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RETINOIC ACID AND α-GALACTOSYLCERAMIDE REGULATE ANTIGEN PRESENTING CELL FUNCTIONS

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ABSTRACT

Antigen presenting cells (APCs)—particularly macrophages, dendritic cells, and B cells—play an important role in the immune system. APCs augment the immune response by activating T cells through major histocompatibility complex (MHC) class II molecules present on the APC cell surface. APCs also augment the immune response by activating invariant natural killer T (iNKT) cells through CD1d, an MHC class-I-like molecule present on the surface of certain APCs that presents lipid antigens. The focus of this thesis will be on the regulation of CD1d expression in APCs and the activation of B cells through CD1d after the addition of all-*trans* retinoic acid (RA) and α-galactosylceramide (αGalCer).

Previously, it was reported that the expression of CD1d in monocytes was upregulated by RA. However, it is unknown whether αGalCer, a ligand for CD1d, is also able to regulate CD1d or whether it is able to synergize with RA to further upregulate CD1d. We show here using the THP-1 human monocytic cell line that αGalCer is unable to regulate the expression of CD1d. Moreover, we have clarified the kinetics of CD1d protein upregulation by showing that RA maximally upregulates the CD1d protein ($p<0.001$) within 24 hours.

Also, it was reported that αGalCer was able to activate splenic B cells through CD1d in mice. However, it is unknown whether αGalCer is able to activate human B cells or whether it is able to synergize with RA to further induce activation. We show here using the CL-01 human B cell line that αGalCer is able to activate human B cells by upregulating the proliferation marker Ki67. We also show that the mechanism of human B cell activation occurs through the upregulation of the B cell transcription factor Pax5 ($p<0.05$) and the B cell coreceptor CD19 ($p<0.05$).

These findings suggest that RA and αGalCer are potent regulators of APCs and could be useful as adjuvants to boost immunity after vaccination.

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ABBREVIATION LIST

αGalCer = α-galactosylceramide

APC = antigen presenting cell

BCR = B cell receptor

CD = cluster of differentiation

DPBS = Dulbecco's phosphate-buffered saline

ERK = extracellular signal-regulated kinase

IFN = interferon

IL = interleukin

iNKT = invariant natural killer T cell

LPS = lipopolysaccharide

MAPK = mitogen-activated protein kinase

MHC = major histocompatibility complex

MFI = mean fluorescent intensity

NFκB = nuclear factor κ-light-chain-enhancer of activated B cells

PI3K = phosphatidylinositol 3-kinase

RA = retinoic acid

RT-PCR = real-time polymerase chain reaction

STAT = signal transducer and activator of transcription

TNF = tumor necrosis factor

1. INTRODUCTION

1.1 The Immune System

The immune system is a network of organs and cells that help fight against disease. The immune system comprises of anatomical barriers, preformed innate immune responses, induced innate immune responses, and adaptive immune responses[1]. Anatomical barriers are the first lines of defense, and they prevent pathogens from entering the body. They include the skin, tears and saliva, stomach acid, and mucus and cilia in the lungs. Components of the innate immune system are present in the serum and all body secretions, and they help rapidly fight pathogens that enter the body. Some effector molecules of the preformed innate immune response include complement proteins, antimicrobial peptides, and lysozymes.

Induced innate immune responses take a few hours to activate, but they are much more effective at clearing infections. Inflammation and phagocytes are hallmarks of the induced innate immune response. Cells of the innate immune system know when to respond to infection because they possess pattern recognition receptors that bind to pathogen associated molecular patterns. Essentially, these cells react to entities that are detected as non-self. After encountering these foreign entities or pathogens in the tissue, macrophages initiate inflammation and produce antimicrobial peptides, cytokines, and chemokines to recruit neutrophils and other cells from the blood[1]. This results in a concentrated series of processes that serve to clear the pathogen from the site of the infection. Induced innate immune responses are considered to be nonspecific.

If the infection persists, the adaptive immune response takes over. This response is only present in vertebrates, and it is known for its specificity, efficiency, and memory. The two major effector cells of the adaptive immune system are B cells and T cells. B cells are important for humoral immunity—fighting infections present in the serum. B cells produce antibodies that

neutralize, opsonize, and synergize with complement to eliminate pathogens[1]. T cells are important for cell-mediated immunity—fighting infections present inside the cell. T cells have two subdivisions: cytotoxic T cells and helper T cells. Cytotoxic T cells kill infected cells by inducing apoptosis, and helper T cells activate other cells to augment the immune response.

B cells and T cells mature in primary lymphoid organs, which include the bone marrow and the thymus[1]. B cells mature in the bone marrow, and T cells mature in the thymus. During maturation, B cells and T cells achieve diversity in their receptors by gene rearrangement. After a functional receptor is made, B cells and T cells undergo negative selection to eliminate cells that are self reactive. In the case of T cells, positive selection also takes place to ensure that T cells can respond to self MHC molecules. Mature B cells and T cells then migrate to secondary lymphoid organs, where they await antigenic stimulation. Examples of secondary lymphoid organs include lymph nodes, the spleen, and Peyer's patches[1].

The adaptive immune response has memory, which allows secondary immune responses to be more potent than primary immune responses. This is the basis of vaccination—introducing attenuated pathogens to an organism early on so that subsequent infections are met with greater resistance. Secondary immune responses are stronger and faster than primary immune responses mainly because, in a secondary immune response, memory B cells and T cells already circulate in the blood[1]. This means that B cells and T cells do not have to undergo clonal expansion, class switching, or affinity maturation as they did during a primary immune response.

1.2 Antigen Presenting Cells

APCs are cells that display antigens complexed with MHC molecules on their surface[1]. Most cells are able to present endogenous peptides via MHC class I molecules; however, the

term APC is limited to cells that are also able to present exogenous peptides via MHC class II molecules[1,2]. Also, APCs characteristically express the costimulatory molecules B7.1 (CD80) or B7.2 (CD86). These costimulatory molecules bind to the T cell CD28 receptor during activation, which prevents the T cell from becoming anergic or unresponsive[3].

There are three types of professional APCs: macrophages, B cells, and dendritic cells. Dendritic cells are the most effective at processing and presenting antigen out of the three[1]. In addition, dendritic cells constitutively express B7 and MHC class II, unlike macrophages and B cells[2]. Macrophages do not constitutively express either B7 or MHC class II, and B cells only constitutively express MHC class II. Only after macrophages and B cells encounter antigen do they begin expressing B7 and MHC class II. After they mature, APCs lose their ability to engulf pathogens, but they develop a greater ability to communicate with and activate T cells.

The most notable function of APCs is serving as the bridge between innate and adaptive immunity. The adaptive immune response cannot take place without APCs because T cells can only be activated by APCs. T cells cannot bind to free, unprocessed antigen since their receptors only recognize linear epitopes[1]. In addition, T cells require costimulatory signals from APCs, otherwise they will become anergic[5]. T cells are key in inducing class switching in B cells and in licensing APCs to activate other T cells and B cells[4]. Thus, in the absence of APCs, the entire adaptive immune response will fail to take place in the organism[2].

There are two main types of antigens that are presented to T cells: peptide antigens and lipid antigens. Peptide antigens help activate conventional T cells, and lipid antigens help activate iNKT cells. iNKT cells are a subset of T cells that express the invariant T cell receptor α chain. iNKT cells are the most studied and best characterized NKT cell population in mice and humans. iNKT cells develop in the thymus similar to conventional T cells. They segregate from

conventional T cells during the double-positive thymocyte stage, which is largely believed to be a random event[7]. In mice, the invariant T cell receptor α chain is formed after the Vα14 gene segment rearranges to the Jα18 gene. The iNKT cell then undergoes positive and negative selection to ensure that it can function properly. The cell then expands and matures, which leads to the acquisition of NK cell phenotypes.

Before T cells and iNKT cells can be activated by APCs, APCs must first take up antigens and present them on their surface. Protein antigens can be engulfed by APCs via phagocytosis (macrophages and dendritic cells) or via receptor mediated endocytosis (B cells)[1]. Protein antigens are then digested by lysozome proteasomes and are presented on the surface of the cell by an MHC class II molecule. Lipid antigens are presented on the surface of the cell by a CD1 antigen presenting molecule. The nonclassical family of CD1 proteins is a third lineage of antigen presenting molecules that presents lipid and glycolipid antigens. CD1 molecules are structurally simiar to MHC class I molecules in regard to their antigen binding site, which is comprised of two α helices (α_1 and α_2)[3]. Lipid antigens can bind directly to surface-bound CD1 molecules or be internalized and digested in late endolysosomal compartments before being presented by CD1 molecules[3].

CD1d is one of the five isotypes of CD1 that have been characterized, and it is the only Group 2 CD1 molecule. Group 2 CD1 molecules are expressed in a greater range of cells, but their natural antigens are not as well characterized. In fact, scientists are still searching for the natural ligand for CD1d. Scientists have discovered that the synthetic glycolipid antigen αGalCer has a very high affinity for CD1d and is able to activate iNKT cells[8]. However, no lipid antigen with α-anomericity has been identified in mammals, so lipid antigens with β-anomericity have been suggested to be the natural ligand for CD1d[8].

1.3 B Cell Activation and CD19

B cells are unique from the other APCs in that they are already (and inherently) part of the adaptive immune system. Activation of B cells results in not only the presentation of antigens to T cells and iNKT cells, but also the production of antibodies to eliminate pathogens.

One of the earliest B cell markers is CD19, a surface protein that interacts with CD21 and CD81 to lower the threshold for stimulation[1,9]. Defects in CD19 expression result in impaired antibody production by B cells[10]. CD19 expression is controlled by the transcription factor Pax5, which is a member of the paired box family of transcription factors that determines B cell lineage differentiation, maintains B cell identity, and increases B cell expansion. Overexpression of Pax5 can increase the expression of recombination enzymes, suggesting a role for Pax5 in early B cell activation and proliferation[11].

When CD19 is activated, its cytosolic tail becomes phosphorylated, allowing src family tyrosine kinases to bind and recruit phosphoinositide 3-kinase (PI3K)[9]. PI3K is an important intracellular signal transducer that is involved in cell proliferation, differentiation, and survival, among other tasks. PI3K is associated with the serine/threonine kinase AKT (also known as protein kinase B), which is known to activate transcription factors such as NFκB (nuclear factor κ-light chain enhancer of B cells) and GSK3β (glycogen synthase kinase 3β).

A mechanism of B cell activation involving CD19 has been proposed by Chung *et. al.*[12]. Here, scientists measured B cell activation by measuring the level of Ki67, a regulatory gene that controls cell proliferation. It was discovered that the mechanism of Ki67 gene regulation was controlled by the Pax5 target CD19 through the PI3K-AKT-GSK3β pathway. Ki67 levels were drastically reduced in CD19-deficient mice, and they were significantly increased in mice overexpressing CD19. It was also found that Pax5 controls Ki67 protein stability and steady-state

levels. This promoter-independent, post-translational mode of Ki67 regulation was independent of immunoreceptor tyrosine activation motif or B cell receptor (BCR) activity. Thus, scientists concluded that Pax5 and CD19 are independent activators of B cell activation.

Transcription of the Ki67 gene typically occurs through the mitogen-associated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway[13]. Ligand binding results in the recruitment of a GTP-GDP exchange factor, which activates the G protein Ras. Activated Ras is then able to phosphorylate Rac, which phosphorylates Mek, which phosphorylates Erk. Erk then enters the nucleus and phosphorylates the Ki67 transcription factor, resulting in cell proliferation. There is cross-talk between the MAPK/ERK and the PI3K/AKT pathways, which supports the mechanism proposed by Chung *et. al.*[14].

1.5 Retinoic Acid and the Immune System

RA is the metabolite of vitamin A that is involved in regulating immune function. Vitamin A is typically ingested either as preformed vitamin A or as provitamin A. Upon entering the small intestine, most forms of vitamin A are converted into retinol, which can passively diffuse into enterocytes[15]. Retinol is then esterified and incorporated into chylomicrons, which travel to the liver through the lymphatic system. The liver controls the release of retinol so that plasma levels remain between 1-3 μM. Before being released into the bloodstream, retinol is bound to retinol-binding protein (RBP). RBP is taken up by receptors, and once inside the cell, retinol is converted into RA by the retinal dehydrogenase enzyme.

RA has an isoprenoid structure with a carboxylic functional group at the end of its hydrocarbon chain (Figure 1.1). As an isoprenoid, RA is able to diffuse across the cell membrane and directly influence the transcription of target genes. RA acts by binding to the retinoic acid

receptor (RAR), which is bound to retinoic acid response elements complexed with the retinoid X receptor[16,17]. Binding of RA to its receptor causes RAR to change conformations. As a result, inhibitory co-repressors are released, exposing regions of DNA. This allows co-activators to bind to the DNA and induce transcription.

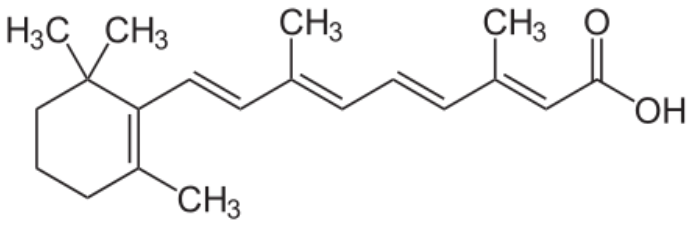

Figure 1.1 Structure of retinoic acid. RA has an isoprenoid structure, with a molecular formula of $C_{20}H_{28}O_2$ and a molecular weight of 300 g/mol.

The antigen presenting functions of monocytes has been shown to be increased by RA. In a previous study by Chen *et. al*, RA rapidly (1 hour) increased CD1d mRNA in THP-1 human monocytes[19]. The effect was RA dependent and RAR specific. This study provides evidence of a previously unknown mechanism of CD1d gene regulation, and it suggests that RA can induce monocytes to become more potent activators of iNKT cells.

The mechanism behind the effects of RA on monocytes has been studied by Chen *et. al*[20]. Scientists studied the ability of RA to induce STAT-1 activation as well as its ability to modulate the effects of various immune stimuli on STAT-1 activation. It was found that RA alone had no effect on STAT-1 activation. However, RA enhanced STAT-1 activation induced by interferon β (IFNβ) and interferon γ (IFNγ). Conversely, RA decreased STAT-1 activation induced by tumor necrosis-factor α (TNFα) and lipopolysaccharide (LPS) by 50-70%. This suggests that RA may differentially regulate STAT-1 dependent responses.

RA also has potent effects on B cells in RA-inadequate and RA-adequate individuals. The main effect of RA on B cells is that it inhibits growth and proliferation, but increases the rate of maturation and differentiation[11,18,21-24]. Scientists discovered that proliferation was blocked at the mid-G1 phase of the cell cycle[18]. Thus, neither the regulatory gene Ki67 nor the activation antigen 4F2 were affected by RA. Also, RA was shown to increase the number of $CD19^+$ B cells in the bone marrow and spleen while decreasing the number of CLPs in C57BL/6 mice[11,24]. RA was also shown to promote B cell maturation at the population level by increasing the number of sIgG1 and CD138 expressing cells[33]. RA is likely to induce maturation in B cells by increasing the level of recombination enzymes and decreasing the level of Pax5, an explanation proposed by Ross *et. al.*[11,21]. Increased levels of recombination enzymes are typical of class switching events, and loss of Pax5 promotes plasma cell differentiation[21].

Also, RA has been shown to affect B cell proliferation differentially, in a manner that depends on the B-cell subpopulation as well as the stimulus[25]. RA was inhibitory on B cell proliferation stimulated by BCR ligation and LPS, but it increased proliferation in memory B cells stimulated by CpG DNA[26]. Ross *et. al.*[26] also observed that RA increases the proliferation of murine splenic B cells stimulated by αGalCer, which was correlated with B cell differentiation, evidenced by sIgG1 and CD138 expression. At the same time, RA reduced the proliferation of identical B cells stimulated by LPS[25]. It is still unknown whether it is the stage of B cell activation (naive or memory) or the stimulus itself, or both, that determines whether RA promotes or inhibits B proliferation.

1.6 α-Galactosylceramide and the Immune System

αGalCer is a synthetic glycolipid antigen that is derived from the marine sponge *Agelas mauritanius*. αGalCer was found to have a very high affinity for CD1d, allowing it to activate iNKT cells and elicit an immune response[10]. This compound is unique in that it has an α-linkage connecting the galactose and the ceramide groups (Figure 1.2). Most glycolipids found in nature are connected by a β-linkage, with the exception of a few glycolipids from microbes. αGalCer is usually internalized before it is presented by CD1d and typically does not bind to surface-bound CD1d before being presented[8].

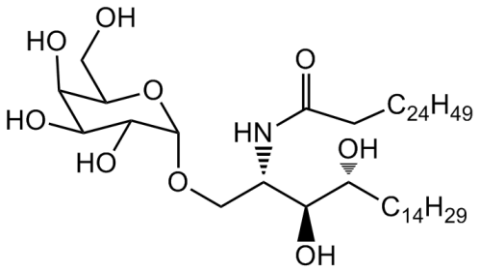

Figure 1.2 Structure of α-galactosylceramide. αGalCer is comprised of a galactose connected to a ceramide via an α-linkage. It has a molecular formula of $C_{50}H_{99}NO_9$ and a molecular weight of 858 g/mol.

αGalCer has been shown to increase primary and secondary antibody responses to T cell dependent antigens[27]. Scientists discovered that the α-gal epitope is commonly incorporated into endogenous glycolipids and glycoproteins in non-primate mammals[28]. While humans do not produce the α-gal epitope, all humans are reactive to it and can produce anti-gal antibodies. 1% of all circulating IgG antibodies recognize this epitope, making anti-gal antibodies one of the most common antibodies in the body. Incorporating αGalCer or conjugating the α-gal epitope to antigens in vaccines has been shown to increase the efficacy of these vaccines.

αGalCer has also been shown to independently regulate B cell functions. In a previous study by Chen *et. al.*[22], αGalCer was shown to increase CD1d mRNA in murine splenic B cells. This suggests that αGalCer can increase the ability of B cells to become activated and present antigens to iNKT cells. Furthermore, αGalCer was also shown to induce proliferation in murine splenic B cells, similar to the natural glycolipid antigen LPS. αGalCer differed from LPS based on its interaction with RA. αGalCer synergized with RA, while LPS antagonized with RA in inducing B cell proliferation. These results suggest that αGalCer may be a more useful adjuvant than LPS in conjunction with RA.

1.7 Rationale, Hypotheses, and Aims

Regulation of monocyte activation and maturation by aGalCer

Rationale: Chen *et. al.*[22] reported that the expression of CD1d was increased by αGalCer in murine splenic B cells. However, it is unknown whether αGalCer is able to upregulate CD1d in THP-1 human monocytes or whether it is able to synergize with RA. It is also unknown when the CD1d protein is maximally upregulated or which signaling pathway is involved in its regulation.

Hypothesis: We hypothesize that αGalCer will be able to upregulate CD1d and synergize with RA in THP-1 human monocytes based on its activity in murine B cells. We also hypothesize that CD1d will be maximally upregulated after 24 hours and that the regulation of CD1d will occur through the PI3K, NFκB, or MAPK pathways.

Aim 1: To detect CD1d protein level in THP-1 cells after treatment. We treated THP-1 human monocytes with RA (20 nM) and αGalCer (200 ng/ml) and harvested the cells after 24, 48, and 72 hours. We stained the cells with fluorescent labeled CD1d antibody and used flow cytometry to detect the CD1d protein expression.

Aim 2: To determine the involvement of PI3K, NFκB and MAPK pathways in RA and aGalCer induced monocyte activation. We treated THP-1 monocytes with RA and αGalCer and harvested the cells after 2 hours. We extracted the proteins from the cells and performed a western blot with the PI3K, NFκB, and ERK antibodies.

Regulation of B cell activation and proliferation by αGalCer

Rationale: Chen *et. al.*[22] reported that the activation of murine splenic B cells was induced by αGalCer and LPS. RA differentially regulated B cell activation by increasing proliferation induced by αGalCer and inhibiting proliferation induced by LPS. It is unknown whether αGalCer, LPS, and RA have the same effects in human B cells. It is also unknown how αGalCer regulates B cell proliferation.

Hypothesis: We hypothesize that αGalCer, LPS, and RA will have the same effects in CL-01 human B cells as they did in murine splenic B cells. We also hypothesize that αGalCer will be able to induce B cell activation by upregulating the B cell transcription factor Pax5 and the B cell coreceptor CD19, a mechanism proposed by Chung *et. al.*[12].

Aim 1: To detect Ki67, Pax5, and CD19 protein levels in CL-01 cells after treatment. We treated CL-01 human B cells αGalCer, LPS, and RA and harvested the cells after 24 hours. We then stained the cells with fluorescent labeled anti-Ki67, anti-Pax5, and anti-CD19 antibodies and used flow cytometry to detect their expression.

Aim 2: To determine the gene regulation of Pax5 and CD19. We treated CL-01 human B cells with αGalCer, LPS, and RA and harvested the cells after 24 hours. We then extracted the RNA from the cells and performed RT-PCR to detect their RNA levels.

2. MATERIALS AND METHODS

2.1 Monocyte and B Cell Culture

THP-1 human monocytes and CL-01 human B cells were both maintained in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100 ug/ml streptomycin, and $5x10^{-5}$ M β-mercaptoethanol. Cell density was measured using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). Monocytes and B cells were sedimented into a pellet using centrifugation at 1500 rpm at 4°C for 5 minutes. Monocytes were resuspended in RPMI-1640 supplemented with 3% FBS to give a final concentration of $5x10^5$ cells/ml. B cells were resuspended in RPMI-1640 supplemented with 10% FBS to give a final concentration of $2x10^5$ cells/ml. Cells were then plated in Falcon Tissue Culture Plates (BD Biosciences, Franklin Lakes, NJ), and treatments were added appropriately. Cells were then incubated at 37°C with 5% CO_2/95% air in a water jacketed incubator (Thermo Fisher Scientific, Waltham, MA) until the proper time points, after which they were harvested.

2.2 Treatments

All treatments were diluted in RPMI-1640 growth media prior to application to cells. The final concentration for RA (Sigma-Aldrich, St. Louis, MO) was 20 nM, reflecting a physiological level of RA; for αGalCer (Alexis Biochemicals, San Diego, CA), 0.2 ug/ml; for TNFα (R&D Systems, Minneapolis, MN), 10 ng/ml; for IFNγ (R&D Systems, Minneapolis, MN), 0.2 ug/ml; and for LPS (*E. coli*, Sigma-Aldrich, St. Louis, MO), 0.5 ug/ml.

2.3 Flow Cytometry

Cells were harvested 24-72 hours after treatment by sedimenting the cells into a pellet at 1500 rpm at 4°C for 5 minutes. The cells were then stained with antibodies to detect surface expression. All antibodies were obtained from BD Biosciences (Franklin Lakes, NJ). Each sample was stained with 0.1 ug of antibody in a Dulbecco's phosphate-buffered saline (DPBS) wash buffer (Invitrogen, Carlsbad, CA) supplemented with 1% BSA (Sigma-Aldrich, St. Louis, MO) and 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO). Monocytes were stained with the PE anti-human CD1d antibody, and B cells were stained with the PerCP Cy5 anti-human CD19 antibody. Cells were incubated at room temperature for 40 minutes in the dark and washed once with wash buffer. The cells were then fixed and permeabilized using a Fixation and Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ). Cells were washed once again, and antibodies were then added to detect intracellular expression. B cells were stained with APC anti-human Pax5 and PE anti-human Ki67 antibodies. Cells were then incubated at 4°C overnight and analyzed with an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). An unstained sample was used to set background levels to <0.5% and single stainings were used to compensate for any spectral overlap from the different fluorescent channels.

2.4 Protein Isolation and Western Blot

Cells were harvested 2 hours after treatment by transferring the cells into tubes with 12 ml of DPBS (Invitrogen, Carlsbad, CA). The cells were then sedimented into a pellet at 1500 rpm at 4°C for 5 minutes. Next, the cells were lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO) supplemented with sodium orthovanadate (Sigma-Aldrich, St. Louis, MO) and a protease inhibitor cocktail (Roche, Germany) on ice for 30 minutes. The lysed cell solution was then

centrifuged at 13000 rpm at 4ºC for 20 minutes, and the supernatant was collected. Next, protein concentrations were measured with a spectrophotometer (λ_{max} = 595 nm) and equalized in DPBS. A 2xSDS sample buffer was then added, and the protein lysate was incubated at 95ºC for 10 minutes to fully denature the proteins. The proteins were then loaded onto a 10% polyacrylamide gel, which was run at 90V for 1 hour and 120V for 4 hours. The protein was then transferred onto a PVDF membrane (Invitrogen, Carlsbad, CA) at 20V overnight.

The membrane was then blocked in an Odyssey blotting solution (LI-COR Biosciences, Lincoln, NE) and gently rocked for 30 minutes. The blotting solution was poured out, and the primary antibody solution was then added to the membrane. All antibodies were obtained from BD Biosciences, Franklin Lakes, NJ. Monocytes were stained with PI3K (200 ug/ml), NFκB (200 ug/ml), and ERK (200 ng/ml), respectively. The membrane was then gently rocked for 30 minutes and incubated at 4ºC overnight. The membrane was then washed with TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20), and the secondary antibody mix was added. The secondary antibody mix contained 800CW anti-rabbit (1:7500) and 680RD anti-mouse (1:7500) secondary antibodies. The membrane was rocked gently for 30 minutes in the dark, and after a series of washes, the film was developed in 800CW and 680RD light using an Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The membrane was then stained using Ponceau S to serve as an internal control.

2.5 RNA Isolation and Real-Time PCR

Cells were harvested 2 and 24 hours after treatment by sedimenting the cells as a pellet at 1500 rpm at 4°C for 5 minutes. RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Limburg). Cells were lysed using RLT buffer and 70% ethanol, after which they were transferred

into a spin column. The RNA solution was spun at 10000 rpm for 15 seconds, and the flow through was discarded. The column was then washed using RW1 buffer and centrifuged at 10000 rpm for 15 seconds. The column was then washed again with RPE buffer at 10000 rpm for 2 minutes. Finally, RNase free water was added to the spin column and centrifuged at 10000 rpm for 2 minutes to elute the RNA.

Next, the concentration of the extracted RNA was measured using a NanoDrop UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA) and the RNAs were equalized in RNase free water. 1 ug of RNA from each sample was collected and added to a tube containing master mix solution for reverse transcription. The master mix solution contained M-MLV reverse transcriptase buffer, oligo(dT) primers, deoxynucleotides (dNTPs), RNase inhibitors, and reverse transcriptases (Life Technologies, Carlsbad, CA). The samples were then incubated in a PTC-100 Thermal Cycler (Bio-Rad, Hercules, CA), where the temperature was held at 42°C for 30 minutes, 37°C for 30 minutes, and 940°C for 5 minutes to obtain complementary DNA (cDNA).

Lastly, the cDNA was subjected to real-time PCR (RT-PCR) to determine the amount of RNA per sample. cDNA from each sample was added to a 96-well plate containing the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and Pax5 or CD19 primers (sequences on next page). 18S RNA levels were also measured to serve as an internal control. A cDNA standard was then set up by diluting the cDNA in known quantities of RNase free water. The 96-well plate was then incubated in a DNA Engine Opticon 2 Real-Time Cycler (Bio-Rad, Hercules, CA), in which the samples were cycled between 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds for 30 cycles.

RT-PCR Primer Sequences

Pax5 Forward	5' GGTCAGCCATGGTTGTGTCAG 3'
Pax5 Reverse	5' CGGCACTGGAGACTCCTGAAT 3'
CD19 Forward	5' TCTCCTGGACCCATGTGCA 3'
CD19 Reverse	5' GGGTCAGTCATTCGCTTTC 3'

Table 1.1. RT-PCR Primer Sequences. The table above shows the sequences of the primers used to amplify the RNA of Pax5 and CD19.

2.6 Statistical Analysis

Prism 5 software was used to calculate means, standard errors, and p values. The data were analyzed using one-way ANOVA with Tukey's post test. Any differences were considered significant if $p<0.05$, with $*p<0.05$, $**p<0.01$, and $***p<0.001$ as levels of significance.

3. RESULTS

3.1 Regulation of Monocyte Activation and Maturation by αGalCer

3.1.1 RA markedly increases CD1d expression, while αGalCer is unable to regulate CD1d in human monocytes

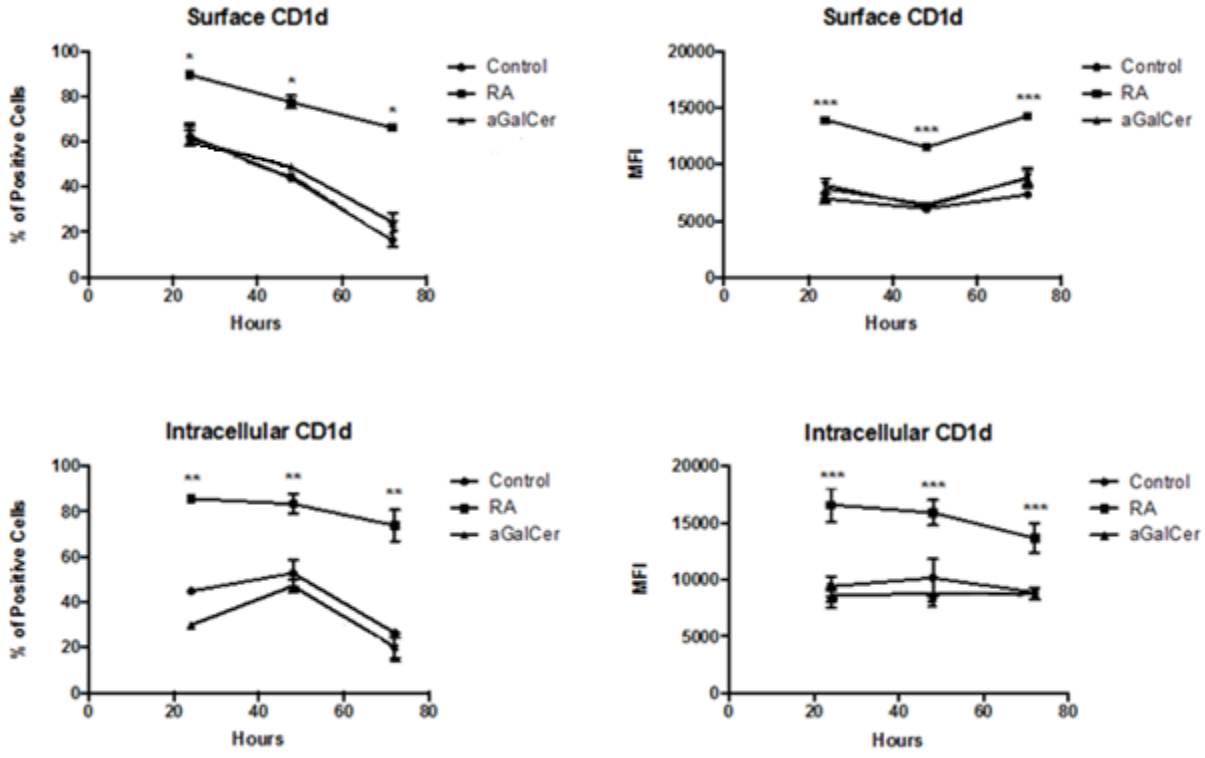

Figure 3.1. The Regulation of Surface and Intracellular CD1d by RA and αGalCer. THP-1 cells were treated with RA and αGalCer for 24, 48, and 72 hours, then stained with the PE anti-human CD1d antibody, and analyzed using flow cytometry. Line graphs represent six independent experiments (means ± SEM, n=3). Significance was determined using one-way ANOVA, with $p<0.05$ considered as significant.

Figure 3.1 shows detection of CD1d protein expression represented by the percentage of $CD1d^+$ cells and the mean fluorescent intensity (MFI) of CD1d after 24, 48, and 72 hours of treatments. It appears that RA is the only treatment to have significantly upregulated the level of CD1d ($p<0.001$). αGalCer alone showed no statistical difference from the control, and αGalCer in conjunction with RA showed no statistical difference from RA alone (data not shown). This suggests that αGalCer is unable to independently regulate or modulate the expression of CD1d in human monocytes.

It appears that αGalCer slightly downregulates intracellular CD1d, though insignificantly. The reason for this may be that αGalCer promotes faster recycling of the CD1d molecule. By binding to its receptor CD1d, αGalCer may stimulate the cell to increase the rate at which CD1d is transported to the membrane. This would result in fewer CD1d molecules to be present inside the cell, resulting in downregulated intracellular CD1d.

Regarding the kinetics of CD1d regulation, it appears that RA significantly upregulates CD1d across all time points—24, 48, and 72 hours. The level of upregulation is about the same across these time points for both surface and intracellular expression, though the 24 hour time point slightly shows the most upregulation. This suggests that RA is able to upregulate CD1d and rapidly boost immunity long-term.

3.1.2 RA and aGalCer in the regulation of monocyte signaling pathways

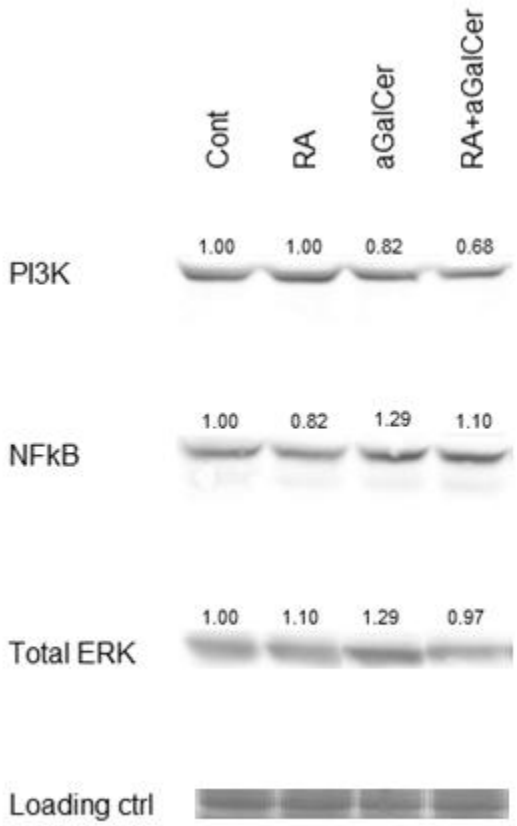

Figure 3.2. The Regulation of Human Monocyte Signaling by RA and αGalCer. THP-1 cells were treated with RA and αGalCer for 2 h, and then lysed to extract proteins. 150 ug of total cellular protein was subjected to western blot analysis using the PI3K, NFκB, and ERK antibodies. A Ponceau S stain was used as a loading control for each sample. The gel above is representative of four replicate experiments. The values shown above each band represent the relative signal intensities collected from the Odyssey CLx Infrared Imaging System.

Figure 3.2 shows the relative band intensities of PI3K, NFκB, and ERK 2 hours after treatment. From our analysis, it appears that αGalCer increased NFκB and ERK band intensities, while RA had no effect on PI3K, NFκB, or ERK band intensities. A Ponceau S stain is shown at the bottom, and it shows that all samples have about the same amount of total protein.

While αGalCer does not regulate CD1d expression in THP-1 human monocytes, αGalCer may upregulate NFκB and ERK signaling in THP-1 cells to affect the rate of CD1d trafficking. The NFκB and MAPK signaling pathways do play a role in regulating the expression and trafficking of certain proteins and receptors[1,13], which supports this conclusion. These signaling pathways also play a role in regulating cell proliferation and survival, which may suggest other functions for αGalCer in human monocytes.

RA alone did not affect the PI3K, NFκB, or MAPK signaling pathways in THP-1 cells. It is possible that, because RA is a lipid molecule that can freely cross the cell membrane, it does not need to activate these pathways in order to initiate its effect. Further analysis should be done before any conclusions are drawn.

3.2 Regulation of B Cell Activation and Proliferation by αGalCer

3.2.1 Highly proliferating cells express higher levels of CD19

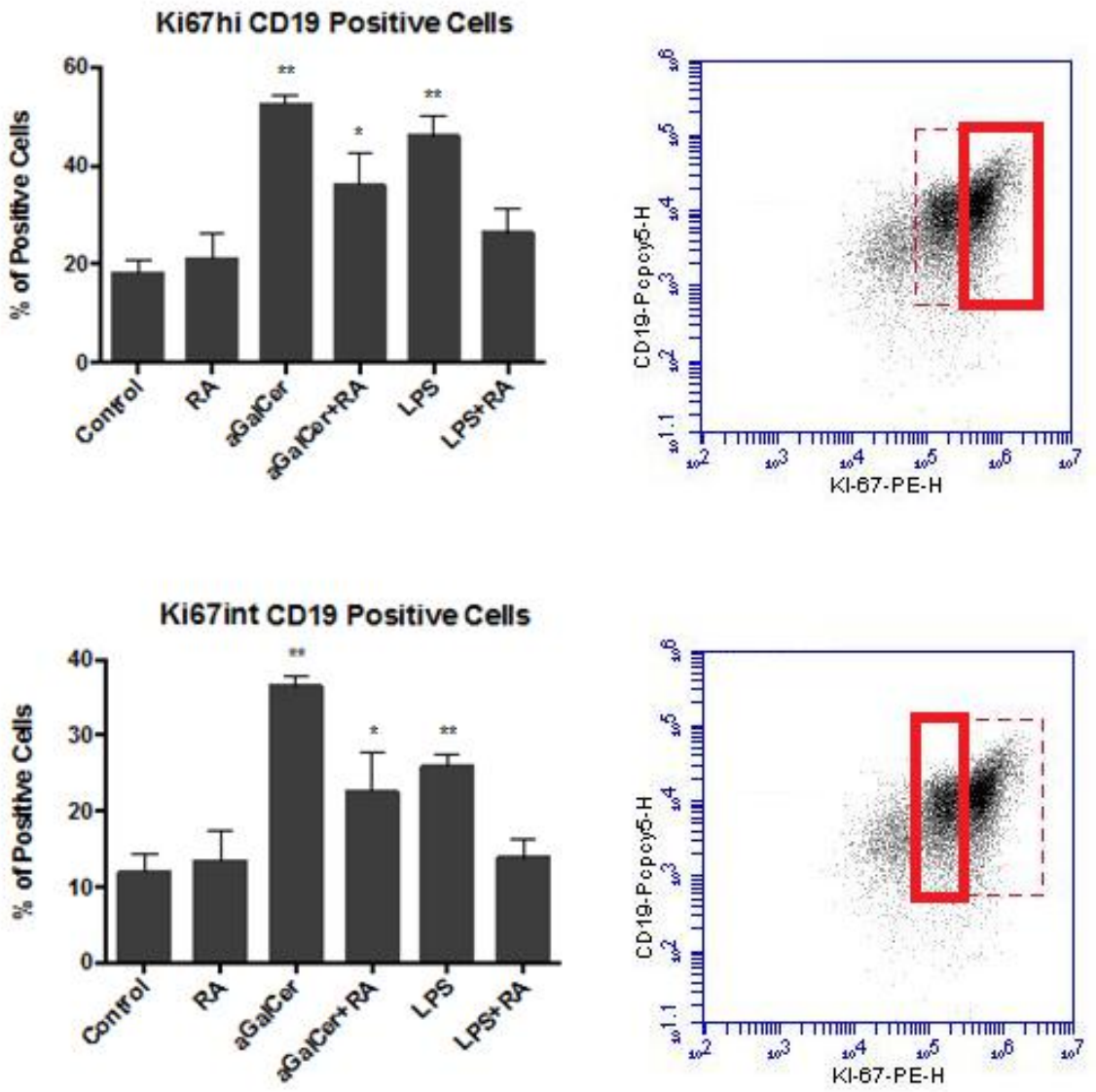

Figure 3.3. The Regulation of Ki67 and CD19 Expression on B Cells by αGalCer, LPS, and RA. CL-01 cells were treated with αGalCer, LPS, and RA, harvested after 24 hours, stained with the PerCP Cy5 anti-human CD19 and PE anti-human Ki67 antibodies, and analyzed using flow cytometry. Bar graphs represent four independent experiments (means ± SEM, n=3). Scatter plots show the gating of cells used in the flow cytometry analysis. Significance was determined using one-way ANOVA, with $p<0.05$ considered as significant.

Figure 3.3 shows the percentage of $CD19^+$ B cells gated based on Ki67 expression after 24 hours. It appears that highly proliferating cells—cells that express higher levels of Ki67—also express higher levels of CD19. For example, in the Ki67hi population, 20% of the control group was $CD19^+$, while in the Ki67int population, only 10% of the control group was $CD19^+$. Because Ki67 is correlated with CD19, it can be concluded that Ki67-driven proliferation is controlled by CD19, supporting the model of B cell activation proposed by Chung *et. al.*[12].

Because Ki67 is constitutively expressed in cells, no treatment group showed significant upregulation of the percentage of $Ki67^+$ B cells (data not shown). However, the percentage of Ki67hi and Ki67int $CD19^+$ B cells was upregulated by both αGalCer and LPS ($p<0.01$). This suggests that αGalCer and LPS are both capable of inducing Ki67-driven proliferation by upregulating CD19 in human B cells.

RA alone had no effect on the percentage of Ki67hi and Ki67int $CD19^+$ B cells, but it reduced the upregulation of CD19 when treated in conjunction with αGalCer and LPS. It is currently believed that RA differentially regulates the proliferation of B cells, but it is unknown whether the effects are dependent on the B cell subpopulation or the type of stimulus, or both[25], which will need further investigation. Our results suggest that RA's effects do not depend on the stimulus used since it decreased proliferation induced by both αGalCer and LPS, but this contradicts the findings from mouse splenocytes by Chen *et. al.*[22]. Further studies need to be done before any conclusions can be drawn.

3.2.2 αGalCer and LPS increase the percent of Pax5$^+$ and CD19$^+$ B cells

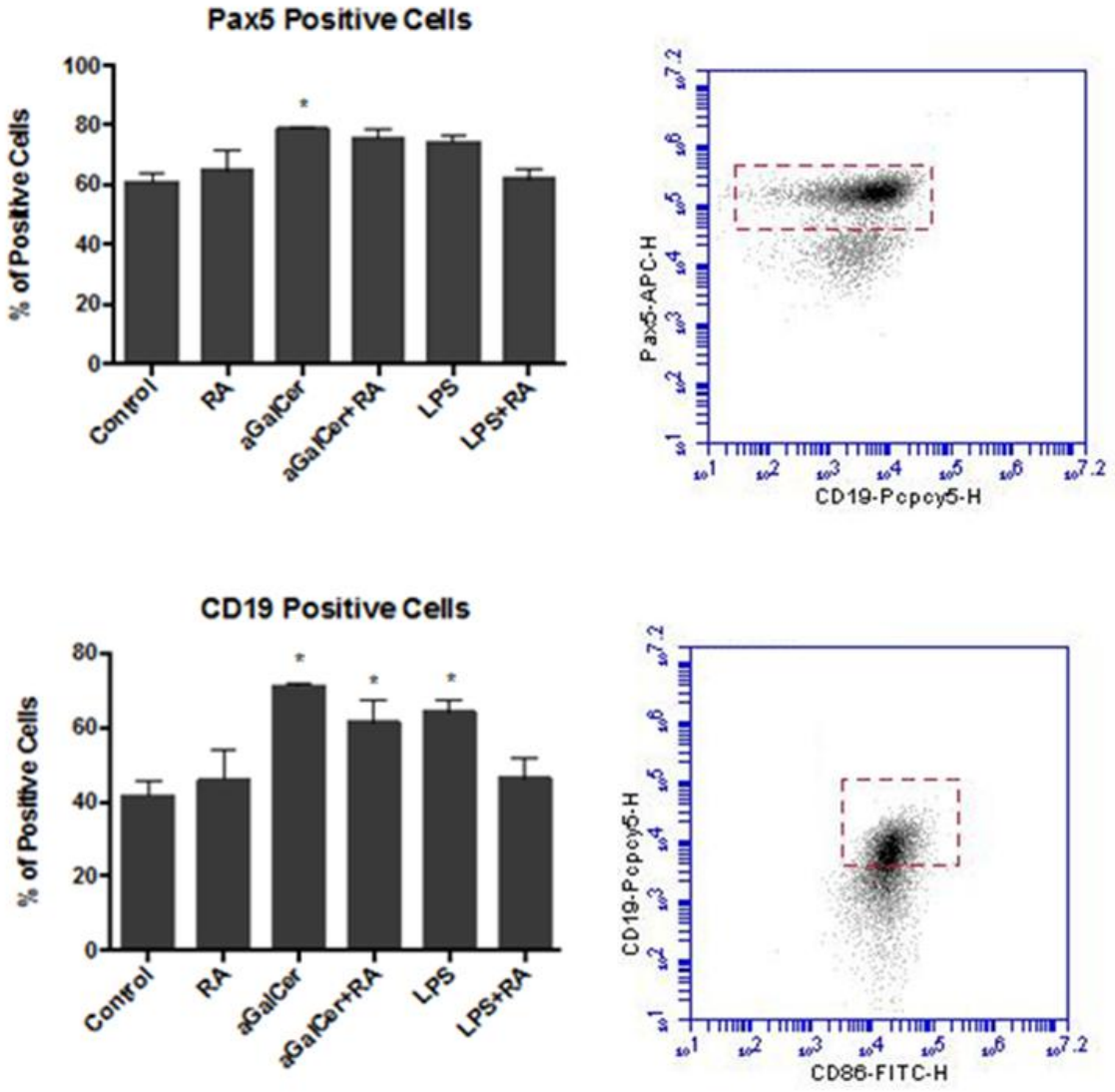

Figure 3.4. The Regulation of Pax5 and CD19 Expression on B Cells by αGalCer, LPS, and RA. CL-01 cells were treated with αGalCer, LPS, and RA, harvested after 24 hours, stained with the APC anti-human Pax5 and PerCP Cy5 anti-human CD19 antibodies, and analyzed using flow cytometry. Bar graphs represent four independent experiments (means ± SEM, n=3). Scatter plots show the gating of cells used in the flow cytometry analysis. Significance was determined using one-way ANOVA, with $p<0.05$ considered as significant.

Figure 3.4 shows the percentage of $Pax5^+$ and $CD19^+$ B cells after 24 hours. It appears that the expression of Pax5 and CD19 is upregulated by αGalCer and LPS in all B cells, not just B cells undergoing proliferation. The upregulation of Pax5 was rather small (about 30% of the control), while the upregulation of CD19 was quite large (about 75% of the control). Again, RA alone was shown to have no effect on the expression of Pax5 and CD19, but it reduced the upregulation of Pax5 and CD19 induced by αGalCer and LPS.

3.2.3 αGalCer increases CD19 but not Pax5 gene expression

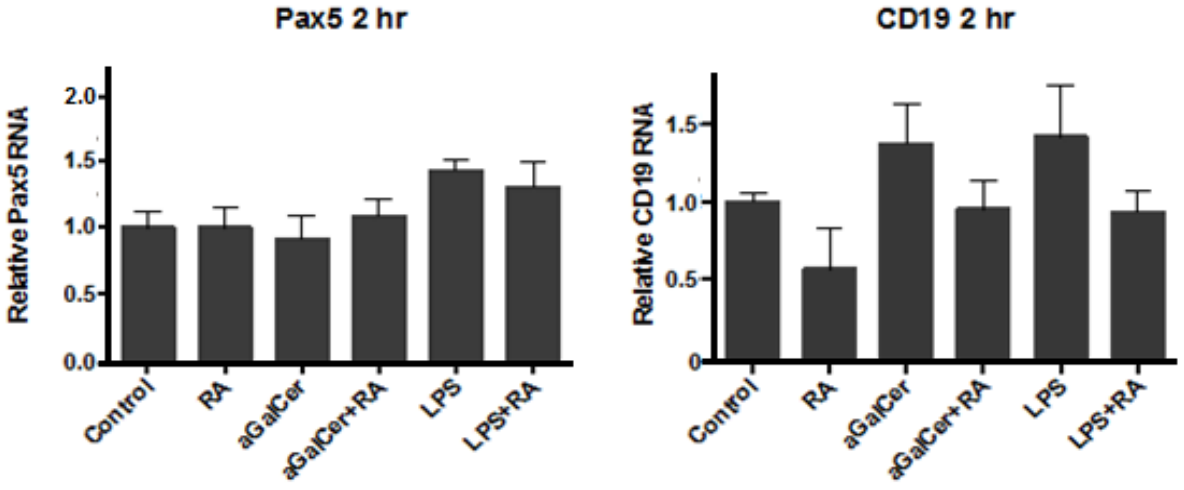

Figure 3.5. The Regulation of Pax5 and CD19 Gene Expression by αGalCer, LPS, and RA. CL-01 cells were treated with αGalCer, LPS, and RA, harvested after 2 hours, lysed to collect their RNA, and analyzed using real-time PCR. Bar graphs represent three independent experiments (means ± SEM, n=3). Significance was determined using one-way ANOVA. While none of the samples showed any significant change from the control, the trends above were consistent over three experiments.

Figure 3.5 shows the relative Pax5 and CD19 RNA levels after 2 hours. It appears that αGalCer and LPS both upregulate the level of CD19 RNA. This suggests that αGalCer and LPS both increase the expression of CD19 at the transcriptional level. However, there is a difference in Pax5 gene regulation induced by αGalCer and LPS. It appears that αGalCer does not affect the level of Pax5 RNA, while LPS does. This suggests that LPS increases Pax5 expression at the transcriptional level, while αGalCer increases Pax5 expression at the post-transcriptional level, probably by stabilizing the protein or by inhibiting its degradation.

RA alone appears to have no effect on Pax5 or CD19 RNA levels. While the figure above shows that RA decreased the level of CD19 RNA, previous experiments have shown that RA really has no effect on CD19 gene expression. Again, RA reduced the upregulation of Pax5 and CD19 RNA levels induced by αGalCer and LPS.

RNA levels were also measured 24 hours after treatment (data not shown), but most of the RNA levels were restored to about the same level as the control. This suggests that αGalCer and LPS rapidly affect gene expression, but their effects are only temporary.

4. DISCUSSION

4.1 Regulation of Monocyte Activation and Maturation by αGalCer

Our data shows that the expression of CD1d in THP-1 human monocytes is upregulated by RA, but not by αGalCer. RA is able to upregulate CD1d long-term (72 hours) and induce maximal expression within 24 hours. This suggests that RA can be useful as an adjuvant to boost the APC functions of monocytes after infection. αGalCer was shown to slightly downregulate intracellular CD1d. We believe that this is a result of αGalCer accelerating the trafficking of the CD1d molecule. By increasing the rate at which CD1d is transported to the surface, αGalCer may help increase the efficiency of antigen presentation.

It is ultimately our hope that an increase in CD1d expression would lead to an increase in iNKT cell activation. Therefore, we should combine monocytes treated with RA with iNKT cells to see if the iNKT cells are more likely to become activated. Theoretically, increased CD1d expression should lead to increased iNKT cell activation since CD1d presents lipid antigens to iNKT cells. Further studies are needed to test this hypothesis.

It is unknown why the expression of CD1d was unaffected by αGalCer. Previous studies have shown that αGalCer is able to upregulate CD1d in splenic B cells from mice[22], but our current study shows that αGalCer is unable to upregulate CD1d in human monocytes. It is possible that the two cell types used do not respond to αGalCer in the same way. This may also hint at the inherent differences between B cells and monocytes. Because B cells were able to upregulate CD1d expression in response to αGalCer, it is possible that B cells may be the predominant presenter of lipid antigens.

It may be worthwhile to study the rate of CD1d trafficking in response to αGalCer. While αGalCer was unable to upregulate the expression of CD1d, it may still be able to affect the CD1d

molecule in an alternative way. The most likely way that αGalCer would affect CD1d is by increasing the rate at which CD1d is transported to the surface. Fluorescent tagging of the CD1d molecule and microscopic analysis would allow us to determine if αGalCer does accelerate the trafficking of CD1d. Observing an increase in CD1d near the surface would suggest αGalCer is able to increase the APC functions of monocytes by accelerating the trafficking of CD1d.

There are many future implications to this study. The main implication is the use of RA as an adjuvant to boost the APC function of monocytes. By inducing the upregulation of CD1d in monocytes, RA makes it easier for monocytes to present lipid antigens to iNKT cells, thereby activating them. Finding ways to augment the activation of iNKT cells is of the utmost importance and should be carefully studied. iNKT cells play an important role in the immune system by releasing cytokines and by activating other cells, and being able to augment their activation can be very useful.

Scientists can also study whether other molecules are able to increase the expression of CD1d in monocytes. It is possible that CD1d can be regulated by many other stimuli, and comparing these various stimuli may be instrumental in developing an effective vaccine to boost the APC function of monocytes.

An additional study that can be done is to measure the expression of CD1d in response to RA or αGalCer in other cell lines or *in vivo*. This current study focuses on CD1d regulation in monocytes. However, the majority of antigen presentation is done by macrophages or dendritic cells, which monocytes later differentiate into. It is possible that the kinetics and dynamics of CD1d regulation differs between these cell lines, so it would be worthwhile to eliminate any doubt by testing the effects of RA and αGalCer in these cell lines.

4.2 Regulation of B Cell Activation and Proliferation by αGalCer

Our data shows that αGalCer and LPS are both able to induce proliferation in human B cells by upregulating Pax5 and CD19. An increase in CD19 expression is correlated with an increase in Ki67 proliferation, which suggests that CD19 is able to independently regulate B-lymphomagenesis. The effect of RA on B cells is still unclear, however. Our data here suggests that RA inhibits proliferation induced by αGalCer and LPS, even though it was reported that RA increases B cell proliferation induced by αGalCer[22]. Further studies need to be done to elucidate RA's effects on B cells.

The activation of human B cells by αGalCer is a novel discovery. A model for this mode of activation is depicted below.

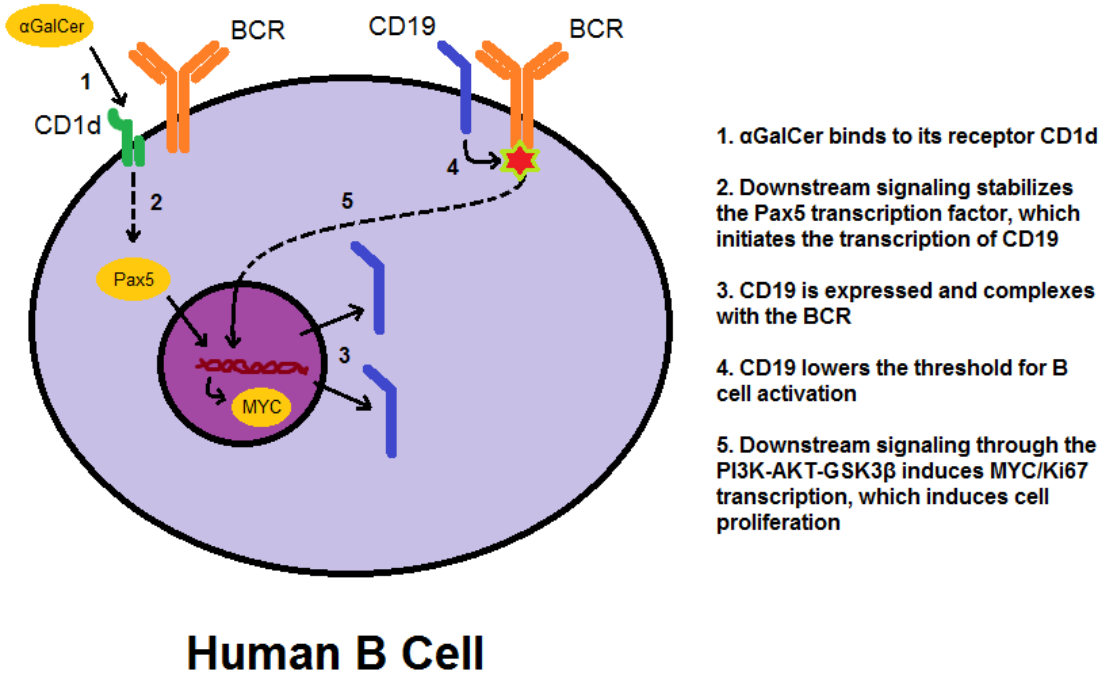

Figure 4.1. The Activation of CL-01 Human B Cells by αGalCer. This diagram depicts the steps involved in the αGalCer-induced activation of human B cells.

The first step of B cell activation is ligand binding. It is known that αGalCer binds to its receptor CD1d to elicit downstream effects. The downstream signaling that takes place after ligand binding is still unknown, but we do know that the final outcome is an increase in Pax5 protein levels, as evidenced by our flow cytometry analysis. From our RT-PCR analysis, we know that αGalCer does not upregulate Pax5 at the transcriptional level. Therefore, αGalCer must increase Pax5 protein levels at the post-transcriptional level by increasing the protein synthesis or by inhibiting its degradation. Moreover, the increased Pax5 protein may enter the nucleus to initiate the transcription of CD19, which is supported by our RT-PCR analysis. It is known that CD19 then complexes with the BCR to lower the threshold for activation, and downstream signaling through the PI3K-AKT-GSK3β pathway[19] occurs to initiate the transcription of Ki67 and induce proliferation.

There are many future implications of this study. The first and foremost implication is the use of αGalCer as an adjuvant in vaccines to activate B cells. B cells play an important role in the immune system by producing antibodies to help neutralize, opsonize, and synergize with complement to eliminate pathogens present in the body[1]. Any way to increase their activity in response to an infection is of the utmost importance, and αGalCer should be carefully studied to see if it is truly an effective adjuvant. The use of RA in conjunction with αGalCer in vaccines is still questionable. It remains unknown whether RA increases or decreases B cell proliferation induced by αGalCer. Further study should be done regarding RA's effectiveness as an adjuvant in conjunction with αGalCer.

Another implication of this study is the ability to activate B cells without BCR ligation. Our study reveals a novel mode of B cell activation through the coreceptors CD1d and CD19. Downstream signaling through these coreceptors result in increased expression of Ki67 without

antigen even binding to the BCR. This could be vitally important for immunocompromised individuals since their B cells may be less responsive, and finding alternative ways to activate their B cells may help them better respond to infection and disease. Scientists can study whether other B cell coreceptors are able to independently regulate B cell proliferation or whether any other stimuli are able to upregulate the expression of these coreceptors.

One of the main sources of error that could have affected our results is the viability of the CL-01 human B cell line. This cell line is very sensitive to overcrowding, and the cells can easily become non-responsive to treatments if they are cultured for a long time. The reason for this is that the cells start out as naive, but they can eventually become activated by cell-to-cell contact if the cell density is too high. When the cells activate each other, they will no longer be naive, and they will not be subsequently activated after treatments of αGalCer, LPS, or RA. As a result, it is possible that some of the cells used may have already been activated by cell-to-cell contact, resulting in decreased upregulation of Pax5 and CD19.

Another source of error to consider is the sensitivity of the RNA extraction and the RT-PCR technique and the difficulty to obtain consistent replicates. The RNA extraction needs to be very careful since most contaminants in nature have RNases that can degrade the RNA we are hoping to quantify. There are many steps along the way that can lead to the introduction of contaminants into the samples, which will affect our data. Also, the quantification of the RNA extracted is very sensitive. RT-PCR relies heavily on a standard, which is created with a serial dilution. The volume used to make the standard is very small, only about 10 µl per well, meaning the smallest error in mixing or aliquoting can lead to large deviations in the data. The culmination of all these factors make RT-PCR a very difficult technique to obtain consistent and reliable replicates.

There are many future studies that would be of interest. First and foremost, these experiments should be repeated to improve the consistency of the data. Also, the B cells should be counted after treatment to determine whether and how much proliferation has occurred. Without this data, we cannot be certain that the upregulation of Pax5 and CD19 is, in fact, correlated with proliferation. The cells can be counted using a flow cytometer or using a thymidine uptake assay.

Another future study that can be done is to test the combination of αGalCer, LPS, and RA treatment in other B cell lines. It remains unknown whether RA's effects on B cells are more dependent on the stage of B cell activation (naive or memory) or the stimulus used, or both[25]. The results of this study are contradictory to the results of some previous studies. Thus, it would be beneficial to test the effects of RA in other B cell lines to see how RA affects proliferation differentially in B cells.

4.3 Summary

The culmination of all our results suggests that RA and αGalCer could independently be useful as adjuvants to boost immunity after vaccination. RA helps upregulate CD1d expression in monocytes, thereby helping to activate iNKT cells, and αGalCer helps activate human B cells by upregulating the expression of Pax5 and CD19.

When deciding whether to incorporate RA and αGalCer separately or together into vaccines, we believe that it is best to include RA and αGalCer together. While the synergy between RA and αGalCer was not clearly demonstrated in this study, we still believe that RA and αGalCer work differentially to boost the APC functions of monocytes and the proliferation of B cells. In terms of CD1d regulation in monocytes, αGalCer may still possibly synergize with RA by accelerating the trafficking of CD1d, increasing antigen presentation efficiency. In terms of B cell activation, RA may still possibly increase proliferation induced by αGalCer (contrary to our study, but in line with numerous other studies).

Regarding the kinetics of a vaccine, it may be best if the vaccine is administered shortly after infection for immunocompromised individuals. It appears that the upregulation of CD1d in monocytes and the activation of B cells induced by RA and αGalCer is only temporary. This may help an individual quickly clear an infection, while also allowing him avoid any autoimmune attacks due to a prolonged heightened immune response.

While the potential use of RA and αGalCer as adjuvants is promising, there is still a lot of work that needs to be done before they may be effectively implemented into vaccines.

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