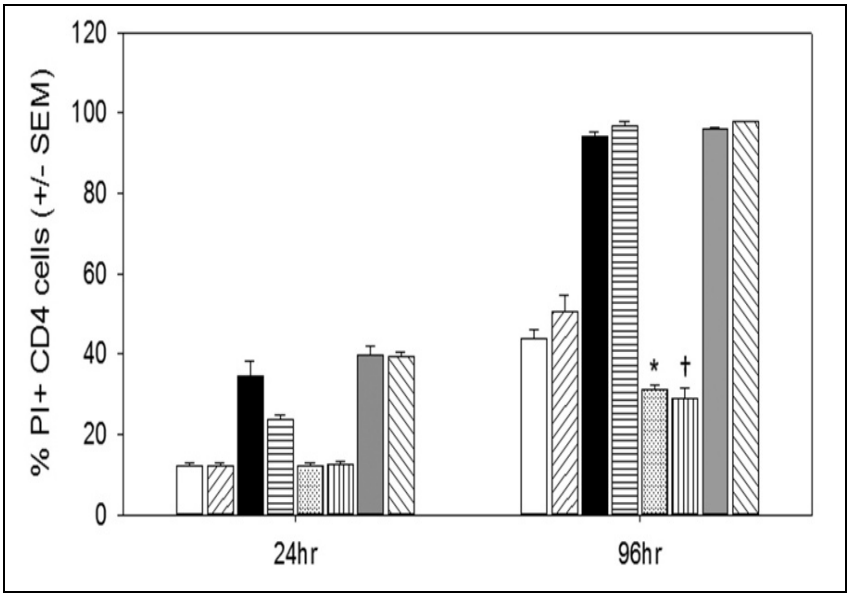

Fig 6: *L. japonica* increases CD4+ T cell death independent of CD178. Total splenocytes were stimulated with 0.5 µg/mL αCD3 and 1.0 µg/mL αCD28 in the presence or absence of 1000 µg/mL *L. japonica*, with or without 15 µg/mL αFas antibody. At each time point, cells were harvested and stained with propidium iodide. Percentages of CD4+, PI+ cells in each treatment group were determined to assess cell death. *****p ≤ 0.05 vs ctrl, † p ≤ 0.05 vs αCD3 as determined by one-way ANOVA with a Holm-Sidak post-hoc analysis. Treatment groups are designated as Control (white), αCD3 (right angle hash), *L. japonica* (black), αCD3+*L. japonica* (horizontal hash), αFas Control (dots), αFas αCD3 (vertical hash), αFas *L. japonica* (grey), and αFas αCD3+*L. japonica* (left angle hash).

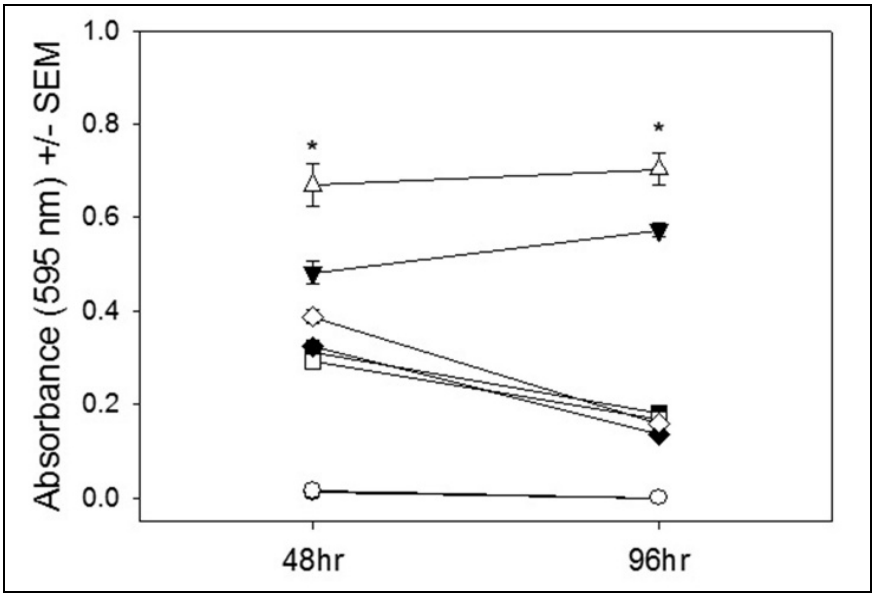

Fig 7: *L. japonica* suppresses T cell proliferation independent of CD152. Isolated T cells were stimulated with 0.5 μg/mL αCD3 and 1.0 μg/mL αCD28 in the presence or absence of 1000 μg/mL *L. japonica* with or without 10 or 20 μg/mL αCD152 antibody. The amount of proliferation was determined at indicated time points using the MTT assay. The absorbance (+/- SEM) was normalized to unstained controls. *****$p \leq 0.05$ vs αCD3 as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks. 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).

4. Conclusions

The purpose of this study was to delineate the ability of an herbal powder, extracted from the flowers of *L. japonica*, to alter T cell function. Findings from this study demonstrated that *L. japonica* inhibited the proliferation of antigen-stimulated T lymphocytes, concomitantly reducing CD25 and increasing CD152 expression but did not alter IL-2 production. Furthermore, treatment of CD4+ T cells with *L. japonica* increased cell death which paralleled but was independent of increased CD95 and CD178 expression. Together these data demonstrate a profound suppressive effect of *L. japonica* on T lymphocyte function through inhibition of proliferation and increased cytotoxicity, which to the best of our knowledge is a novel finding regarding the characteristics of *L. japonica*.

Flavonoids, which are present in high concentration in extracts isolated from the flowers of *L. japonica*, have been previously demonstrated to suppress T lymphocyte proliferation in response to mitogen stimulation or co-culture assay but they were not found to be cytotoxic [12, 13, 23]. Furthermore, an investigation examining the effect of *L. japonica* in combination with other herbs demonstrated similar suppression of TCR-stimulated T cell function. Yin Zhi Huang (YZH), an injectable herbal medicine used to treat liver disease in China, has four major herbal components, one being the extract from the flower buds of *L. japonica*. Treatment of mouse splenocytes with YZH resulted in inhibited TCR-stimulated T cell proliferation and decreased expression of the activation proteins CD25 and CD69 and costimulatory proteins PD-1 and ICOS on CD4+ T cells but cytotoxic effects were not observed[11].Our data parallels these findings and extends the understanding of the effect of *L. japonica* on T cell activation and cell survival, demonstrating its ability to increase the expression of CD152, CD95 and CD178 and induce cytotoxicity. In TCM, *L. japonica* is often administered as a tea in combination with other herbs. Our finding, that treatment with *L. japonica* alone enhances the cell death of T cells, provides evidence supporting the use of *L. japonica* in decoctions and not as a treatment on its own.

Alternatively, the cytotoxic effect of *L. japonica* on T cells may be an important attribute to its ability to treat 'heat' conditions and serve as an anti-inflammatory. Th1 cells are a subset of CD4+ T cells that enhance inflammation through their secretion of IFNγ and its subsequent activation of macrophages, which are the main mediators of inflammation [24]. *L. japonica* may decrease 'internal heat' by blocking the positive feedback loop between Th1 cells and inflammation through its cytotoxic effect on CD4+ T cells. Indeed, some TCM herbal medicine texts recommend limited (no more than 7 days) and not continual use of *L. japonica* [3]. This possibility is further supported by an investigation into the effects of Jin Ying Tang (JYT), an herbal concoction that consists of nine different plant extracts including *L. japonica*, on *Staphylococcus aureus*-induced mastitis in rabbits. Treatment with JYT reduced lymphocyte counts and TNF-α and IL-6 levels in the infected mammary tissue and blood and decreased the degeneration of the infected epithelial mammary tissue during inflammation [25]. Although this study identified a reduction in lymphocytes rather than T cells, specifically, it highlights the *in vivo* anti-inflammatory effects of decreasing lymphocyte numbers.

In conclusion, the experimental evidence obtained from this study demonstrated that treatment with *L. japonica* alone, and not in combination with other herbs, alters T cell activation, suppresses proliferation, and importantly, enhances cell death. These findings suggest that this effect of *L. japonica* may be beneficial when the herb is used for a short duration, such that it reduces the number of T cells that may be promoting the inflammatory response. But alternatively, these results may suggest that prolonged or aberrant use of *L. japonica* may hinder the generation of an appropriate and effective T cell response.

5. Acknowledgments
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