THE EFFECT OF RED YEAST RICE 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

PETER JOSHUA FORD

ADVISOR: HEATHER BRUNS

BALL STATE UNIVERSITY

MUNCIE, INDIANA

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Committee Approval:

Committee Chairperson	Date
Committee Member	Date
Committee Member	Date

Departmental Approval:

Departmental Chairperson	Date
Dean of Graduate School	Date

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ABSTRACT

Red yeast rice (RYR) is a traditional Chinese medical agent that is produced by fermenting rice with *Monascus purpureus*, and has been used as an alternative treatment for a variety of diseases, including dyslipidemia and coronary heart disease. Statins are HMG-CoA reductase inhibitors that are most commonly used for the treatment of hyperlipidemia. In addition to their lipid-lowering properties, statins also display anti-inflammatory abilities and act by inhibiting T-cell activation and recruitment. RYR contains a group of naturally occurring polyketides known as monacolins that are similar in structure and are HMG-CoA reductase inhibitors. Monacolin K, also known as lovastatin, is in the highest concentration in RYR, which can reduce serum cholesterol levels similarly to statins. However, endogenous lovastatin levels in RYR are significantly lower than that of a prescribed statin regiment. Therefore, the goal of this study was to investigate the ability of RYR to alter T-cell functions, including proliferation, protein expression, and cytokine production.

Our results show that RYR treatment significantly decreased the proliferation of activated T-cells as compared to untreated controls. Additionally, results showed the RYR treatment may alter expression of key proteins involved in T-cell activation, stimulation, and regulation. A significant decrease in the expression of CD25 was observed in RYR treated T-cells. A possible decrease in expression of CD45R was observed and a possible increase in expression of CD95 and CD178, though these results were not significant.

INTRODUCTION

The immune system is a multi-layered defense system which provides an organism protection against an array of potentially harmful microorganisms, including bacteria, viruses, fungi, and parasitic worms [1]. The two components of the immune system are the innate immune response and the adaptive immune response. The innate immune response, mediated by phagocytes and granulocytes, is the body's first line of defense against invading pathogens [2,3]. Additionally, epithelial cells, endothelial cells, and fibroblasts, also contribute to innate immunity. The adaptive response is the body's second line of defense and is characterized by the activation of antigen-specific B- and T-lymphocytes.

Cells of the innate immune system recognize pathogens through germline-encoded pattern-recognition receptors (PRRs), which include toll-like receptors (TLRs), RIG-I-like receptor, NOD-like receptors, and C-type lectin receptors, that recognize and bind pathogen-associated molecular patterns (PAMPs), which include carbohydrates, lipids, proteins, and nucleic acids [4,5]. After ligand binding, TLRs will dimerize and undergo conformational changes that act to recruit adaptor molecules, namely MyD88 and TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), that activate signal pathways, which lead to the production of pro-inflammatory cytokines and type I interferons (IFNs). Pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12, IFN-γ, and tumor necrosis factor-α (TNF-α), which are produced mainly by activated macrophages, induce an inflammatory response; which is characterized by pain, redness, swelling, and heat caused by increased blood flow to the site of infection, and is a defining feature of the innate immune response [5,6]. The release of these cytokines acts to increase vascular diameter and permeability, and to activate endothelial cells to express adhesion molecules, such as P-selectin and ICAM-1 [6]. These events allow for the

recruitment of immune cells to the site of infection, which leads to pathogen destruction and clearance.

In addition to inducing an inflammatory response, the innate immune system is also responsible for activating the adaptive immune response. In contrast to the innate response, which is mediated by phagocytes and granulocytes and operates through recognition of non-specific PAMPs, the adaptive response is mediated by B- and T-lymphocytes and is antigen-specific [1]. The main functions of the adaptive response are recognition of "self" versus "non-self" antigens, generation of a response tailored to specific antigens on pathogens or pathogen-infected cells, and development of immunological memory thorough generation of long-lived, memory B- and T-lymphocytes [1].

B-lymphocytes have two functions in the adaptive response. First, they secrete antigen-specific antibodies, which (depending on the specific subclass of antibody) act to opsonize pathogens, increase cellular cytotoxicity, and induce degranulation and release of histamines via mast cell activation [7]. Secondly, they activate T-cells by binding and internalizing pathogens via receptor-mediated endocytosis and then displaying processed antigen on major histocompatibility complex I or II (MHCI/II) [7].

T-lymphocytes can be divided into two subsets based on their function in the adaptive response and what surface markers they express. They can either act as directors of the immune response, which govern the response via cytokine secretion, or act as cytotoxic cells that directly kill abnormal or infected cells [8,9]. Directors, which include T_H1, T_H2, T_H17, and T_{reg} cell subsets, express CD4 and respond to antigens presented on MHC-II, while cytotoxic T-cells (T_c) express CD8 and respond to antigens presented on MHC-I.

Generating an effective and robust immune response is dependent upon the activation and generation of T-cells. Activation of most T-cells occurs within secondary lymphoid tissues and is achieved via the interaction of naïve T-cells with antigen presenting cells (APCs), which include B-cells, dendritic cells, and macrophages [3,8,9]. Activation requires engagement of the TCR with MHC-I or MHC-II-antigen complex, recognition of MHC-I/-II via CD8/CD4, and co-stimulation via engagement of T-cell CD28 with APC-expressed CD80 or CD86 [9]. Naïve T-cells will become anergic if the activating signals do not occur simultaneously.

The process of activation occurs over several hours and a number of proteins are involved in maintaining and stabilizing the activation process [8]. These include integrins such as leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), as well as CD2, which bind to their respective ligands intercellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM), and CD58 on the APC [8]. Activation will then induce signaling cascades that involve CD3 proteins and CD45R, a transmembrane receptor-like protein tyrosine phosphatase (PTPase) required for TCR-mediated signaling and activation. Activation of CD3 and CD45R within the T-cell leads to the release of interleukin-2 (IL-2) and the expression of high affinity IL-2 receptor (IL-2R) on the cell surface [8,10]. High affinity IL-2R is a multi-subunit complex consisting of a common cytokine receptor γ-chain (CD132), IL-2Rβ chain (CD122), and IL-2Rα chain (CD25). The release and binding of IL-2 occurs in both a paracrine and autocrine fashion and acts to induce a rapid proliferative burst [10]. Expression of CD25 is regulated by the combination of TCR stimulation and IL-2, which creates a positive-feedback loop that involves binding of signal transducer and activator of transcription (STAT5) to *Cd25.* Signal transduction occurs through several intracellular pathways including, Janus-Kinase (JAK)-STAT pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway and the mitogen-

activated protein kinase (MAPK) pathway. Following binding of IL-2 to the high affinity IL-2R, the cell will then internalize the IL-2/IL-2R complex. IL-2, CD122, and CD132 will then undergo proteasome-mediated degradation, while CD25 is recycled back to the cell surface. This recycling provides a supply of CD25 for continuous stimulation [10]. IL-2 production is regulated by several mechanisms, including the binding of transcription factor B-lymphocyte-induced maturation protein (BLIMP1/PRDM1) to *Il2*. Differentiated, long-lived effector T-cells maintain low expression levels of BLIMP1, thus maintaining the cell's IL-2 production ability. Prolonged antigen-exposure induces up-regulation of BLIMP1 and decreases IL-2 production and can induce expression of death receptor FAS (CD95) and FAS-ligand (CD178) which act to promote apoptosis. There are also several other mechanisms that act to downregulate the T-cell response. T-cell activation is terminated 24-48 hours after activation, primarily by the activation of cytotoxic T-lymphocyte associated antigen-4 (CTLA-4/CD152) [8]. CD152 expression is maximized during this time and down-regulates activation through inhibition of CD28 [11]. Specifically, CD152 will bind to CD80 and CD86 with a higher affinity than CD28.

IL-2 is important for both CD8 and CD4 T-cells and acts in all stages of the immune response, including primary expansion, contraction, and memory generation [12]. CD8 T-cells can differentiate into short-lived effector cells or long-lived memory cells. Differentiation is determined by the strength and duration of IL-2 stimulation. During primary expansion and following an initial increase in the expression of CD25, a small subset of CD8 T-cells will downregulate CD25 expression. This downregulation of is then followed by an upregulation of IL-7Rα (CD-127) and CD62L (L-selectin), which is a phenotype that is a characteristic of memory cells. In contrast, $CD25^{Hi}$ T-cells received strong IL-2 stimulation and will differentiate

into short-lived effector cells. These effector cells will sustain expression of CD25 for an additional 1-2 days post antigen-priming and then die via apoptosis.

IL-2 also has important roles during antigen-driven CD4 T-cell differentiation. Proliferation stimulated by IL-2 is the first step in the differentiation of CD4 T-cells into T_H1, T_H2, T_H17, and T_{reg} subsets. Which subset develops is dependent on the presence of specific cytokines, and the subsequent downstream activation of specific transcription factors. T_H1 subsets are dependent upon binding of IL-2 and stimulation by IL-12 [12]. This leads to an upregulation in the IL-12R subunits (IL-12Rβ1 and IL-12Rβ2) which increases affinity for IL-12. IL-12 stimulation then acts to increase expression of transcription factors STAT4 and T-box transcription factor (T-bet), which increases transcription of IFNγ. Production of IFNγ acts to enhance inflammation and pathogen clearance by activating macrophages, increasing expression of FcRs on NK cells and macrophages, and induces class switching in B-cells from IgG1 to IgG3 [12,13]. STAT4 is necessary for appropriate IL-12 stimulated T_H1 differentiation, as previous studies show that STAT4-deficient mice display a loss of IL-12 mediated immune function, including T_H1 differentiation [13,14,15].

T_H2 differentiation is stimulated by IL-4 and induces an increase in IL-4Rα, which associates with CD132 to form the IL-4 receptor. Binding of IL-4 stimulates transcription factors STAT6 and GATA-3 and induces expression of T_H2-specific cytokines IL-4, IL-5, and IL-13 [11]. STAT6 is essential for IL-4 dependent T_H2 differentiation [16]. Other transcription factors, such as c-MAF, jun-B, and nuclear factor of activated B-cells (NFAT), play critical roles in the regulation of T_H2 differentiation and act downstream of STAT6. addition to inducing T_H2 differentiation, IL-4 also stimulates activated B-cells to differentiate into plasma cells and

induces class switching to IgE, up-regulates MHC-II production, and acts to suppress T_H1 activation. IL-5 secretion acts to stimulation B-cell proliferation and increase Ig secretion and is a key mediator in eosinophil activity. IL-13 secretion also acts to stimulate B-cell proliferation [11].

In contrast to T_H1 and T_H2 T-cells, T_H17 differentiation is instead driven by IL-6 and is inhibited by IL-2 [11]. The IL-6R is composed of two subunits, IL-6Rα and IL-6Rβ. Binding of IL-6 drives expression of transcription factors STAT3 and RORγt and induces IL-17 and IL-22 secretion. IL-17 and IL-22 act cooperatively with each other to induce and mediate pro-inflammatory responses by stimulating expression of cytokines (IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, and TNF-α), chemokines (IL-8, GRO-α, and MCP-1), and prostaglandins [17]. IL-2-mediated activity of STAT5 limits responsiveness of T_H17 cells to IL-6 by downregulating expression of IL-6Rβ. STAT5 acts to inhibit T_H17 differentiation by competing with STAT3 for binding to *Il17* gene locus [11]. IL-2 also plays a major role in T_{reg} homeostasis. IL-2 maintains high levels of CD25 and forkhead box P3 (FOXP3) expression by T_{reg} cells and enhances their suppressive capacity.

In summary, the immune response is complex and multilayered; involving numerous different cell types, activation cascades, and molecules and cytokines. An effective response to pathogen invasion involves activation of innate immune cells, initiation of an inflammatory response, activation of antigen-specific antibody-producing B-cells, activation of T-cells and subsequent direction and regulation of the adaptive response via T-cells.

Statins are a class of lipid-lowering medications that are primarily used in the prevention and treatment of cardio vascular disease [18,19]. Mechanistically, statins act to reduce endogenous cholesterol by competitively binding to the active-site of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway, thus preventing binding of the target substrate HMG-CoA. In addition to their lipid-lowering properties, statins have also shown to have immunomodulatory and anti-inflammatory functions [19]. These effects are attributed to the ability of statins to inhibit activation of T-cells and decrease recruitment of monocytes and T-cells.

The anti-inflammatory effects of statins are due to mechanisms related to their lipid-lowering ability and to lipid-independent mechanisms. Hypercholesterolemia up-regulates CD40/CD40L, enhances NF-κB activation, adhesion and chemoattractant expression, and facilitates macrophage infiltration. In animal models, statins decrease macrophage infiltration, reduce leukocyte rolling and adherence, and reduce leukocyte transmigration. These effects are related to the inhibition of HMG-CoA reductase and decrease in isoprenylation of small G proteins, such as Ras, Rac, and Rho, during lipid biosynthesis [19].

More specifically, the anti-inflammatory effects of statins are due to their ability to inhibit IFN-γ-dependent MHC-II expression [20,21,22]. MHC-II is essential for the activation of $CD4^+$ T-cells and IFN-γ increases expression of MHC-II on APCs and several studies have shown that statins inhibit the immunological functions of IFN-γ, which decreases MHC-II expression and activation of $CD4^+$ T-cells [20]. Importantly, the presence of mevalonate reverses IFN-γ-dependent MHC-II expression, demonstrating that inhibition of MHC-II activation is due to the inhibitory effects statins have on HMG-CoA reductase. Statin treatment is thus able to

reduce inflammation by decreasing the ability of APCs to effectively present antigens via the inhibition of MHC-II.

Lovastatin is a naturally occurring statin found at low levels in oyster mushrooms, red yeast rice and Pu'er [23,24,25]. Lovastatin has been shown to have a direct effect on the functions of lymphoid cells by suppressing proliferation [19]. Lovastatin also exerts immunomodulatory effects by inhibiting leukocyte adhesion and T-cell activation by binding to LFA-1. More specifically, lovastatin inhibits production of pro-inflammatory cytokines IL-2, IL-4, and IFN-γ in T-cells and acts on lipopolysaccharide-stimulated B-cells by inhibiting proliferation and differentiation, and inducing apoptosis [26,27].

Red yeast rice (RYR) is a traditional Chinese cuisine and medicinal agent, with earliest uses dating back to the Ming dynasty (1368-1644) and has been used as an alternative to statins for the treatment of dyslipidemia, coronary heart disease, diabetes, and liver disease [24,25]. RYR is rice fermented by using *Monascus purpureus*, a homothallic and teleomorphic fungi [24,28]. RYR extracts contain a vast number of compounds, including a variety of different pigments, starches, sterols, isoflavones, and monounsaturated fatty acids [29,30]. Principle among these extracts is the monacolin family, which contains monacolin K, the naturally produced form of the commercially produced drug, lovastatin. RYR has been shown to reduce serum total cholesterol levels in a manner similar to statin drugs, despite doses of RYR having a significantly lower concentration of HMG-CoA reductase inhibitors (approximately 0.4%, 9.6 mg lovastatin in 2.4 g RYR), compared to therapeutic dosages of lovastatin (typically 20 – 40 mg) [31, 32].

While the main component of RYR is starch, there are metabolic byproducts present in RYR due to the fermentation process, primarily fatty acids and polyketides [30]. Principle among those byproducts are six aromatic polyketide pigments: monascin, ankaflavin, rubropunctatin, monascorubin, rubropunctamine, monascorubramine [31]. These pigments have well-characterized structures, however, the specific biosynthesis pathways that give rise to these pigments remains unclear. The pigments show anti-microbial, anti-tumor, and anti-inflammatory effects. Rubropunctatin and monascorubin display anti-microbial activity. Rubropunctatin, monascorubin, monascin, and ankaflavin all show significant immunomodulatory effects, specifically on T-cells [31]. Additionally, monascin and ankaflavin display anticholesterolemic effects similar to lovastatin. Specifically, these pigments have been shown to inhibit IgG, IgE, and IgM in a dose-dependent manner in rat spleen lymphocytes, reduce TNF-α-stimulated endothelial adherence, and down regulate NF-κB activation and VCAM-1 expression in human aortic endothelial cells [33, 34, 35].

Monacolins are polyketide-derived compounds that have been shown to lower total blood lipid levels via competitive inhibition of HMG-CoA reductase [30, 36]. Thus far, 14 naturally occurring monacolins (monacolin K, monacolin J, monacolin L, monacolin M, monacolin X, and their hydroxyl acid form, as well as dehydromonacolin K, dihydromonacolin L, compactin, 3α hydroxyl- 3,5-dihydromonacolin L) have been identified in RYR [36]. Monacolins exist in two forms, a lactone form and hydroxyl acid form, that are found in RYR extract and are present at differing concentrations (3.5 μg/mL and 2.7 μg/mL, respectively). Importantly, the biologically active form of monacolin K is the hydroxyl acid form. Given the low concentration of monacolins in RYR it is unlikely that the immunomodulatory effects of RYR are due solely to monacolins. With the known effects of the other constituents found in RYR, the

immunomodulatory effects are likely due to the combined effects of monacolins, pigments, starches, sterols, isoflavones, and monounsaturated fatty acids

Given the ability of RYR to lower cholesterol in a manner similar to statin drugs and the biological activities of several constituents of RYR [24, 25, 31, 32], the overarching goal of this study was to investigate the immunomodulatory ability of RYR. Because T-cells are the directors of the immune response, this project investigated the effects of RYR extract on T-cell activation (as demonstrated by surface-protein expression), proliferation, and cytokine production.

Therefore, we hypothesized that RYR extract would alter stimulated T-cell functions. Specifically, we hypothesized that RYR extract would reduce anti-CD3-stimulated proliferation and IL-2 cytokine production. Additionally, we hypothesized that RYR extract would increase IL-4 cytokine production while simultaneously suppressing IFN-γ production. Finally, we hypothesized that RYR extract would dampen expression of the activation-induced proteins CD25 and CD45R.

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 soluble Red Yeast Rice Extract: Soluble red yeast rice extract was prepared by dissolving 1,200 mg of red yeast rice extract (Solaray, Park City, UT) in 10 mL of methanol (UN1230, Mallinekrodt, Paris, KY) for 5-10 minutes. Following incubation, samples were centrifuged at 2,800 x g for 10 minutes at room temperature. The supernatant was then evaporated to dryness using gaseous nitrogen. Leftover residue was resuspended in 1 mL RPMI-1640 (R0883, Sigma, St. Louis, MO) /1% methanol and stored at -80°C. The concentration of hydrolyzed lovastatin in the RYR extract was determined to be 28.625 μg/μL by reverse-phase HPLC analysis. Experiments were performed with either 1, 2.5, 5, 10, or 20 μL of RYR extract, which corresponds to 28.6, 71.6, 143.1, 286.3, and 572.5 μg of hydrolyzed lovastatin, respectively.

Lymphocyte Isolation: Lymphocytes were harvested from the spleen by maceration in complete RPMI-1640 culture medium (Sigma) supplemented with 10% heat-inactivated FBS (S11050H, Atlanta Biologicals, Lawrenceville, GA), penicillin-streptomycin (30-002-CI, Sigma), sodium pyruvate (SH3023901, Sigma), non-essential amino acids (116-078-721 Sigma), L-glutamine (56-

85-9, Sigma), HEPES (SH30237.01, Sigma), and $5x10^{-5}$ M 2-mercaptoethanol (M3148, Sigma) Lymphocyte suspensions were then treated with RBC lysis buffer with 0.83% NH_4Cl (114110712, MP Biomedicals, Santa Ana, CA), 0.01 M Tris (77-80-1, Sigma), pH 7.4, then washed and resuspended in complete RPMI-1640 (Sigma). Lymphocyte count was determined by trypan blue exclusion.

In Vitro Stimulation and Cell Surface Marker Expression Analysis: Total lymphocytes were plated on 24-well plates at $2x10^6$ cells/mL, divided into six treatment groups, and plated in triplicate. Stimulated T lymphocytes were plated in wells coated with 2 μg/mL anti-CD3 antibody (40-0281-V100, eBioscience, San Diego, CA). Wells were coated for 24 hours before T lymphocyte stimulation. Stimulated cells were treated with 1.0 μg/mL anti-CD28 (40-0031-V500, eBioscience). Stimulated cells were then treated with either 20 μM Lovastatin (75330-75-5, Sigma), 100 μM mevalonate (32451-23-3, Sigma), 20 μL red yeast rice (Solaray), 20 μM Lovastatin and 20 μL red yeast rice, or 100 μM mevalonate and 20 μL red yeast rice. Unstimulated cells were plated on uncoated plates and treated with either 20 μM Lovastatin, 100 μM mevalonate, or 20 μL red yeast rice. Cells were incubated for 24 hours at 37ºC (5% CO_2).

Cell surface marker expression was then assessed using a MACSquant Analyzer 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Following *in vitro* stimulation, cells were washed and resuspended in FACS buffer (1X PBS with 2% BSA, 0.1% NaN_3). Cells were then incubated for 10 minutes at 4°C in the dark with the following fluorochrome-conjugated anti-mouse antibodies: CD3e-APC-Vio770 (130-117-788, Miltenyi), IL-2Rα(CD25) (12-0252-81, eBioscience), CD45R-VioGreen (130-110-852, Miltenyi). Experiments were performed using 3 replicates for each treatment group for a total of 30 samples. To determine relative

fluorescence intensity, the mean fluorescence intensity (+/- SEM) of CD25 and CD45R on CD3-gated lymphocytes was normalized to unstained controls. One-way ANOVA with Tukey multiple comparison test was used for statistical analysis of mean fluorescence intensity of treatment groups using GraphPad Prism software.

Intracellular Cytokine Staining: Total lymphocytes were isolated, plated, and stimulated as previously described. Following *in vitro* stimulation, cells were washed and resuspended in FACS buffer. Cells were then stained with anti-CD3 antibody. After a five-minute incubation, cells were centrifuged and resuspended in fixative. Cells were then be permeabilized with permeabilization buffer (FACS/0.1% saponin [8047-15-2]) and treated with anti-IL2, anti-IL4, and anti-IFN-γ antibodies. Cytokine production was assessed using a MACSquant Analyzer 10 flow cytometer. The mean fluorescence intensity (+/- SEM) of IL2, IL4, and IFN-γ on CD3-gated lymphocytes was normalized to unstained controls. One-way ANOVA with Tukey multiple comparison test was used for statistical analysis of mean fluorescence intensity of treatment groups using GraphPad Prism software.

In Vitro Stimulation and Proliferation Assay: T-lymphocytes were first isolated by magnetic sorting using anti-CD90.1 microbeads (130-095-523, Miltenyi Biotec), according to manufacturer's protocol. Isolated T-cells were then plated on a 96-well plate at $2x10^6$ cells/mL, divided into five treatment groups, and plated in quadruplicate. Stimulated cells were plated in wells previously coated with 2 μg/mL anti-CD3 (eBioscience), followed by 1 μg/mL anti-CD28 (eBioscience). Stimulated and unstimulated cells then received either 5 μL red yeast rice, 10 μL

red yeast rice, 20 μL red yeast rice, 20 μM Lovastatin (Sigma). Cells were then incubated for 24 hours at 37ºC (5% CO_2).

Proliferation was assessed using the MTT Cell Proliferation Assay kit (30-1010K, ATCC, Manassas, VA) according to the manufacturer's guidelines. Absorbance was determined using a BIO-RAD Model 680 microplate reader at 570 nm. The absorbance (+/- SEM) was normalized to unstained controls. One-way ANOVA with Tukey multiple comparison test was used for statistical analysis of treatment groups using GraphPad Prism software.

Results

Preliminary Data: In the course of investigating possible cytotoxic effects of RYR extract on T lymphocytes, it was observed that an increased number of T cells were present in samples stimulated with anti-CD3 with increasing concentrations of RYR extract (Figure 1, Jenna Boyd, unpublished data). Additionally, it was observed that increased RYR extract concentrations showed no cytotoxicity to anti-CD3 stimulated T cells (Figure 2, Jenna Boyd, unpublished data). These data were the basis for the current investigation on the effect of RYR extract on T lymphocyte function.

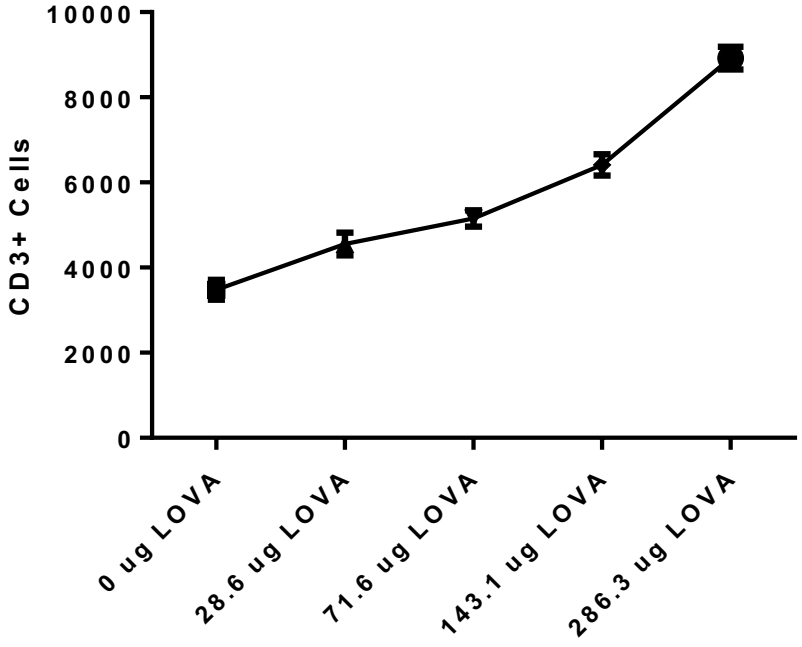

Figure 1: *RYR extract in increasing concentrations results in increased total number of T-Lymphocytes*. 24-well plates were coated with 4 μg/mL anti-CD3 for 24 hours. Total splenocytes were plated at $4x10^6$ cells/mL and treated with RYR extract (0 μg, 28.6 μg, 71.6 μg, 143.1 μg, and 286.3 μg endogenous hydrolyzed lovastatin) in triplicate. Cells were incubated for 24-hour hours at 37°C. Cell counts were determined with a BD Accuri C6 flow cytometer. Activated T-lymphocytes show significant differences in proliferation when incubated with RYR with increasing concentrations of endogenous lovastatin ($p<0.05$). Mean fluorescent intensity (MFI) was normalized compared to the background control. Error bars represent standard error of the mean. Data is representative of three experiments.

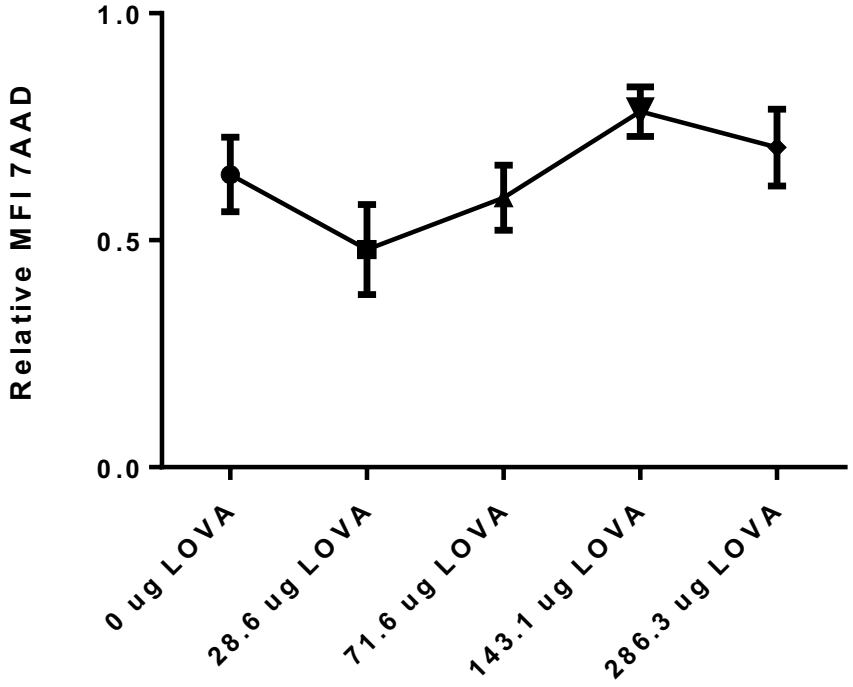

Figure 2: *RYR extract in increasing concentrations was not cytotoxic to the T-Lymphocytes.* 24-well plates were coated with 4 μg/mL anti-CD3 for 24 hours. Total splenocytes were plated at $4x10^6$ cells/mL and treated with RYR extract (0 μg, 28.6 μg, 71.6 μg, 143.1 μg, and 286.3 μg endogenous hydrolyzed lovastatin) in triplicate. Cells were then incubated for 24 hours at 37°C (5% CO_2), and analyzed using a BD Accuri C6 flow cytometer to determine cytotoxicity levels. Activated T-lymphocytes show no significant differences in cytotoxicity when incubated with RYR with increasing concentrations of endogenous lovastatin. Mean fluorescent intensity (MFI) was normalized compared to the background control.

Proliferation Studies: In order to further elucidate the potential effects of RYR extract on T-cell function, lymphocyte proliferation following RYR treatment was investigated. Critical to an effective and robust immune response is the activation and proliferation of antigen-specific T-cells. Results of previous studies by the Bruns lab found that treatment of stimulated T-cells with RYR extract resulted in an increase in total T-cell numbers (Figure 1) and that RYR extract was non-cytotoxic to T-cells (Figure 2). These results were the basis for current the proliferation study. Total lymphocytes were isolated from the spleens of male and female C45BL/6 mice, stimulated with anti-CD3/anti-CD28 and treated with increasing concentrations of RYR extract. Following treatment, relative levels of proliferation between treatment groups were determined using an MTT Cell Proliferation Assay Kit.

Based on our previous results (Figure 1 and Figure 2), we hypothesized that treatment of stimulated T-cells with RYR extract would result in an increase in proliferation with increasing concentrations of RYR extract. Contrastingly, these results show that RYR treatment inhibited the proliferation of stimulated T-cells in a significant manner when compared to untreated/stimulated controls (Figure 3). However, though results were significant, the degree of variability observed led us to question the validity of our results and if other cells present in the total splenocyte population were skewing our results. We hypothesized that the chemical constituents of RYR extract could be binding to receptors of non-T-cells and leading to some degree of stimulation. For this reason, protocols were modified to include a T-cell purification step, in which total T-cells were separated via magnetic sorting. Prior to plating cells, total splenocytes were incubated with magnetic microbeads specific to CD90.2, a T-cell-specific marker, and passed through a magnetic column, separating +CD90.2 and -CD90.2 cells. These

results show that RYR treatment inhibited the proliferation of purified, stimulated T-cells in a significant manner when compared to stimulated/untreated controls (Figure 4).

Results also show that the degree of proliferative inhibition increased with increasing concentrations of RYR extract, with 20 μL RYR/572.5 μg hydrolyzed lovastatin inhibiting proliferation in a manner similar to the negative control, lovastatin. These results refute our original hypothesis and were the basis for future gene expression and cytokine analysis studies; with the goal of determining RYR extract's possible effects on stimulatory proteins and cytokines that are key to T-cell and immune function.

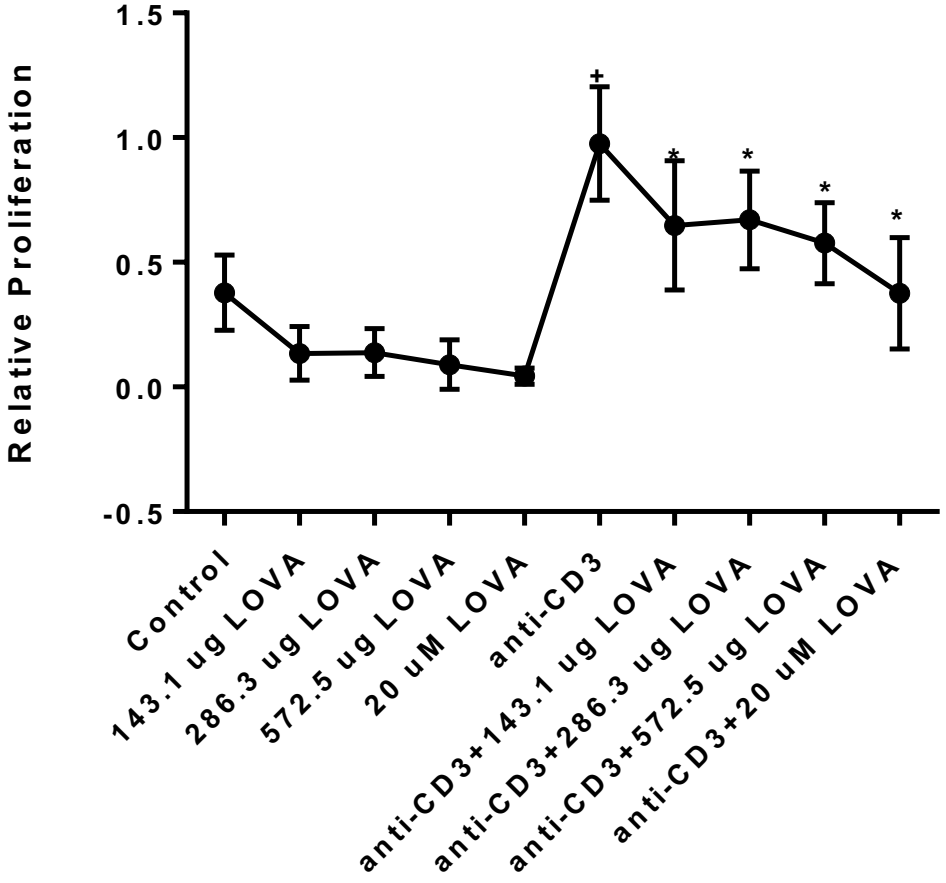

Figure 3: *RYR extract may suppress T-lymphocyte proliferation.* Cells were then plated at 2 x 10^6 cells/mL. Unstimulated groups were then treated with RYR extract (143.1, 286.3, or 572.5 µg hydrolyzed lovastatin), or 20 µM LOVA. Stimulated groups were treated with 2 µg/mL anti-CD3 and 1 µg/mL anti-CD28 and with RYR extract (143.1, 286.3, or 572.5 µg endogenous hydrolyzed lovastatin), or 20 µM LOVA. Cells were then incubated for 24 hours at 37°C (5% CO_2). Proliferation was assessed using MTT Cell Proliferation Assay according to manufacturer's protocol and absorbance was measured at 595 nm using a BioRad iMark plate reader. All biological replicates were done in quadruplicate. Significant differences compared to stimulated control is indicated by * ($p<0.05$). Significant differences compared to unstimulated control is indicated by + ($p<0.05$). Results are pooled, normalized data of three separate experiments.

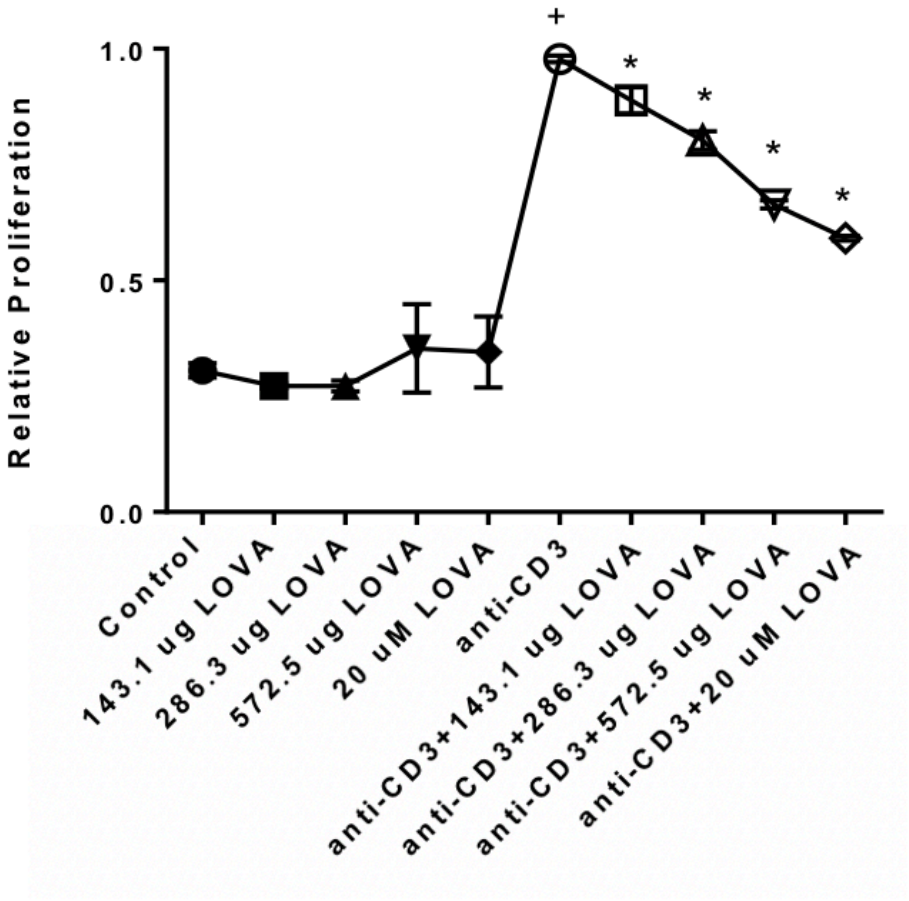

Figure 4: *RYR extract suppresses T-lymphocyte proliferation.* T-lymphocytes were first isolated from total lymphocytes via magnetic sorting. Cells were then plated at 2 x 10^6 cells/mL. Unstimulated groups were then treated with RYR extract (143.1, 286.3, 572.5 µg endogenous hydrolyzed lovastatin), or 20 µM LOVA. Stimulated groups were treated with 2 µg/mL anti-CD3 and 1 µg/mL anti-CD28 and with RYR extract (143.1, 286.3, 572.5 µg hydrolyzed lovastatin), or 20 µM LOVA. Cells were then incubated for 24 hours at 37°C (5% CO_2). Proliferation was assessed using MTT Cell Proliferation Assay according to manufacturer's protocol and absorbance was measured at 595 nm using a BioRad iMark plate reader. All biological replicates were done in quadruplicate. Significant differences compared to stimulated control is indicated by * ($p<0.05$). Significant differences compared to unstimulated control is indicated by + ($p<0.05$).

Gene Expression Studies: To further elucidate the effects of RYR extract on the immune response, specifically effects on T-cell activation, proteins critical for appropriate signaling and are expressed on the cell surface following activation, were examined: CD25/IL-2Rα, CD45R/PTPRC, CD95/Fas, CD178/FasL, and CD152/CTLA-4. Total lymphocytes were isolated from the spleens of adult male and female C57BL/6 mice. Unstimulated and anti-CD3/anti-CD28-stimulated cells were then treated with varying concentrations of RYR extract, incubated for 24 hours, and stained with the appropriate fluorochrome-conjugated antibodies. Samples were then analyzed using a MACSquant Analyzer 10 flow cytometer.

IL-2 is an important immune-stimulatory cytokine that acts in all stages of the immune response, including primary expansion, contraction, and memory generation [8,10]. Critical to these processes is the increase in affinity of T-cells to IL-2, which is achieved through the expression of the high-affinity IL-2R. CD25 is a transmembrane protein expressed on non-activated T-lymphocytes as the low-affinity IL-2 receptor, and is a constituent of the high-affinity IL-2R present on activated T-cells [10]. Given the function of CD25 and taken together with the data from our proliferation studies, we hypothesized that treatment of stimulated T-cells purified from a total splenocyte population with RYR would result in a decrease in CD25 expression.

Our results show that RYR extract significantly decreased cell surface expression of CD25 in anti-CD3/anti-CD28 stimulated T-cells, relative to the untreated/stimulated group (Figure 5). Additionally, the observed decrease in CD25 expression in the stimulated/RYR-treated group was similar to what was observed in the lovastatin-treated stimulated group.

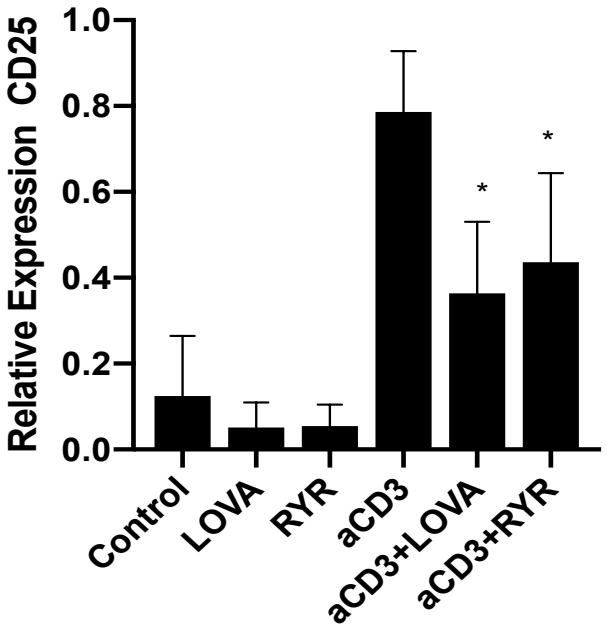

Figure 5: *RYR extract decreases expression of CD25/IL2RA in anti-CD3-stimulated T-lymphocytes*. 24-well plates were coated with 2 μg/mL anti-CD3 for 24 hours. Total lymphocytes were plated at 1x 10^6 cells/mL. Unstimulated cells were treated with 20 μM LOVA, or RYR extract (572.5 μg hydrolyzed lovastatin). Stimulated groups were treated with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 and either 20 μM LOVA or RYR extract (572.5 μg endogenous hydrolyzed lovastatin). Cells were then incubated for 24 hours at 37°C (5% CO_2). Cells were stained with CD3e-APC-Vio77o and CD25-PE. Cells were then analyzed using a MACSquant Analyzer 10. All biological replicates were done in triplicate. Significant differences compared to stimulated control is indicated by * ($p<0.05$). Results are pooled, normalized data from four separate experiments.

CD45R is a transmembrane receptor-like protein tyrosine phosphatase, receptor type-C (PTPRC) that is found on all hematopoietic cells and is an essential regulator of T-cell activation, TCR-mediated signaling, and T-cell regulation [8]. Binding of CD45 to CD45R results in the dephosphorylation of inhibitory tyrosine residues in Src family kinases (SFK), which leads to further downstream activation cascades and T-cell activation. Given the function of CD45R and taken together with the data from our proliferation studies, we hypothesized that treatment of stimulated T-cells with RYR extract would result in a decrease in CD45R expression.

Our results show that RYR extract may have an inhibitory effect on the expression of CD45R in stimulated T-cells (Figure 6). A decrease in the expression of CD45R was observed in the stimulated, RYR-treated group when compared to the stimulated, untreated group. However, the observed differences were not statistically significant as determined by one-way ANOVA.

CD95/Fas is a transmembrane protein expressed on the cell surface and a death receptor that, upon binding of CD178/FasL, induces apoptosis via formation of the death-inducing signaling complex (DISC) [8]. Fas/FasL triggered apoptosis is critical in the regulation of the immune system. Expression of CD178/FasL is induced in T-cells upon their activation. Activated T-cells undergoing clonal expansion are initially resistant to the apoptotic effects of Fas/FasL. However, as the immune response progresses activated T-cells become more and more sensitive to Fas/FasL binding. Ultimately, this process leads to activation-induced cell death (AICD) and acts to inhibit an excessive immune response.

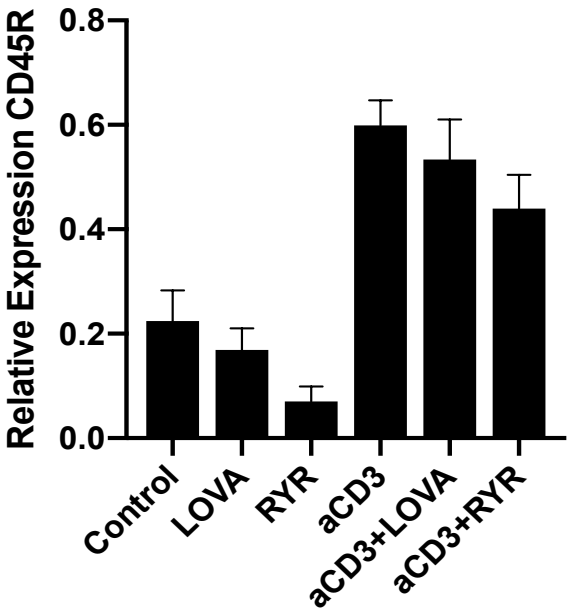

Figure 6: *RYR extract may decrease expression of CD45 in anti-CD3-stimulated T-lymphocytes*. Total lymphocytes were plated at 1x 10^6 cells/mL. Unstimulated cells were treated with 20 µM LOVA, or RYR extract (572.5 µg endogenous hydrolyzed lovastatin). Stimulated groups were treated with 2 µg/mL anti-CD3 and 1 µg/mL anti-CD28 and either 20 µM LOVA or RYR extract (572.5 µg endogenous hydrolyzed lovastatin). Cells were then incubated for 24 hours at 37°C (5% CO_2). Cells were stained with CD3e-APC-Vio77o and CD25-PE. Cells were then analyzed using a MACSquant Analyzer 10. All biological replicates were done in triplicate. Significant differences compared to stimulated control is indicated by * ($p<0.05$). Results are pooled, normalized data from three separate experiments.

Unfortunately, we are unable to draw any solid conclusions from our results, as a higher than acceptable level of variability was observed for both CD95 and CD178 (Figure 7). Though differences were not significant, it appears that RYR extract may indeed have an effect on expression of both CD95 and CD178 in unstimulated and stimulated T-cells. A higher expression of CD95 and CD178 was observed in unstimulated, RYR-treated groups, relative to unstimulated controls. Additionally, though not statistically significant, it appears that expression of CD95 and CD178 may be increased in stimulated, RYR-treated groups, as compared to stimulated, untreated groups.

CD152 is a receptor expressed on activated T-cells that plays a key role in the downregulation of the secondary immune response [40]. Downregulation is achieved via competitive inhibition, as CD152 binds CD80 and CD86 with a greater affinity than that of CD28. Upon ligand binding, CD152 will induce inhibitory cascades that leads to the cessation of the immune response. Our results show no significant differences in CD152 expression between any treatment groups (Figure 8).

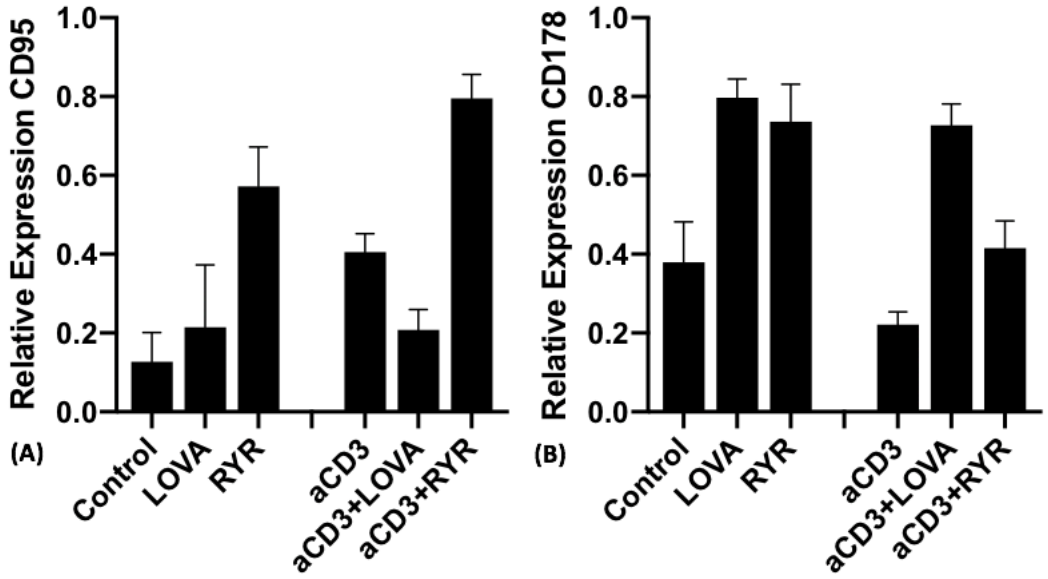

Figure 7: (A) *RYR extract may increase expression of CD95 in anti-CD3-stimulated T-lymphocytes*. 24-well plates were coated with 2 μg/mL anti-CD3 for 24 hours. Total splenocytes were then plated at $2x10^6$ cells/mL. Unstimulated cells were treated with 20 μM LOVA, or RYR extract (572.5 μg endogenous hydrolyzed lovastatin). Stimulated groups were treated with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 and either 20 μM LOVA or RYR extract (572.5 μg endogenous hydrolyzed lovastatin). Cells were then incubated for 24 hours at 37°C (5% CO_2). Treatments were performed in triplicate and cells were incubated for 24-hours at 37°C. Following stimulation, cells were stained with fluorochrome-conjugated anti-CD95 antibody and analyzed using a MACSquant Analyzer 10 flow cytometer. Error bars represent standard error of the mean. Data were normalized to unstained controls and are representative of two experiments. **(B)** *RYR extract may increase expression of CD178 in anti-CD3-stimulated T-lymphocytes*. 24-well plates were coated with 2 μg/mL anti-CD3 for 24 hours. Total splenocytes were then plated at $2x10^6$ cells/mL. Unstimulated cells were treated with 20 μM LOVA, or RYR extract (endogenous 572.5 μg hydrolyzed lovastatin). Stimulated groups were treated with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 and either 20 μM LOVA or RYR extract (572.5 μg endogenous hydrolyzed lovastatin). Cells were then incubated for 24 hours at 37°C (5% CO_2). Treatments were performed in triplicate and cells were incubated for 24-hours at 37°C. Following stimulation, cells were stained with fluorochrome-conjugated anti-CD178 antibody and analyzed using a MACSquant Analyzer 10 flow cytometer. Error bars represent standard error of the mean. Data were normalized to unstained controls and are representative of three experiments

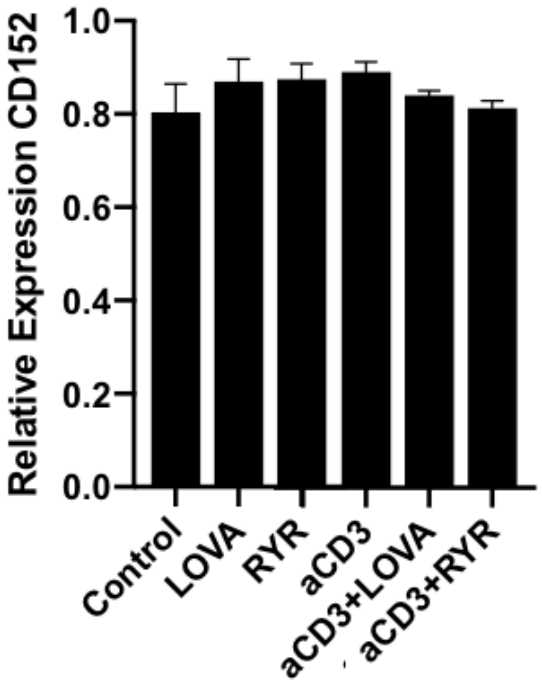

Figure 8: *RYR extract appears to have no effect on expression of CD152 in anti-CD3-stimulated T-lymphocytes.* 24-well plates were coated with 2 μg/mL anti-CD3 for 24 hours. Total splenocytes were then plated at $2x10^6$ cells/mL. Unstimulated cells were treated with 20 μM LOVA, or RYR extract (572.5 μg endogenous hydrolyzed lovastatin). Stimulated groups were treated with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 and either 20 μM LOVA or RYR extract (572.5 μg endogenous hydrolyzed lovastatin). Cells were then incubated for 24 hours at 37°C (5% CO_2). Treatments were performed in triplicate and cells were incubated for 24-hours at 37°C. Following stimulation, cells were stained with fluorochrome-conjugated anti-CD152 antibody and analyzed using a MACSquant Analyzer 10 flow cytometer. Error bars represent standard error of the mean. Data were normalized to unstained controls and are representative of two experiments.

In addition to investigating how RYR extract affects the expression of genes critical to a T-cell response, we also wanted to determine which of the many constituents of RYR extract may be contributing to the altered protein expression during activation. Figure 9 shows an overview of the cholesterol biosynthesis pathway and the effects of statin drugs on cholesterol biosynthesis. Statins are HMG-CoA Reductase inhibitors and prevent the conversion of HMG-CoA to mevalonate, a cholesterol intermediary, thus preventing cholesterol biosynthesis. Additionally, many of the anti-inflammatory effects on statins are attributed to mechanisms related to their lipid lowering abilities.

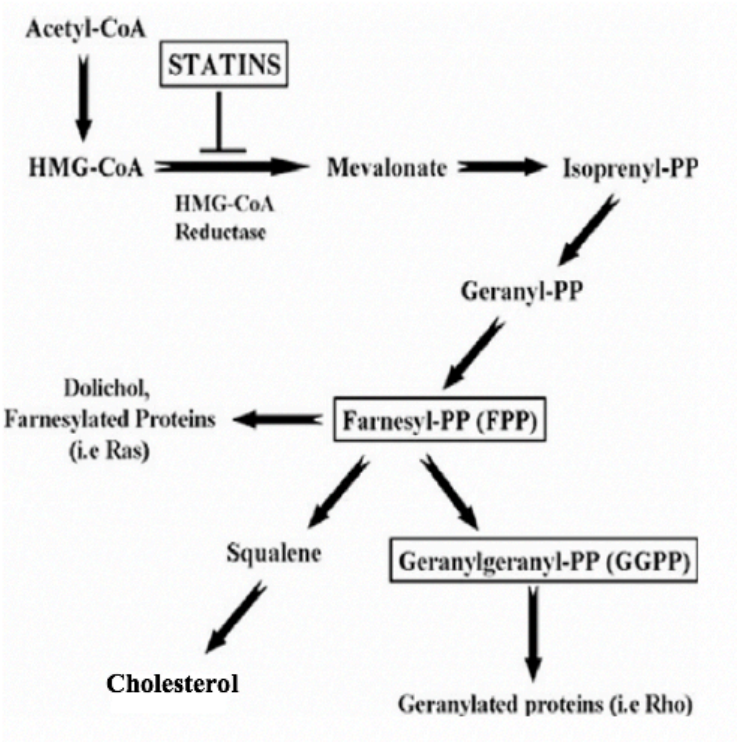

Figure 9: Overview of effects of statins of cholesterol biosynthesis pathway. Statins act via inhibition of HMG-CoA Reductase. Inhibition blocks production of mevalonate, an intermediary for cholesterol and several isoprenoid intermediaries.

Therefore, we hypothesized that if the observed effects of RYR extract on T-cells are due primarily to endogenous monacolins, then the addition of mevalonate should reverse the effects of statins, restore normal cholesterol biosynthesis, and subsequent T-cell activation. Total lymphocytes were isolated from the spleens of adult male and female C57BL/6 mice. Unstimulated and anti-CD3/anti-CD28-stimulated cells were then treated with varying concentrations of RYR and 100 μM mevalonate, incubated for 24 hours, and stained with the appropriate fluorochrome-conjugated antibodies. Samples were then analyzed using a MACSquant Analyzer 10 flow cytometer.

Our results show that addition of mevalonate restored CD25 expression in both lovastatin- and RYR-treated, stimulated groups. However, treatment with mevalonate was unable to fully restore CD25 expression to levels observed in the stimulated/untreated group, as significant differences are observed between treatment groups (Figure 10). Additionally, results show that RYR extrtact may have an inhibitory effect on the expression of CD45R in stimulated T-cells (Figure 11). A decrease in the expression of CD45R was observed in the stimulated, RYR-treated group when compared to the stimulated, untreated group. However, the observed differences were not statistically significant as determined by one-way ANOVA. Results also show that mevalonate appears to have little to no effect on CD45R expression, as no statistically significant differences were observed between stimulated and RYR-treated and stimulated groups.

In summary, although initial studies to assess cytotoxicity of RYR indicated that RYR might enhance proliferation, these studies have demonstrated that RYR inhibits proliferation (Figure 3 and Figure 4). This may be due, in part, to the suppression of proteins involved in T cell activation, CD25 and CD45R, following RYR treatment. Although monacolin K (lovastatin) is one of the most abundant chemical constituents in RYR, data from this study suggests that other chemical constituents contribute to the altered expression of activation proteins in T cells observed following treatment with RYR (Figures 10 and 11). If the immunomodulatory effects of RYR are primarily due to the endogenous monacolins (HMG-CoA reductase inhibitors), then addition of mevalonate should reverse effects of RYR, which was not observed.

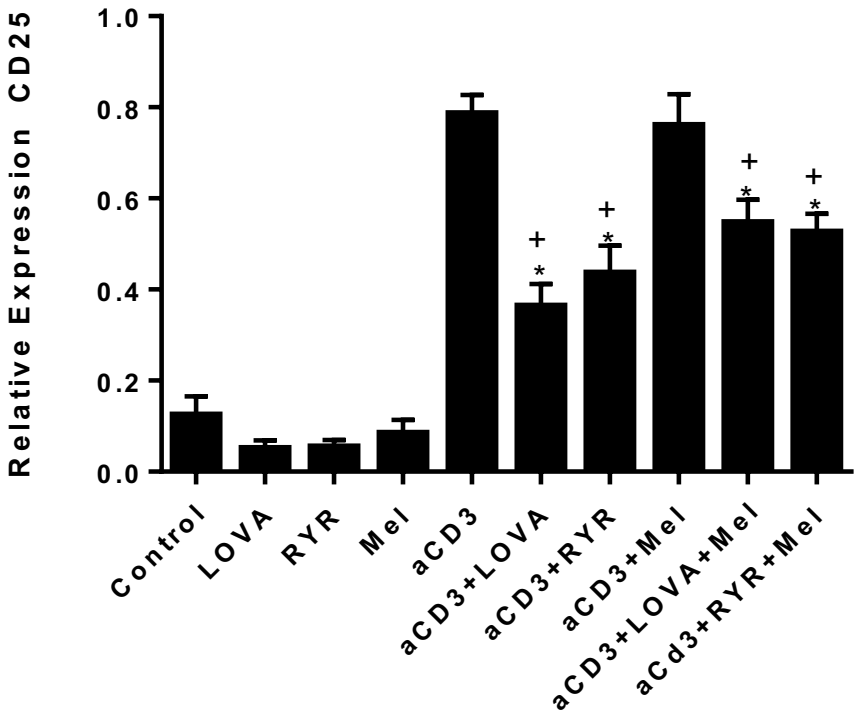

Figure 10: *RYR extract decreases expression of CD25/IL2RA in anti-CD3-stimulated T-lymphocytes and mevalonate increases expression of CD25/IL2RA in RYR-treated anti-CD3-stimulated T-lymphocytes*. 24-well plates were coated with 2 μg/mL anti-CD3 for 24 hours. Total lymphocytes were plated at $2x10^6$ cells/mL. Unstimulated cells were treated with 20 μM LOVA, RYR extract (572.5 μg endogenous hydrolyzed lovastatin), or 100 μM mevalonate. Stimulated groups were treated with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 and either 20 μM LOVA, RYR extract (572.5 μg endogenous hydrolyzed lovastatin), 100 μM Mevalonate, 20 μM LOVA+100 μM Mevalonate, or RYR extract (572.5 μg hydrolyzed lovastatin) +100 μM Mevalonate. Cells were then incubated for 24 hours at 37°C (5% CO_2). Cells were then stained with fluorochrome-conjugated anti-CD25. Cells were then analyzed using a MACSquant Analyzer 10. All groups were done in triplicate. Significant differences compared to stimulated control is indicated by * ($p<0.05$). Significant differences compared to aCD3+Mel is indicated by + ($p<0.05$). Results are pooled, normalized data from four separate experiments.

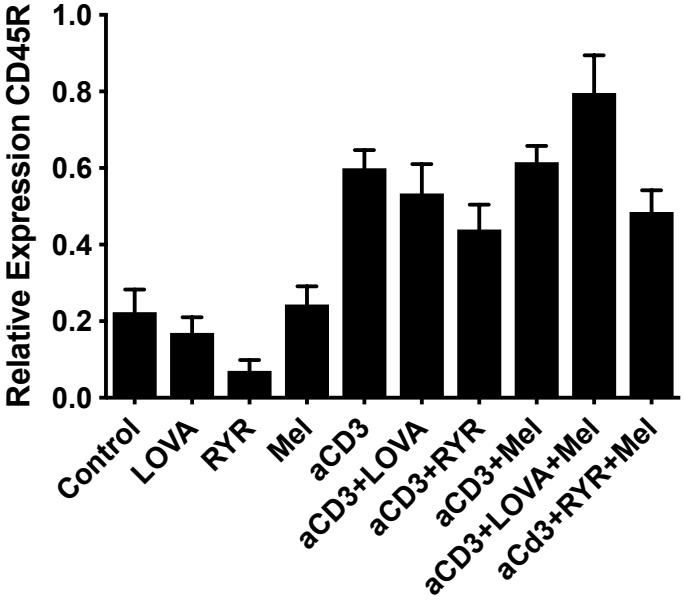

Figure 11: *RYR extract may decrease expression of CD45R in anti-CD3-stimulated T-lymphocytes and mevalonate may increase expression of CD45R in RYR-treated anti-CD3-stimulated T-lymphocytes*. 24-well plates were coated with 2 μg/mL anti-CD3 for 24 hours. Total splenocytes were then plated at $2x10^6$ cells/mL. Unstimulated cells were treated with 20 μM LOVA, RYR extract (572.5 μg endogenous hydrolyzed lovastatin), or 100 μM mevalonate. Stimulated groups were treated with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 and either 20 μM LOVA, RYR extract (572.5 μg endogenous hydrolyzed lovastatin), 100 μM Mevalonate, 20 μM LOVA+100 μM Mevalonate, or RYR extract (572.5 μg hydrolyzed lovastatin)+100 μM Mevalonate. Treatments were performed in triplicate and cells were incubated for 24-hours at 37°C. Following stimulation, cells were stained with fluorochrome-conjugated anti-CD45R antibody and analyzed using a MACSquant Analyzer 10 flow cytometer. Error bars represent standard error of the mean. Data were normalized to unstained controls and are representative of two experiments.

Discussion

T-cell activation is a key component of the adaptive immune response and leads to a proliferative burst of antigen-specific T-cells that direct the adaptive response. Activation is a complex process that involves a number of recognition, adhesion, stimulatory, and signaling molecules. Following activation, signaling cascades that involve CD3 proteins and CD45R lead to the release of IL-2 and the expression of high affinity IL-2R on the cell surface [8,10]. High affinity IL-2R is a multi-subunit complex consisting of CD132, CD122, and CD25. The release and binding of IL-2 acts to induce a rapid proliferative bust. Following binding of IL-2 to the high affinity IL-2R, the cell will then internalize the IL-2/IL-2R complex, degrade the complex, and recycle CD25 back to the cell surface. This recycling provides a supply of CD25 for continuous stimulation and proliferation, which will continue for 24-48 hours after activation.

Our results show that treatment of activated, anti-CD3/anti-CD28 stimulated T-cells with increasing concentrations of RYR extract was able to significantly lower proliferation in total splenocyte population and purified T-cell populations, as assessed by MTT proliferation assay (Figure 3 and Figure 4). Initially, these results were surprising as preliminary data showed an increase in the number of CD3+ cells with increasing concentrations of RYR extract (Figure 1) while determining that the RYR extract was not cytotoxic to T-cells (Figure 2). Additionally, previous studies that investigated the immunosuppressive roles of RYR extract on human peripheral blood T-cells have shown that RYR was non-cytotoxic and that proliferation was significantly lowered [37].

We believe that this seemingly contrasting data could possibly be attributed to the cells that compose a total splenocyte population, which include T- and B-cells, macrophages, and dendritic cells, and their possible effects on each other in response to RYR treatment. RYR extract's mechanism of action could be acting in a way that decreases the proliferative ability of cells, while simultaneously altering the ratio of specific cells present in a splenocyte population, possibly via induction of survival signals that allow for T-cells to survive but not proliferate. Indeed, previous studies have demonstrated the influence and importance of other cell types for T-cell survival; for example, it has been shown that T-cells that have prolonged exposure to immature and mature dendritic cells have increased fitness and resistance to death [38].

Additionally, the MTT proliferation assay utilized in this study is a colorimetric assay that measures the activity of mitochondrial-associated enzymes that are able to reduce the MTT reagent; it is not an assay that is utilized to measure total cell counts. We mention this, not to question the validity of our results or experimental design, but to question the relationship between total cell counts and proliferation. We assumed that a cell population with a low metabolic rate would produce a lower cell count than a population with a higher metabolic rate. Though logical, our assumption could very well be wrong; and as we previously mentioned, the MTT assay provided us with a measure of metabolic activity of a cell population, not the total population size. In the future, an addition of a cell counting step would aid us in better understanding the relationship between the preliminary data that measured population size of CD3+ cells in response to RYR extract and to this study's proliferation data.

Following proliferation studies, our next goal was to investigate the possible effects of RYR extract on surface proteins key for appropriate signaling, activation, and regulation. Given the results of proliferation studies, we hypothesized that we would observe lower expression of signaling and activator proteins, CD25 and CD45R, and that we would observe an increase in the expression of regulator proteins CD95, CD178, and CD152 following RYR treatment.

Our results show that treatment of activated, anti-CD3/anti-CD28 stimulated T-cells with RYR extract was able to significantly decrease expression of CD25 as compared to stimulated, untreated controls (Figure 5). This observed effect could be a possible explanation for the results obtained from proliferation studies. As stated previously, CD25 is a key component of the high affinity IL-2R and is upregulated in response to activation [8, 10]. This results in an increase in affinity to IL-2 and ultimately leads to a proliferative bust. Given this information, a decrease in a component of the high-affinity IL-2R would likely result in a decrease in the responsiveness of T-cells to IL-2, which would likely diminish the proliferative ability of a T-cell population. Previous studies have indeed demonstrated that T-cell proliferation is inhibited when IL-2R expression is inhibited [39]. A pattern that mirrors the data for CD25 was observed for CD45R, as it appears that RYR extract decreases expression of CD45R in activated, anti-CD3/anti-CD28 stimulated T-cells; however, results were not significant (Figure 6).

Conversely, it appears that RYR treatment may increase expression of CD95 and CD178 (Figure 7A and Figure 7B). These proteins are key in maintaining T-cell homeostasis and are upregulated after prolonged antigen exposure [10]. Subsequent binding of CD95/CD178 induces apoptosis via formation of the death-inducing signaling complex [DISC]. Given this, if CD95 and CD178 are playing a role in diminishing T-cell activation and proliferation in response to RYR treatment, then we would expect an increase in these proteins. However, these results were also statistically insignificant and a high level of variability was observed. Additionally, no difference was observed in CD152 expression, contrary to anticipated results (Figure 8). Previous studies investigating CD152 expression have demonstrated that stimulated T-cells express the highest amount of CD152 at 48 hours, post-stimulation [40]. Therefore, in order to accurately assess the potential effects of RYR on CD152 expression in the future, flow analysis will be performed after a 48-hour incubation.

A number of compounds with characterized immunomodulatory properties have been identified in RYR [29-36]. Thus, the goal of this study was to not only determine how RYR extract affects genes critical to T-cell functions, but to also determine which of the many constituents of RYR extract may be contributing to any alterations in protein expression. Due to the known anti-inflammatory effects of statins and the presence of endogenous monacolins (naturally occurring statins) in RYR extract, we hypothesized that the immunoregulatory effects of RYR extract are attributed primarily to endogenous monacolins. If true, then the addition of mevalonate should reverse the observed effects of statins, restore normal cholesterol biosynthesis, and subsequent T-cell functions.

Our results show that addition of mevalonate increased CD25 expression in both lovastatin- and RYR-treated, stimulated groups but was unable to fully restore CD25 expression to levels observed in the positive control stimulated group (Figure 10). Given that mevalonate did not fully restore expression of CD25 in the lovastatin-treated stimulated group, it is possible that the optimal mevalonate concentration was not used. Thus, we cannot conclude if the observed immunomodulatory effects of RYR are due solely to endogenous monacolins. Future studies will be focused on determining the optimal concentration of mevalonate to use so that the involvement of the monacolin family members in altering gene expressing can be accurately assessed.

When taken together, these results suggest that RYR may decrease proliferation by acting to decrease expression of activation/stimulatory proteins, while simultaneously acting to increase expression, and sensitivity of T-cells, to regulatory proteins. Previous studies have demonstrated that RYR lowers proliferation of T-cells and that RYR is non-cytotoxic [37], while others have shown the immunomodulatory effects of many of the separate, individual chemical constituents of RYR [30, 31, 34, 35, 36]. Results of this study provide support for the findings generated by previous studies, while simultaneously providing novel findings that further expand the understanding of the immunomodulatory effects of RYR. Our results provide direct support for the ability of RYR to suppress T-cell proliferation, while demonstrating that suppressed proliferation may be due to the modulation of activating proteins CD25 and CD45R, and regulatory proteins CD95 and CD178, despite having a significantly lower concentration of endogenous monacolins than a typically prescribed statin regiment.

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