Lake Forest College
Lake Forest College Publications

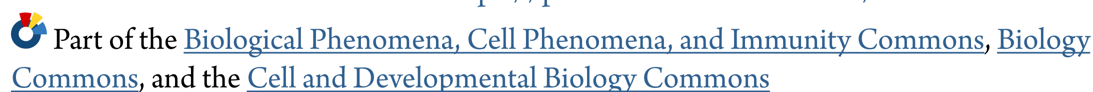
Senior Theses | Student Publications

4-19-2017

Toll-Like Receptor 2 Dependent Signaling of Low Molecular Weight Hyaluronan Upregulates T-Helper 17 Differentiation and Promotes the Expression of Hyaluronidase 2

Kaitlyn M. Woodman
Lake Forest College, woodmankm@lakeforest.edu

Follow this and additional works at: https://publications.lakeforest.edu/seniortheses


Part of the Biological Phenomena, Cell Phenomena, and Immunity Commons, Biology Commons, and the Cell and Developmental Biology Commons

Recommended Citation
Woodman, Kaitlyn M., "Toll-Like Receptor 2 Dependent Signaling of Low Molecular Weight Hyaluronan Upregulates T-Helper 17 Differentiation and Promotes the Expression of Hyaluronidase 2" (2017). *Senior Theses.*

This Thesis is brought to you for free and open access by the Student Publications at Lake Forest College Publications. It has been accepted for inclusion in Senior Theses by an authorized administrator of Lake Forest College Publications. For more information, please contact levinson@lakeforest.edu.

Toll-Like Receptor 2 Dependent Signaling of Low Molecular Weight Hyaluronan Upregulates T-Helper 17 Differentiation and Promotes the Expression of Hyaluronidase 2

Abstract

Immune cells have the capacity to differentiate and proliferate after stimulation through pattern recognition receptors (PRRs). In the case of potentially autoreactive T-cells, signaling from self molecules through PRRs can stimulate these cells to attack otherwise healthy tissue leading to autoinflammatory diseases such as multiple sclerosis. One self molecule that may induce this effect is hyaluronan, a structural polysaccharide found in the extracellular matrix. We initially hypothesized that low molecular weight hyaluronan, indicative of injury, could signal through toll-like receptor 2, a PRR, to upregulate Th17 cell differentiation and proliferation. Naïve CD4+ murine T-cells were isolated and exposed to low and high molecular weight hyaluronan to determine the molecule's effect. Surprisingly, our results showed evidence for both hyaluronan and hyaluronidase II in upregulating T-helper 17 cell differentiation and proliferation. Further research is necessary to describe the mechanism for this effect, as well as to establish its physiological relevance.

Document Type

Thesis

Distinguished Thesis

Yes

Degree Name

Bachelor of Arts (BA)

Department or Program

Biology

First Advisor

Douglas B. Light

Second Advisor

Ann B. Maine

Third Advisor

Elizabeth W. Fischer

Fourth Advisor

Joseph M. Reynolds, Rosalind Franklin University of Medicine and Science

This thesis is available at Lake Forest College Publications: https://publications.lakeforest.edu/seniortheses/109

Subject Categories
Biological Phenomena, Cell Phenomena, and Immunity | Biology | Cell and Developmental Biology

This thesis is available at Lake Forest College Publications: https://publications.lakeforest.edu/seniortheses/109

Lake Forest College Archives

Your thesis will be deposited in the Lake Forest College Archives and the College's online digital repository, *Lake Forest College Publications*. This agreement grants Lake Forest College the non-exclusive right to distribute your thesis to researchers and over the Internet and make it part of the *Lake Forest College Publications* site. You warrant:

- that you have the full power and authority to make this agreement;
- that you retain literary property rights (the copyright) to your work. Current U.S. law stipulates that you will retain these rights for your lifetime plus 70 years, at which point your thesis will enter common domain;
- that for as long you as you retain literary property rights, no one may sell your thesis without your permission;
- that the College will catalog, preserve, and provide access to your thesis;
- that the thesis does not infringe any copyright, nor violate any proprietary rights, nor contain any libelous matter, nor invade the privacy of any person or third party;
- If you request that your thesis be placed under embargo, approval from your thesis chairperson is required.

By signing below, you indicate that you have read, understand, and agree to the statements above.

Printed Name: Kaitlyn M. Woodman

Thesis Title: Toll-Like Receptor 2 Dependent Signaling of Low Molecular Weight Hyaluronan Upregulates T-Helper 17 Differentiation and Promotes the Expression of Hyaluronidase 2

This thesis is available at Lake Forest College Publications: https://publications.lakeforest.edu/seniortheses/109

Lake Forest College

Senior Thesis

Toll-Like Receptor 2 Dependent Signaling of Low Molecular Weight Hyaluronan Upregulates T-Helper 17 Differentiation and Promotes the Expression of Hyaluronidase 2

by

Kaitlyn M. Woodman

April 19th, 2017

The report of this investigation undertaken as a Senior Thesis, to carry two courses of credit in the Department of Biology

Michael Orr
Provost and Dean of the Faculty

Douglas B. Light, Chairperson

Ann B. Maine

Elizabeth W. Fischer

Joseph M. Reynolds
Rosalind Franklin University of Medicine and Science

Abstract

Immune cells have the capacity to differentiate and proliferate after stimulation through pattern recognition receptors (PRRs). In the case of potentially autoreactive T-cells, signaling from self molecules through PRRs can stimulate these cells to attack otherwise healthy tissue leading to autoinflammatory diseases such as multiple sclerosis. One self molecule that may induce this effect is hyaluronan, a structural polysaccharide found in the extracellular matrix. We initially hypothesized that low molecular weight hyaluronan, indicative of injury, could signal through toll-like receptor 2, a PRR, to upregulate Th17 cell differentiation and proliferation. Naïve CD4+ murine T-cells were isolated and exposed to low and high molecular weight hyaluronan to determine the molecule's effect. Surprisingly, our results showed evidence for both hyaluronan and hyaluronidase II in upregulating T-helper 17 cell differentiation and proliferation. Further research is necessary to describe the mechanism for this effect, as well as to establish its physiological relevance.

Acknowledgments

I would like to thank my advisor, Dr. Light, for all his help in writing this work. Also, I would like to thank Drs. Fischer and Maine for agreeing to review my work as members of my thesis committee. Furthermore, I'd like to thank my mentor, Dr. Reynolds, for his patience throughout our experiments and writing, as well as the current and previous members of our lab, Theodore Peterson, Stephanie Flaherty, Sean Schrank, and Kathryne Young, for all their support.

Table of Contents

Abstract	i
Acknowledgements	ii
List of Figures	iv
List of Abbreviations	v
Introduction	1
Materials & Methods	16
Results	29
Discussion	42
Conclusion	55
Literature Cited	57

List of Figures

Figure 1. Functions of the innate immune system. 12
Figure 2. Innate immune cells activate adaptive immune cells. 13
Figure 3. Structure of hyaluronan. 14
Figure 4. A diagram of Th17 activation and differentiation 15
Figure 5. Flow cytometry analysis of cytokine expression following the differentiation assay. 34
Figure 6. qPCR data of cytokine mRNA expression following the differentiation assay. 35
Figure 7. ELISA analysis of supernatant IL-17A levels following the differentiation assay. 36
Figure 8. SDS-PAGE analysis of hyaluronan in the supernatant following the differentiation experiment. 37
Figure 9. Flow cytometry analysis of cytokine expression and staining following the viability assay. 38
Figure 10. Flow cytometry analysis of cytokine expression following the αCD44 treatment. 39
Figure 11. Flow cytometry analysis of cytokine expression following the retroviral transfection. 40
Figure 12. SDS-PAGE analysis of supernatants following the retroviral transfection. 41
Figure 13. A diagram of the revised hypothesis. 56

List of Abbreviations

AIRE	autoimmune regulator
APC	antigen presenting cells
B6	C57BL/6 (wild-type)
Chd7	chromodomain-helicase-DNA-binding protein 7
CNS	central nervous system
DAMP	danger associated molecular pattern
EAE	experimental autoimmune encephalitis
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
GMCSF	granulocyte macrophage colony-stimulating factor
HMW	high molecular weight
Hyal2	hyaluronidase 2
IFNγ	interferon gamma
IL-17A	interleukin 17A
IL-17F	interleukin 17F
IL-21	interleukin 21
IL-22	interleukin 22
IL-23	interleukin 23
IL-6	interleukin-6
LMW	low molecular weight
LYVE-1	lymphatic vessel endothelial hyaluronan receptor 1
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
OPC	oligodendrocyte progenitor
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered solution
PBS+	PBS+1% FBS
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RORγt	retinoic acid orphan receptor-gamma 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCR	T-cell receptor
TGF-β	transforming growth factor-beta
Th1	T-helper 1
Th17	T-helper 17
Th2	T-helper 2
TLR	toll-like receptor
TLR2	toll-like receptor 2
TLR2-/-	TLR2 deficient
Treg	T-regulatory cell

Introduction

The immune system, composed of both innate and adaptive defenses, works to protect the body from pathogens, cancer, and foreign materials (Parham, 2009). Innate defenses are not typically specific to any one pathogen, such as an inflammatory response provoked at the site of microbial infection (Alberts et al., 2015). Chemical signals from microbes trigger the expression of signaling peptides termed cytokines, which modulate inflammation at the site of infection. Typically, body temperature rises, permeability of the nearby blood vessels increase, and vasodilation occurs (Parham, 2009). Chemokines are also released and attract phagocytic cells such as macrophages and neutrophils. These phagocytes engulf and then kill microbes using several toxic oxygen-derived compounds (Alberts et al., 2015).

Innate immune cells use pattern recognition receptors to identify pathogens. Most bacteria or pathogen-derived molecules contain certain pathogen-associated molecular patterns (PAMPs) (Tang et al., 2012). These patterns are recognized by pattern recognition receptors (PRRs) and reveal the molecule to be dangerous (Tang et al., 2012). There are five well characterized classes of PRRs: toll-like receptors (TLRs), retinoic-acid-inducible gene-I like receptors, nucleotide-binding domain and leucine rich repeat containing gene family receptors, C-type lectin receptors, and cytosolic DNA receptors (Alberts et al., 2015). Different PRRs recognize different PAMPs.

In addition to destroying pathogens, innate immune cells have additional roles as seen in figure 1. There are also many other components to the innate

immune system, including the complement system and various physical barriers to pathogen entry, but these lay outside the scope of this project.

Bridging the gap between the innate and adaptive immune cell types are dendritic cells (Sloane et al., 2010). These cells will phagocytize microbes and help activate adaptive immune cells. Following phagocytosis, dendritic cells cleave microbial peptides and display them on their surface along with major histocompatibility complex (MHC) proteins, thereby giving them the name of antigen presenting cells (APCs) (Parham, 2009). In doing so, the APCs activate immature adaptive immune cells, or lymphocytes, residing in the lymph nodes. In this activation process, discussed in greater depth later, adaptive immune cells recognize the antigen and MHC protein and begin differentiating into effector cells (Romagnani et al., 2009).

The adaptive immune system is comprised mostly of two main classes: B-cells and T-cells, collectively called lymphocytes (Alberts et al., 2015). Both cell types are responsible for remembering specific substances, termed antigens, but produce different immune responses. B-cells drive the humoral response by producing proteins complementary to specific antigenic determinants, termed antibodies. (Alberts et al., 2015) After activation with an antigen complimentary to its B-cell receptor, B-cells transform into plasma cells which produce antibodies that are distributed through the blood and lymph to bind to pathogens expressing the same antigen. These antibodies may neutralize the pathogen directly, by impairing its mobility, or indirectly, by allowing it to be phagocytized more easily in a process called opsonization (Parham, 2009). After the infection is contained,

the long-lived memory B-cells wait for stimulation with the same initial antigen and will produce antibodies once again. In contrast, T-cells generate a cell-mediated response whereby they fill a number of roles, ranging from the direct killing of microbes by releasing cytotoxic chemicals to stimulating a greater phagocytic cell response by releasing pro-inflammatory cytokines or helping in the activation of B-cells (Alberts et al., 2015).

T-cells develop from hematopoietic stem cells in the bone marrow. After the stem cell develops into a lymphoid progenitor cell, the cell migrates to the thymus gland, where it matures and becomes immunocompetent (Alberts et al., 2015) Immunocompetency refers to ability of lymphocytes to distinguish between self and non-self cells, and thereby generate an immune response; this vital property means that no cells from the individual should trigger an immune response against cells originating from its host (self-cells)(Carpenter & Bosselut, 2010). However, errors in the maturation of T-cells can lead to self-reactive T-cells being created, which can trigger some autoimmune diseases (Shah & Zuniga-Pflucker, 2014).

Initially, the progenitor cells are termed double negative because they express neither CD4 nor CD8, two receptors vital to T-cell activity (Parham, 2009). After signaling through Notch receptors, the progenitor cells become committed to being T-cells, and transition to double positive cells (CD4+CD8+) (Carpenter & Bosselut, 2010). Rearrangement at the T-cell receptor (TCR) locus results in unique α and β chains that construct a TCR specific for a single antigen (Burkett et al, 2015). Finally, the immature cells undergo positive selection,

transition to single positive cells (CD4+CD8- or CD4-CD8+), and then undergo negative selection.

In both positive and negative selection, the cells are exposed to a self-antigen, generate a signal, and the intensity of that signal is evaluated (Shah & Zuniga-Pflucker, 2014). Positive selection ensures that all cells have a functional T-cell receptor (TCR); cells that do not generate a sufficient signal are neglected and allowed to die as they would be unable to generate a sufficient immune response to an actual antigen. In negative selection, cells are forced to undergo apoptosis (programmed cell death) if they generate an abnormally high signal; this oversensitivity suggests the cell could be autoreactive and lead to an autoimmune response later (Carpenter & Bosselut, 2010). Self-antigens can be obtained one of two ways: they are carried to the thymus by recirculating dendritic cells or are generated by the thymic epithelial cells. The transcription factor autoimmune regulator (AIRE) can induce thymic epithelial cells to express tissue-specific antigens not typically found in the thymus, so that they can be used in for testing in selection (Shah & Zuniga-Pflucker, 2014).

As such, any autoreactive T-cells should be destroyed before ever leaving the thymus. However, errors in the selection process can occur; alternatively, there are some self-antigens that are not induced by AIRE and are not brought by dendritic cells. If this occurs, these antigens are not present in the thymus during negative selection, so T-cells cannot be tested for reactivity against these antigens. T-cells that are reactive to these self-antigens cannot be identified, and may be activated by the self-antigen later in the cell's development.

After selection, the immature, or naïve, cells are categorized by their expression of the cellular markers CD4 and CD8 (Jin et al., 2012). Eventually, after activation and proliferation, the naïve CD4-CD8+ T-cells become cytotoxic T-cells and the naïve CD4+CD8-T-cells become helper or regulatory T-cells (Parham, 2009). Cytotoxic T-cells kill target cells, such as cancer cells and virus-infected cells by releasing cytotoxic chemicals (Alberts et al., 2015). T-helper cells direct other immune cells to mount an immune response.

T-cells are characterized by the CD4 and CD8 receptors because they are crucial for the activation of T-cells. There are two types of MHC proteins (I and II) expressed on the surface of APCs (Alberts et al., 2015). MHC protein I interacts only with CD8 and MHC protein II interacts only with CD4 (Parham, 2009). Once an APC has phagocytized a pathogen and broken down its proteins, the fragments will bind to a MHC protein; MHC I binds to endogenous peptides (i.e. viral peptides), whereas MHC II will bind to exogenous peptides, such as viral, bacterial, fungal, or parasitic peptides (Parham, 2009). Once bound, the MHC-peptide complex is expressed on the exterior of the APC where it can be recognized by a passing naïve T-cell. The CD receptor on the cell binds to the MHC protein, thereby bringing the peptide fragment within binding distance of the T-cell receptor (TCR). If the peptide fragment can bind to the TCR, the T-cell is activated and stimulated to differentiate and proliferate (Parham, 2009). The peptide fragment helps determine what class of cell it differentiates into, so that the most effective immune response is generated. Figure 2 outlines the process needed for activation.

Of particular interest in this project is the T-helper subset (also called CD4+ T-cells); these cells interact with other cell types directly (through cell-cell interactions) or release cytokines after being activated by an APC (Patel & Kuchroo, 2015). T-helper cells work to direct other immune cells such as cytotoxic T-cells and macrophages to target infected cells and antigens as well as activate and promote the release of antibodies by immature B-cells. Though their approach to pathogen eradication is less direct than most other cell types, their effect is substantial. Virtually all adaptive immune responses are directed by CD4+ T-cells (Louten, et al., 2009).

Within the CD4+ T-cell group, there are several subsets, each with a distinct role within the body. After being released from the thymus, the nonactivated CD4+ T-cells are naïve, or undifferentiated. To activate the naïve cell, and cause them to differentiate and release cytokines, the cell must first be stimulated with cytokines and presented with an antigen (Patel & Kuchroo, 2015). The stimulating cytokines induce the production of transcription factors by binding to receptors which then initiate downsteam signaling. Transcription factors are proteins that bind to various regulatory regions or genes and promote the differentiation towards a particular subset. Some of these subsets include T-helper 1 (Th1), T-helper 2 (Th2), and T-regulatory (Treg) cells (Jin, et al., 2012). These cells are identifiable by their expression of interferon gamma (IFN-γ), interleukin 4, and forkhead box P3, respectively.

One recently characterized subset is the T-helper 17 cell, or Th17 cell. The differentiation of Th17 cells can be stimulated by several sets of cytokines;

interleukin-6 (IL-6) and transforming growth factor-beta (TGF-β) are typically cited (Louten et al., 2009). Differentiation cannot occur without cytokine stimulation. These cytokines trigger the expression of two major transcription factors: signal transducer and activator of transcription 3 (STAT3), and retinoic acid orphan receptor-gamma 2 (RORγt) (Romagnani et al., 2009). Once differentiated, the mature Th17 cell is characterized by the release of specific cytokines such as interleukin 17A (IL-17A), interleukin 17F (IL-17F), interleukin 22 (IL-22), and interleukin 21 (IL-21) (Louten et al., 2009). These pro-inflammatory cytokines released by Th17 cells are used to mount an immune response specifically against extracellular bacteria (Jin et al., 2012).

Microbial products can also affect CD4+ T-cell differentiation by signaling through PRRs (Jin et al., 2012). Typically, PRRs are thought of as a part of the innate immune system and are expressed on macrophages and other monocytes (Tang et al., 2012). Once a monocyte recognizes a PAMP, it is stimulated to act. However, PRRs are not restricted to innate immune cells. For example, TLRs are expressed on both innate and adaptive immune cells. TLR receptors can bind a wide range of ligands ranging from double-stranded RNA to lipopolysaccharides (Miranda-Hernandez & Baxter, 2013). Specifically, TLR2 is expressed on naïve CD4+ T-cells as well as Th17 cells (Bhaskaran et al., 2015). This receptor typically binds to bacterial lipoproteins and lipoteichoic acid from Gram-positive bacteria (Miranda-Hernandez & Baxter, 2013). Signaling through the TLR2 receptor could have consequences for CD4+ T-cell differentiation and proliferation (Nyirenda et al., 2015; Zhao et al., 2015).

It is of interest to note that PRRs do not differentiate between self and foreign materials; instead it recognizes the presence of a PAMP or other danger associated molecular pattern (DAMP). As such, it is possible for self-molecules with structures resembling DAMPs to bind to TLR2 or other PRRs and cause an immune response, contributing to an autoimmune disease. Cases of autoimmune disorders with sterile inflammation such as multiple sclerosis (MS) may be caused in part by inappropriate self-molecule recognition (Hernandez-Pedro et al., 2013).

Multiple sclerosis is a chronic inflammatory disease in which the immune system attacks the myelin sheath and nerve fibers of the central nervous system (CNS) (Jadidi-Niaragh & Mirshafiey, 2011). Symptoms can be severe, and range from blurry vision, to muscle weakness, to paralysis (Russi & Brown, 2015). Though the symptoms can be treated with a variety of medications, there is no reversing the damage to CNS tissue nor safely impairing the destructive immune cells. As such, there is no cure, and current treatments are limited to symptom management. As a disease that affects over 2.3 million individuals, there is a great need for treatments to prevent or stop the inflammation that causes the disease state.

Evidence has implicated Th17 cells in MS (Goverman, 2009). Research has found evidence that Th17 cells and Th1 cells are the main immune cells contributing to central nervous system inflammation in MS (Rostami & Ciric, 2013). Th17 cells and Th1 cells can be found infiltrating central nervous system tissues in diseased individuals, while they are scarcely found in the tissue of

healthy individuals. Thus, researchers inferred that the infiltration of Th17 cells and Th1 cells participate in directing inflammation in the CNS tissues with pro-inflammatory cytokines (El-Behi et al., 2011).

Furthermore, signaling through TLRs on Th17 cells incites an immune response, contributing to an increase in pathogenic Th17 cells in MS (Reynolds et al., 2010). Pathogenicity is the ability of a cell to do harm, or destroy; it is not intrinsically bad. For example, pathogenic T-cells are extremely helpful during infections as these cells are more able to fight off pathogens than typical Th17 cells (Lubberts, 2015). However, when autoreactive T-cells are activated and become pathogenic, a disease state can occur in which inflammation is heightened and more immune cells are recruited activated (Rostami & Ciric, 2013).

Pathogenic Th17 cells can be characterized by increased expression of (IFNγ) or granulocyte macrophage colony-stimulating factor (GMCSF) along with all other typical cytokines such as IL-17A and IL-17F (Reynolds et al., 2010). Signaling through TLR2 or with interleukin-23 (IL-23) can incite pathogenesis in Th17 cells (Lubberts, 2015). Evidence shows that the increase in TLR2 signaling ligands leads to an increase in IL-17A and IL-22, indicating that there are increased numbers of, or more pathogenic, Th17 cells (He et al., 2016; Nishimori et al., 2012). Furthermore, IL-17A leads to greater TLR2 expression, thereby creating a positive feedback loop. (Lee et al., 2009) Other studies show that signaling through TLR2 also upregulates Th17 differentiation, though no definitive mechanism was found (Nyirenda et al., 2011). Finally, MS specific

experiments support the role of TLR2 as being able to provoke an inflammatory Th17 response. Experimental autoimmune encephalitis (EAE) (a murine model of MS) studies demonstrate a decrease in disease severity and progression in TLR2 deficient T-cells (Reynolds & Dong, 2013).

Thus, we know that autoreactive T-cells can initiate autoimmune inflammation amplified by the induction of pathogenicity in T-cells, which can set off a cycle of disease state inflammation leading to increased Th17 cell populations which leads back to increased inflammation. We are still seeking the self-molecule that will signal through TLR2 to trigger pathogenesis in autoreactive T-cells. One clinical goal of this study was to better understand and to prevent the onset of autoimmune disorders from self-molecule signaling through TLRs.

One possible self-molecule that could aggravate autoimmune inflammation is hyaluronan (its anionic form is called hyaluronic acid), a large polysaccharide comprised of repeating disaccharide units of N-acetyl glucosamine linked to glucuronic acid by β 1,3 bonds (figure 3) (Humphrey, 1943). This ubiquitous molecule is typically found within synovial fluid, skin, and connective tissues; it binds to water and is an important gelling compound in the extracellular matrix (Spinelli et al., 2015). Receptors for hyaluronan include CD44, which is prevalent in T-cells, and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (Laurent & Fraser, 1992).

Typically, healthy tissue contains high molecular weight hyaluronan, which is on the magnitude of 10^5 to10^6 kDa. Each molecule of hyaluronan has a

relatively short lifetime for a structural molecule, and must travel through the lymph system to reach the liver for degradation (Gomez-Aristizabal et al., 2016). Alternatively, when tissue is damaged and the risk of infection by bacteria is high, hyaluronan is broken into low molecular weight fragments of anywhere between $10^2 – 10^4$ kDa (Lee-Sayer, et al. 2015). Prior research has shown that hyaluronan production is heightened during inflammation and has confirmed its role in wound healing (Jiang, 2005). Hyaluronan is also involved with T-cell mediated autoimmune disorders (Back et al., 2005; Esser et al., 2012). There is also some speculation that hyaluronan is involved with CD4+ T-cell differentiation (Kuipers et al., 2015). Research has shown that hyaluronan can signal through TLR2 (Shimada et al., 2008). Thus, there is a possibility that hyaluronan signaling through TLR2 leads to an upregulation in Th17 differentiation, which in turn amplifies autoimmune inflammation. Figure 4 describes the entire Th17 system: an autoreactive naïve T-cell is activated by a self-antigen and stimulated by hyaluronan signaling through TLR2, leading to an upregulation of Th17 differentiation, increased cytokine production and possibly pathogenicity.

Here we investigate how differing molecular weights of hyaluronan affect Th17 differentiation. Given that high molecular weight hyaluronan dominates in healthy body tissue, we expect this treatment to minimally influence Th17 differentiation. However, since low molecular weight hyaluronan is indicative of injury and possible bacterial infection, we expect this treatment to act as an endogenous danger signal. In this case, we expect that low molecular weight hyaluronan would promote Th17 differentiation.

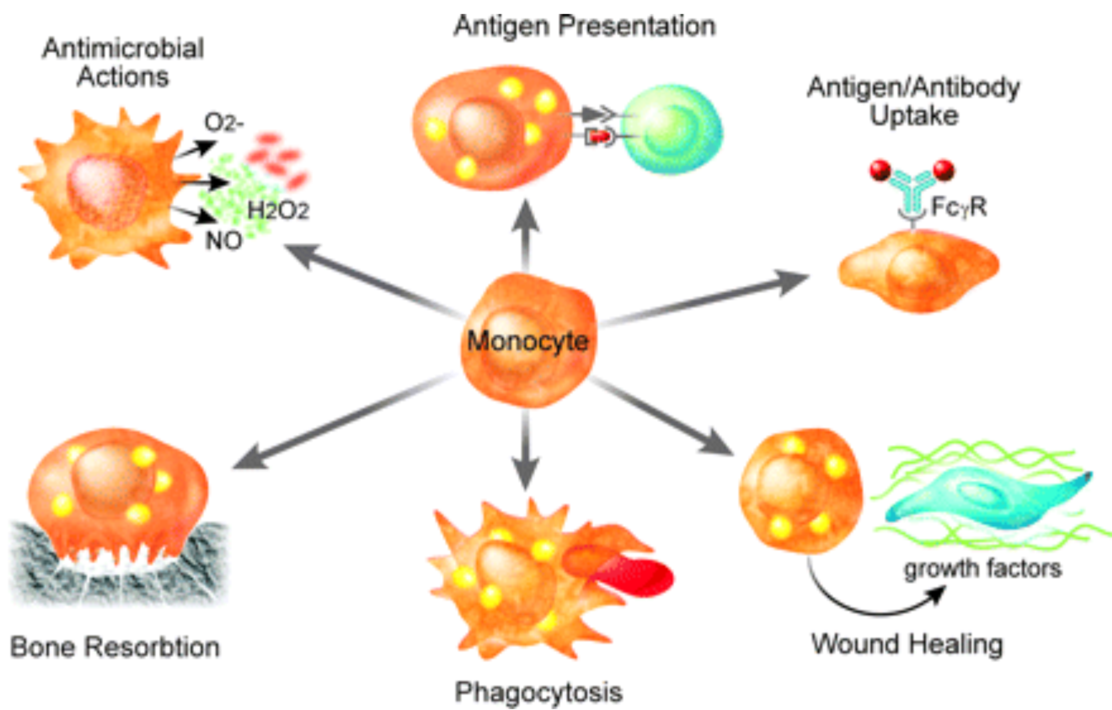


Figure 1. Functions of the innate immune system. Monocytes, an immature macrophage/dendritic cell, can identify pathogens using PRRs, and then use these techniques to defend the body. Image from Chawla, 2010.

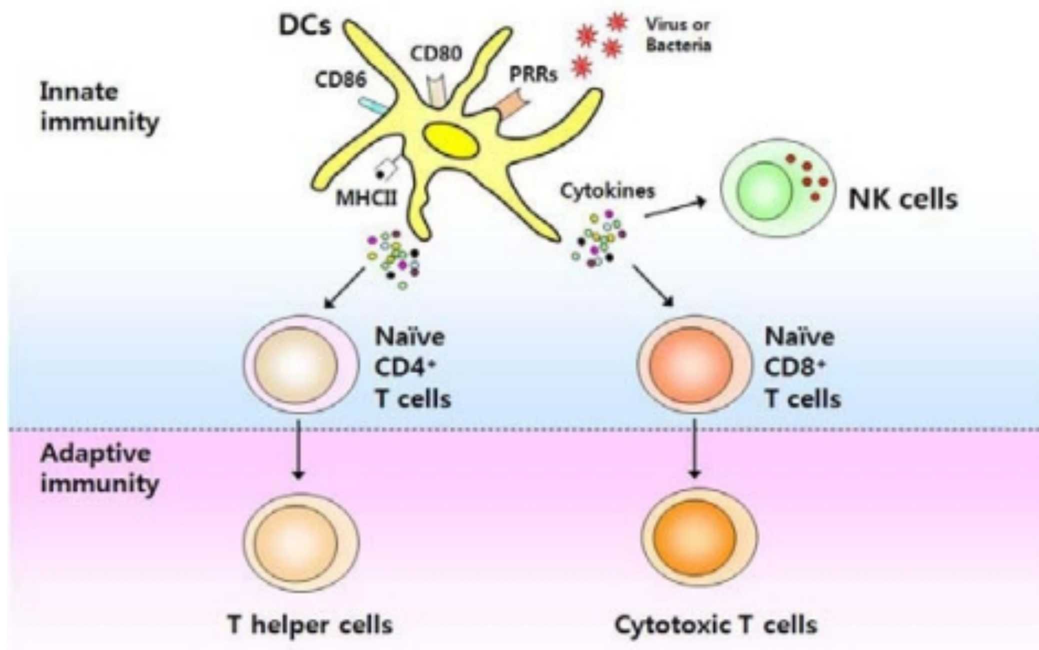


Figure 2. Innate immune cells activate adaptive immune cells. A dendritic cell can carry an antigen on MHC protein I or II to a naïve T-cell and, with stimulatory cytokines, induce the T-cell to differentiate into a mature T-helper cell or cytotoxic T-cell. Image from Gi, et al., 2009.

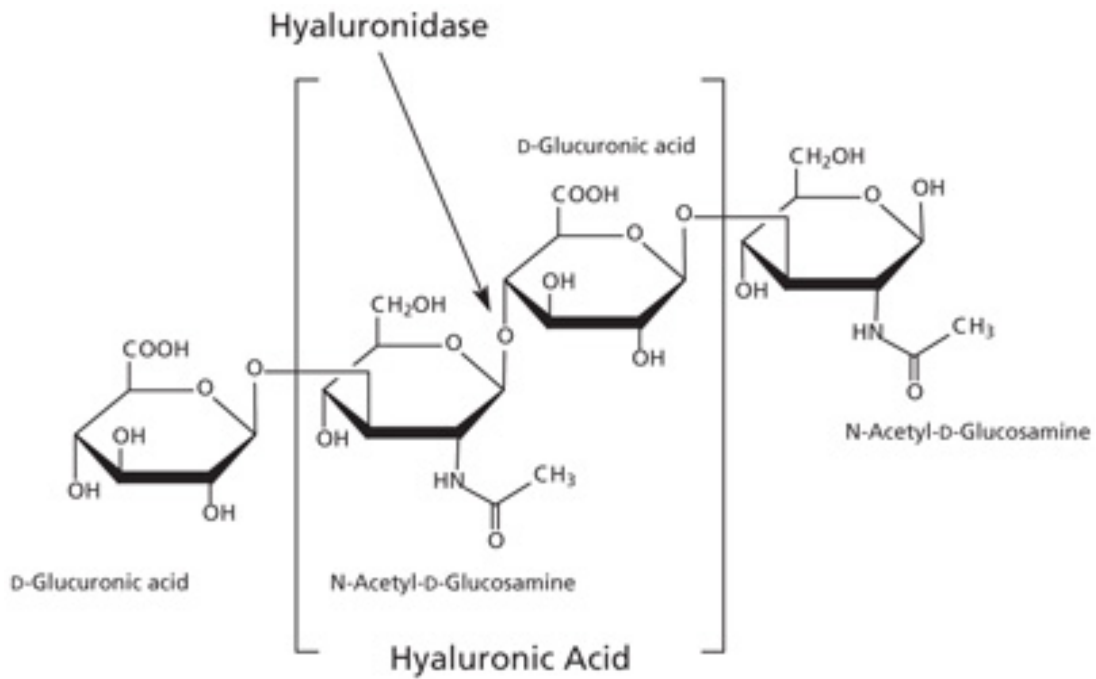


Figure 3. Structure of hyaluronan. Hyaluronan is composed of N-acetyl glucosamine and glucuronic acid linked by β 1,3 bonds. High molecular weight hyaluronan is approximately $1x10^6$ kDa, but can be cleaved into smaller fragments by hyaluronidases. Image from Sigma-Aldrich.

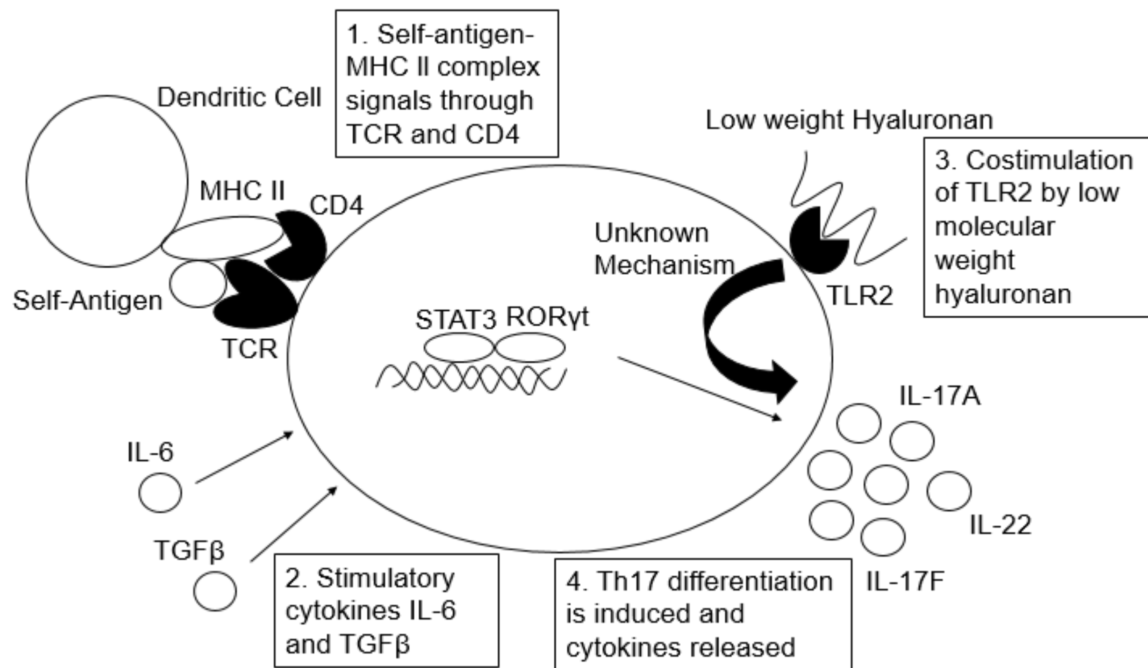


Figure 4. A diagram of Th17 activation and differentiation. 1. An autoreactive naïve T-cell will be activated with signaling through the TCR with a self-antigen and with MHC II protein signaling through CD4. 2. Stimulatory cytokines IL-6 and TGFβ also activate the cell. 3. Stimulation of TLR2 by low molecular weight hyaluronan increases Th17 activation. 4. After steps 1-3, transcription factors STAT3 and RORγ are expressed, leading to differentiation and the release of cytokines. Steps 1 and 2 and imperative for T-cell differentiation; step 3 can increase cytokine production, proliferation, or pathogenicity but differentiation can still continue without it.

Materials & Methods

Experimental Design

To best determine the impact of hyaluronan signaling through TLR2 in Th17 differentiation, four key experiments were performed: a differentiation assay, a viability assay, αCD44 treatment, and Hyal2 retroviral transfection. In each experiment, naïve T-cells from wild-type and TLR2 knock-out mice were obtained, treated with differing amounts and types of hyaluronan, allowed to differentiate, and then compared. The cells were analyzed using flow cytometry, quantitative polymerase chain reaction (qPCR) (mRNA analysis), enzyme linked immunosorbent assay (ELISA), or a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Mice

Experiments were conducted with C57BL/6 (B6) (wild-type) and TLR2 deficient (TLR2-/-) mice, both of which were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed at Rosalind Franklin University of Medicine and Science and given free access to water and dry food. A 12 hour light, 12 hour dark schedule was maintained. At 6-10 weeks of age, the mice were sacrificed using carbon dioxide and secondary cervical dislocation under protocols approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University.

Naïve CD4+ T-cell Collection

Naïve CD4+ T-cells were isolated from female B6 and TLR2-/- mice; approximately 3 x 10^6 cells/mouse were expected. The wild type and TLR2 deficient cells were kept separate throughout this entire procedure. After being sacrificed, the spleen and lymph nodes (inguinal, axillary, brachial, and cervical) were removed. Both sets of tissue were smashed in PBS+ (1x PBS (phosphate buffered solution, pH=7.4; diluted from purchased 10x stock: Life Technologies, Carlsbad, CA) + 1% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA)) using a 3 mL syringe, then filtered through mesh. The spleen solution was centrifuged at 1500 rpm for 5 minutes at 4ºC to pellet the cells. The cells were resuspended in 3 mL/mouse of lysing buffer, allowed to incubate for 1 minute, then quenched with PBS+. The spleen and lymph node solutions were combined and pelleted.

At this point, one of two protocols would be followed. One could separate out the CD4+ cells, stain them, and then sort out the naïve T-cells using the cell sorter machine. Alternatively, one could use a kit to sort out the naïve T-cells using a small magnet. The first protocol, using a cell sorter, is more expensive and time-consuming, but typically provides higher yields and fewer contaminating cells. The second protocol of using a magnet is cheaper and quicker, but has lower yields and often small amounts of magnetic beads or red blood cells would be mixed with the naïve T-cells. Both protocols were used for different experiments, but were expected to yield the same CD4+ T-cell population.

To sort using a cell sorter, we needed to first eliminate the CD4- cells to decrease the volume of the sample going in the sorter. After cells were lysed with lysing buffer and pelleted, they were resuspended in 150 µL/mouse of PBS+ and 15 µL/mouse of anti-CD4-conjugated microbeads (Miltenyi Biotec, Auburn, CA) and incubated for 15 minutes at 4ºC in the dark. The cells were washed, pelleted, resuspended in PBS+, and filtered through a mesh. The cells were sorted on an AutoMacs cell seperator (Miltenyi Biotec, Serial#0095) using the positive selection program; the positive fraction was washed with PBS+ and the negative fraction was discarded.

After washing and pelleting the positive fraction, the cells were stained with anti-CD62L-FITC, anti-CD25-PE, anti-CD4-PerCP, and anti-CD44-APC stains (all obtained from Biolegend, San Diego, CA). The cells incubated for 20 minutes at 4ºC in the dark, then were washed with PBS+, pelleted, resuspended in 300 µL/mouse PBS+, and filtered through a cell strainer mesh. The cells were then sorted on the BD FACS Aria IIu cell sorter for the $CD4^{+}CD62L^{high}CD25^{-}$ $CD44^{low}$ fraction, which corresponds to naïve CD4+ T-cells. After the cells were collected, they were immediately used in one of the following assays.

Alternatively, when one was sorting using the kit magnet, we would determine the concentration of cells after lysing with the ACK lysing buffer and pelleting. The cells were resuspended to a concentration of $1x10^{8}$ cells/mL in PBS+ and processed in 2 mL or less increments. All the following reagents were from the Easy Sep mouse naïve CD4+ T-cell isolation kit (Stem Cell Technologies, Vancouver, Canada). Next, 50 µL/mL of cells of normal rat serum

and 50 µL/mL of CD4+ T-cell isolation cocktail were added to the cells and incubated for 7.5 minutes at room-temperature. Then, 50 µL/mL memory T-cell depletion cocktail were added to the cells and incubated for 2.5 minutes at room-temperature. The streptavidin RapidSpheres 50001 were vortexed and added to the cells at 75 µL/mL to incubate for another 2.5 minutes. The tube containing the cells was then set in the EasySep Magnet (Stem Cell Technologies, Vancouver, Canada) and incubated for 2.5 minutes. The magnet and tube were picked up and the solution inside containing the naïve CD4+ T-cells was carefully decanted. The cells were washed with PBS+ and immediately used for one of the following assays.

Differentiation Assay

This assay was used to determine whether hyaluronan of differing molecular weights affected differentiation rates of CD4+ T-cells. Naïve T-cells from both wild-type and TLR2 deficient mice were isolated using a cell sorter and cultured at $1x10^6$ cells/mL in RPMI 1640 (Life Technologies, Carlsbad, CA) media supplemented with 10% FBS, 1% L-glutamine (Life Technologies, Carlsbad, CA),1% Penicillin/Streptomycin (Life Technologies, Carlsbad, CA), 0.1% β-mercaptoethanol (Life Technologies, Carlsbad, CA), and 0.05% gentamicin (Life Technologies, Carlsbad, CA). The cells were plated in 48 well plates that had been incubated with 1 µg anti-CD28 antibodies (BD, Franklin Lakes, NJ) and 1 µg anti-CD3 antibodies (BD, Franklin Lakes, NJ) overnight at 4ºC and washed with PBS. All cells were induced to differentiate into Th17 cells with mIL-6 at 20 ng/mL, TGF-β at 3 ng/mL, anti-IL-4 monoclonal antibodies

(eBioscience, San Diego, CA) at 5 µg/mL, and anti-IFN-γ monoclonal antibodies (eBioscience, San Diego, CA) at 5 µg/mL. The treatments were as follows: Th17 (no additional components), Th17+500 µg low molecular weight hyaluronan (289 kDa; R&D, Houston, TX), Th17+1000 µg low molecular weight hyaluronan, Th17 +500 µg high molecular weight hyaluronan($1.35x10^5$ kDa; R&D, Houston, TX), and Th17 +1000 µg high molecular weight hyaluronan in both wild-type and TLR2 KO cells.

The cells grew for 4 days at 37ºC with 5% carbon dioxide. On the fourth day, the cells were split 1:2 for flow cytometry analysis and mRNA analysis.

Viability Assay

The viability assay was used to compare the amounts of dying cells with different hyaluronan treatments. Immediately following the isolation of naïve T-cells by cell sorter, the cells were divided and plated on two plates that had been coated overnight with 1 µg anti-CD28 antibodies and 1 µg anti-CD3 antibodies at 4ºC and washed with PBS. All cells were induced to differentiate into Th17 cells with mIL-6 at 20 ng/mL, TGF-β at 3 ng/mL, anti-IL-4 monoclonal antibodies at 5 µg/mL, and anti-IFN-γ monoclonal antibodies at 5 µg/mL. The treatments were as follows: Th17 (no additional components), Th17 +1000 µg low molecular weight hyaluronan, and Th17 +1000 µg high molecular weight hyaluronan in both wild-type and TLR2 KO cells.

The cells grew for 4 days at 37ºC with 5% carbon dioxide. On the fourth day, the cells were taken for flow cytometry analysis. The first plate of cells was prepared for flow cytometry as detailed later.

The second plate of unlabeled cells was stained with Annexin V (fluoresces in the APC channel) for apoptosis peptides and 7-AAD (fluoresces in the PerCP channel) for exposed DNA, two common markers of cellular death. The cells were pelleted and resuspended at 10^6 cells/mL in Annexin V binding buffer (BD, Franklin Lakes, NJ); 10^5 cells were moved to a new tube for staining. Next, 5 µL each of Annexin V stain (BD, Franklin Lakes, NJ) and 7-AAD stain (BD, Franklin Lakes, NJ) was added to the cells and allowed to incubate for 20 minutes at room-temperature in the dark. A volume of 400 µL of Annexin V binding buffer was added to each tube to stop the staining.

All the samples were run on the flow cytometer (BD LSR ll) and the fluorescence emitted by the stain in each sample measured. The data were then analyzed using FlowJo v10 (FlowJo, LLC, Ashland, Oregon) software.

CD44 Antibody Treatment

The differentiation assay was repeated with anti-CD44 antibodies to prevent hyaluronan signaling through CD44. Both WT and TLR2-/- cells were sorted using the magnet and the cells were plated with the stimulating cytokines, as described previously. Two wells of the following conditions were prepared: Th17, Th17 +1000 µg low molecular weight hyaluronan, and Th17 +1000 µg high molecular weight hyaluronan in both WT & TLR2-/- cell types (12 wells total). Then, one well of each condition was treated with 5 µg/mL of anti-CD44 antibodies (Biolegend, San Diego, CA).

The cells were allowed to grow for 5 days at 37ºC with 5% carbon dioxide. Following this, the cells were stimulated and stained for flow cytometry analysis as described later.

Creation of Hyal2 Retroviral Construct

The Hyal2 sequence was cloned out of Th17 cDNA using a Phusion enzyme kit (Fisher, Hampton, NH). The primers used had AgeI and XhoI restriction enzymes sites on the start and end of the sequence, respectively. The primer sequences were as follows: AGAACCGGTATGCGGGCAGGACTAGGT (forward) and TCGCTCGAGTCATAAGGTCCAGGTGAG (reverse). The Hyal2 sequence was then polyA-tailed and ligated into the pGEM-T Easy Vector (Promega, Madison, WI). The Hyal2-pGEM-T Easy construct was sequenced by Northwestern University's NUSeq Core Facility (Chicago, IL). Once the sequence was confirmed, the Hyal2 sequence was cut out using AgeI and XhoI cut sites and ligated into the retroviral pCmmp iRES GFP vector.

Retroviral Transfection

Naïve CD4+ T-cells were transfected with the Hyal2 construct to overexpress Hyal2. First, viral particles were produced by PLAT-E cells after a calcium phosphate transfection. Media was aspirated off 6 plates of PLAT-E cells ($4.5x10^6$ cells/plate) and replaced with 10 mL DMEM+ (DMEM (Life Technologies, Carlsbad, CA) +10% FBS, 1% Penicillin/Streptomycin, 1% L-glutamine) with 1x chloroquine. Two mixtures of 10 µg of DNA per plate of PLAT-E cells (either Hyal2 construct or empty pDES vector) in 0.25 M $CaCl_2$ (diluted with water) were mixed with equal-volume 2x HBSS (Life Technologies,

Carlsbad, CA) and incubated for 5 minutes at room temperature. Then, the mixture was added dropwise to the plates of cells; each mixture was used on 3 plates. The plates incubated at 37ºC with 5% CO_2 for 6 hours, then washed with fresh DMEM+ and allowed to grow for another 48 hours.

After the 48 hour incubation, 6 wells of wild-type naïve T-cells were plated as detailed above. No hyaluronan was added at that time. Also after the 48 hour incubation, the supernatant (filled with copies of the retrovirus) was pulled off the PLAT-E cells and spun at 8000 g at 4ºC overnight. The media was aspirated and the viral pellets resuspended in a quarter of the original volume of RPMI+ with 8 ng/mL polybrene. The naïve CD4+ T-cells were spun down, aspirated, and resuspended in 1 mL of viral mixture; each viral mixture was used to treat half the wells. The naïve T-cells were centrifuged at 1800rpm for 65 minutes at 30ºC with low brake. The cells were then washed with fresh RPMI+ and were treated with hyaluronan. The following conditions were set up: Th17, Th17 +1000 µg low molecular weight hyaluronan, and Th17 +1000 µg high molecular weight hyaluronan with both Hyal2 and empty vector transfection treatments.

The cells were allowed to grow for another 2 days, then the supernatants were taken for SDS-PAGE analysis and the cells taken for flow cytometry analysis as detailed above.

Flow Cytometry Analysis

The cells saved from the differentiation assay were analyzed for cytokine production. On the fourth day following the start of the differentiation assay, the cells were split 1:2 and half remained in the original wells. A mixture of 1 µL

ionomycin (Sigma-Aldrich, St. Louis, MO), 1 μL Brefeldin-A (eBioscience, San Diego, CA), and 0.1 μL PMA (Sigma-Aldrich, St. Louis, MO) were added to each well. The cells were allowed to grow for an additional 5 hours and then they were transferred to tubes and pelleted.

The cells were resuspended in 30% Fc Block in 1x FACS (0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.05 mM ethylenediamine tetraacetic acid (EDTA; VWR, Radnor, PA), 1x sodium azide (Sigma-Aldrich, St. Louis, MO) in PBS) buffer and allowed to incubate for 15 minutes at room temperature. The cells were washed with 1 mL of FACS buffer and pelleted, then fixed with IC Fixation buffer (eBioscience, San Diego, CA)) for 20 minutes at 4ºC in the dark. The cells were washed with 1 mL of permeabilization buffer (eBioscience, San Diego, CA), pelleted, then resuspended in IL-17A-PE (eBioscience, San Diego, CA) and IFN-γ-APC (BD, Franklin Lakes, NJ) stains diluted in permeabilization buffer. The cells incubated for 30 minutes at 4ºC in the dark, then were washed with permeabilization buffer and pelleted. The samples were resuspended in 4% paraformaldehyde.

The cells were then run on the flow cytometer (BD LSR II) and the fluorescence emitted by the stain in each sample measured. The data were then analyzed using FlowJo v10 (FlowJo, LLC, Ashland, Oregon) software.

mRNA Analysis

Cells saved from the differentiation assay were analyzed for relative amounts of cytokine and transcription factor mRNA. On the fourth day following the start of the differentiation assay, the cells were split 1:2 and half were moved

to a new plate that had been incubated with anti-CD3 antibodies overnight at 4ºC. The cells were allowed to grow for an additional 2 hours, then they were transferred to microcentrifuge tubes and pelleted. Then, the cells were resuspended in Trizol (Life Technologies, Carlsbad, CA). Next, chloroform was added and the sample was centrifuged at 13,300 rpm for 15 minutes at 4ºC in a microcentrifuge. The sample will have separated into two distinct layers within the tube; the top layer was carefully pulled off and transferred to a new tube. The bottom layer can be discarded. Then, ice-cold -20ºC isopropanol was added to the sample and centrifuged at the same conditions, except the spin time was only 10 minutes. The sample was decanted carefully, to avoid disturbing the RNA pellet and washed with 70% ethanol in water. The sample was centrifuged was centrifuged at the same conditions, except the spin time was only 5 minutes. Again, the sample was decanted carefully, then the pellet was allowed to dry completely. The RNA pellet was resuspended in 11 µL of nuclease-free water.

Next, the RNA was made into cDNA, both to improve the stability of the sample and to allow PCR to be run on the sample. A volume of 10.5 µL of each sample's RNA was denatured in the thermocycler (Biorad, C1000 Touch, CT009882) using the program as follows: 65ºC for 6 minutes, hold at 4ºC. Next, 4 µL of 5x FS buffer (Invitrogen, Carlsbad, CA), 2 µL DTT (Invitrogen, Carlsbad, CA), 1 µL of dNTPs, 1 µL oligos (Invitrogen, Carlsbad, CA), 0.5 µL RNAse inhibitor (Invitrogen, Carlsbad, CA), and 1 µL M-MLV (Life Technologies, Carlsbad, CA) was added to each sample. The samples were placed in a thermocycler and the following program run: 37ºC for 65 minutes, 70ºC for 10.5

minutes, hold at 12ºC. The samples were then diluted with 80 μL of nuclease-free water.

Finally, qPCR was run on the samples to determine the relative amounts of cytokine/transcription factor mRNA present. A volume of 5 μL per well of cDNA was pipetted into a 96 well qPCR plate, with 6 wells for each sample (each sample had each gene run in duplicate). Then, 12.5 μL of SYBR Green (Life Technologies, Carlsbad, CA), 5.5 μL of water, and 1 μM primers (either Actin, IL-17A, or RORyt) were added to each well. The plate was placed in the real-time PCR machine (ThermoFisher, 7500 Real Time PCR Machine, 27550150) was the following program run: 95ºC for 10 minutes, then 40 cycles alternating between 95ºC for 15 seconds and 62ºC for 1 minute, then 95ºC for 15 seconds, 60ºC for 1 minute, 95ºC for 30 seconds, and then hold at 60ºC.

ELISA Analysis

The cell supernatants saved from the differentiation assay samples were analyzed for cytokine production. A 96 well ELISA plate was coated with IL-17A capture antibodies (BD, Franklin Lakes, NJ) diluted 1:1000 with PBS overnight at 4ºC. The plate was washed twice with washing buffer (1xPBS+0.5% Tween-20) on the ELISA plate washer (BioTek Instruments; Model # 405LSRS; Serial #13041015) then blocked with blocking buffer (1xPBS+10% FBS) for 1 hour at room temperature. The plate was washed three times on the ELISA plate washer with washing buffer and the standards and samples added. The standards were plated in duplicate, and had concentrations ranging from 25ng/mL-0.024ng/mL. The samples were plated in triplicate and diluted 1:3 with blocking buffer. The

plate incubated at 37ºC for 3 hours, and then was washed 6 times on the ELISA plate washer with washing buffer. The plate was then incubated with IL-17A detection antibodies (BD, Franklin Lakes, NJ) diluted 1:1000 with blocking buffer for 1 hour at room temperature. The plate was washed 6 times on the ELISA plate washer with washing buffer and incubated with horseradish peroxidase (Vector Labs, Burlingame, CA) diluted 1:2000 with blocking buffer for 30 minutes at room temperature in the dark. The plate was washed 6 times on the ELISA plate washer with washing buffer and incubated with ELISA substrate (Sigma-Aldrich, St. Louis, MO) until the color development was complete, approximately 15 minutes. The reaction was stopped by addition of 8.0 M sulfuric acid and the absorbance of each well read at 492 nm by a spectrophotometer (Molecular Devices, Spectra Max Plus 384). A 4-parameter fit standard curve was constructed and used to determine the concentration of each of the samples.

SDS-PAGE Analysis

The supernatants of the retrovirally transfected cells were run on a SDS-PAGE to determine if the overexpression of Hyal2 resulted in the cleavage of hyaluronan. An 8% SDS-PAGE gel (all resolving gel-no stacking gel) was made. Approximately 30 µL of cell supernatant or hyaluronan standard was loaded on the gel. Then, the gel was run at 120 V for 4-5 hours. Next, it was fixed in a solution of 50% methanol, 10% acetic acid, and fixative enhancer (Biorad, Hercules, CA) for 20 minutes, washed with water, and stained overnight with 0.005% Stains-all (Sigma-Aldrich, St. Louis, MO). Finally, the gel was washed with water, exposed to light for 30 minutes, then imaged.

Statistical Treatment

Two-tailed t-tests were conducted to determine if there were significant differences between conditions. A P-value of less than or equal to 0.05 was considered significant. Three replicates were needed to determine significance for all experiments except qPCR data; the data collection software is able to calculate standard deviation for two replicates, allowing us to use a t-test. Not all experiments had sufficient replicates to determine significance of differences.

Results

Differentiation Assay

I first determined whether the presence of low molecular weight hyaluronan would upregulate Th17 differentiation using the differentiation assay. Figures 5-7 show the results of the flow cytometry, qPCR, and ELISA analyses. These analysis procedures were used to assess the percentage of intercellular cytokine positive cells, relative cytokine and transcription factor mRNA expression, and amount of cytokines released into the cellular supernatant respectively. Assuming our hypothesis is correct, and low weight hyaluronan signals through TLR2 to upregulate Th17 differentiation and proliferation, we expected to see an increase in Th17 cells between the WT control and hyaluronan treatments, but see no difference between TLR2-/- controls and treatments.

We first determined the percent of Th17 cells present in our samples. After allowing the cells to proliferate, each sample contained between 1-2 x 10^6 cells. The IL-17A+IFNγ- fraction in the flow cytometry analysis corresponded to the Th17 population (Figure 5). The other remaining cells were dead or undifferentiated. The high concentration, low molecular weight hyaluronan treatment provided the greatest increase in Th17 cells when compared to the control (15.6% and 12.2% in WT and TLR2-/-, respectively). However, the high molecular weight hyaluronan treatment caused a similar increase in Th17 concentration (11.5% and 10.6%). The Th17 concentrations in the TLR2-/-

samples were lower than in WT samples, but an increase in concentration was still seen between the control and hyaluronan treatments.

Next we evaluated the relative expression of mRNA of two key Th17 produced molecules to judge the extent of Th17 differentiation. The relative mRNA expression of IL-17A and RORγt compared to actin is shown in Figure 6. The expression of IL-17A was higher in the WT samples indicating that there were more Th17 cells, as we expected based on our hypothesis. Furthermore, the hyaluronan samples had greater IL-17A expression than the respective controls. There was some elevation of RORγt expression in the WT high molecular weight hyaluronan treatments, but these results were less clear-cut.

We then evaluated the level of IL-17A in the cellular supernatant using an ELISA to judge the extent of Th17 differentiation. The levels of IL-17A protein in the cellular supernatant showed a similar trend as RORγt mRNA (Figure 7). Though the WT samples seemed to have had slightly higher levels of IL-17A production, and the hyaluronan treatments had higher expression than the control, there was no significant difference like in the flow cytometry results.

Finally, the cellular supernatants were analyzed for any change in hyaluronan composition. The stock high and low molecular weight hyaluronan were compared to the supernatants of Th17 and high molecular weight condition using a SDS-PAGE analysis (Figure 8). It appeared as though the Th17 cells were digesting the high molecular weight hyaluronan into low molecular weight fragments, which may then have upregulated Th17 differentiation.

Viability Assay

After establishing hyaluronan treatments resulted in the upregulation of Th17 differentiation, I next determined whether hyaluronan treatments would lead to greater cell viability. Figure 9 shows the results of apoptosis associated cellular marker staining analyzed by flow cytometry. If our hypothesis is correct, we would expect the lowest number of dying (7-AAD+AnnexinV+) cells between the control and hyaluronan treated WT cells, with no difference in the TLR2-/- cells.

The first set of staining repeated the differentiation assay (Figure 9) to show that the upregulation of Th17 differentiation was still present. The same trends were still evident; i.e., hyaluronan treatments upregulated Th17 differentiation and the WT cells had a higher level of Th17 differentiation than TLR2-/-. There also was a smaller difference between the WT and TLR2-/- cells compared to what was seen in the differentiation assay, indicating that the cells may have been over activated.

A second set of staining with 7-AAD and Annexin V (in the PerCP and APC channels respectively) was used to visualize apoptosis associated proteins and exposed DNA (Figure 9). Hyaluronan treatments lowered the fraction of 7-AAD+AnnexinV+ cells, indicating that there was greater viability. However, there was no discernable difference between the WT and TLR2-/- cells.

Anti-CD44 Treatment

Because previous experiments in our lab showed some upregulation of Th17 differentiation and proliferation in the TLR2-/- mice with hyaluronan treatment, we needed to isolate the role of TLR2. The CD44 receptor, present in naïve and Th17 cells, can also bind fragmented hyaluronan and transduce a signal (Banerji et al., 1999). To prevent CD44 signaling from interfering with TLR2 signaling, the cells were treated with neutralizing αCD44 antibodies. These antibodies bind to CD44 receptors and prevent the transduction of any signal. Figure 10 shows the effect of this treatment on Th17 differentiation. If the upregulation of Th17 differentiation is mediated solely by TLR2 signaling, there should be no difference between αCD44 untreated and treated conditions. If this effect is regulated by TLR2 and CD44 signaling, TLR2-/- treated cells should show no differences between the control and hyaluronan treatment.

The differentiation assay was repeated (Figure 10) to confirm the cells were still behaving the same way. Surprisingly, there was no difference between WT control and hyaluronan samples this time; however, it also appeared that some cells died in the hyaluronan treatments. This may have been caused by some external factor, possibly in the initial cell sorting, which impaired the WT cell growth. Yet the TLR2-/- cells experienced the same trends as before.

The αCD44 treated cells still showed upregulation of Th17 differentiation in both WT and TLR2-/- samples (Figure 10). For both cell types, Th17 differentiation was highly upregulated by low molecular weight hyaluronan and

moderately upregulated by high molecular weight hyaluronan, meaning the αCD44 treatment had no effect on the differentiation trends.

Retroviral Transfection

Because there was Th17 upregulation by high molecular weight hyaluronan, we chose to investigate the mechanism of this in greater detail. As shown in figure 11, the high molecular weight hyaluronan that was added to naïve T-cells was broken down into smaller fragments. We hypothesized that naïve T-cells or Th17 derived hyaluronidase II (Hyal2) digests the high molecular weight hyaluronan into lower molecular weight fragments, which then upregulate Th17 differentiation. To test this, we transfected naïve T-cells with a plasmid to overexpress Hyal2, then added hyaluronan and analyzed Th17 differentiation through flow cytometry. If this proposed mechanism is accurate, we expected to see greater Th17 differentiation in the overexpressing, high molecular weight condition compared to the control high molecular weight condition. In addition, no difference should be seen between control and overexpressing low molecular hyaluronan conditions.

The retroviral transfection was successful, as seen by high percentages of GFP+, or plasmid containing, cells (Figure 11). However, there was substantial cell death in all conditions, indicating that the procedure was too stressful for the cells. Unfortunately, high cell death obscured the effect of increased Hyal2 and hyaluronan on Th17 differentiation. SDS-PAGE analysis (Figure 12) confirms that high molecular weight hyaluronan digestion was higher in the Hyal2 overexpression condition as opposed to the control condition.

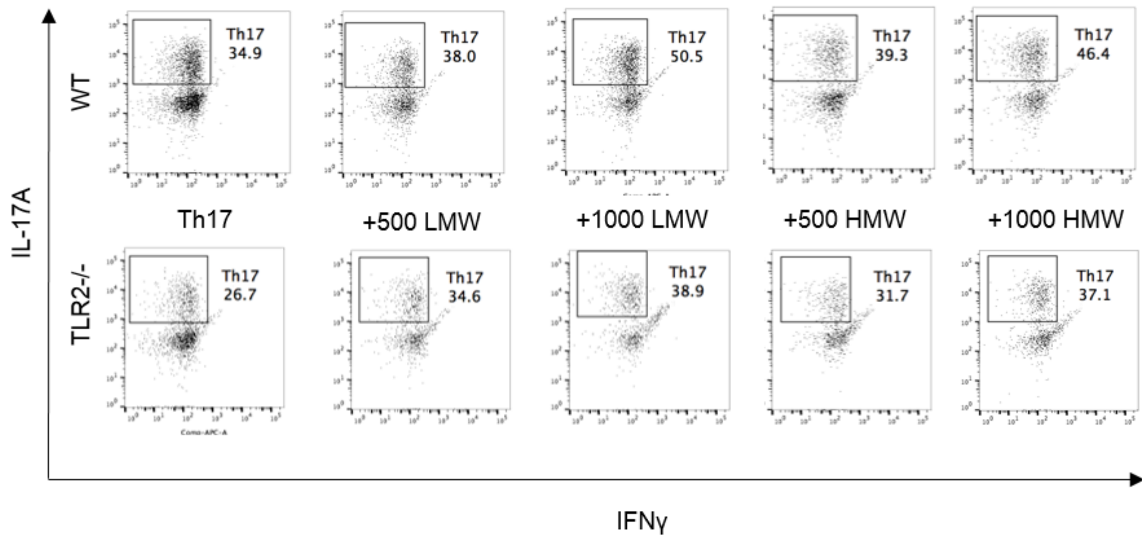
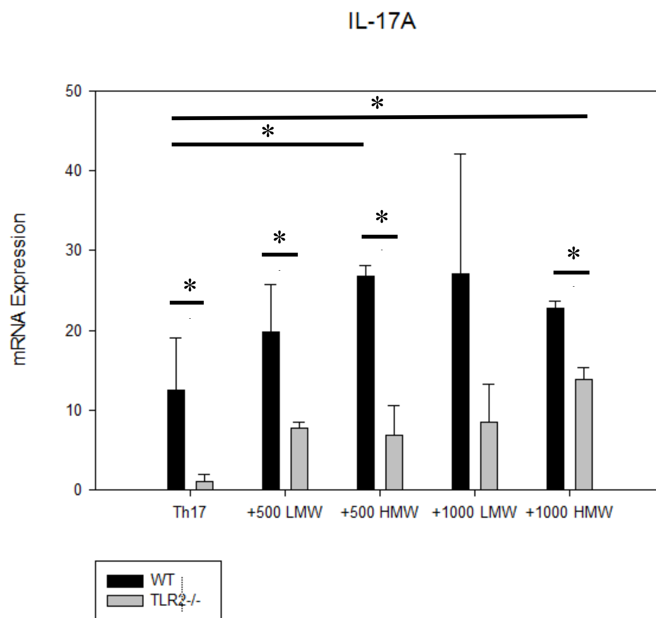


Figure 5. Flow cytometry analysis of cytokine expression following the differentiation assay. There was a significant difference between the WT Th17 and both the high concentration low molecular weight (LMW) and high molecular weight (HMW) hyaluronan treatments (n =3, P ≤ 0.05). No significant difference was seen between the WT and TLR2-/- conditions.



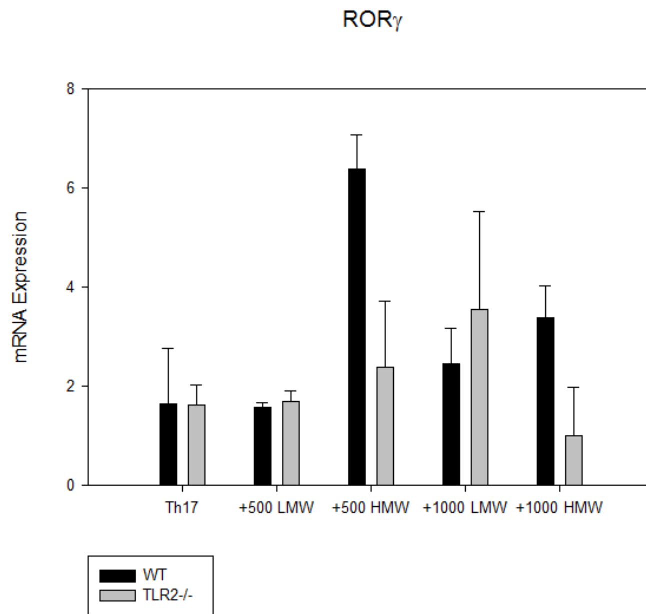


Figure 6. qPCR data of cytokine mRNA expression following the differentiation assay. There was a significant difference in IL-17A expression between WT Th17 and both high molecular weight hyaluronan treatments (n = 2, P ≤ 0.05). Furthermore, all samples except the high concentration, low molecular weight hyaluronan treatments showed significant differences between WT and TLR2-/- samples (n = 2, P ≤ 0.05). The RORγt results showed no consistent trends.

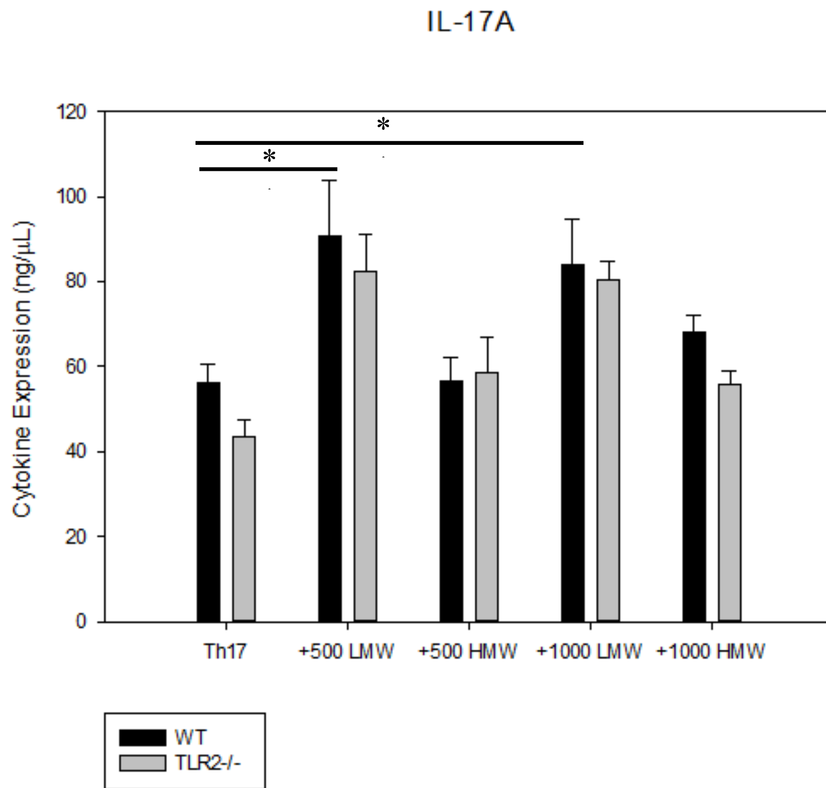


Figure 7. ELISA analysis of supernatant IL-17A levels following the differentiation assay. There was a significant difference between some samples, such as WT Th17 and the low molecular weight hyaluronan conditions ($n = 3$, $P \leq 0.05$). However, there were no consistent trends.

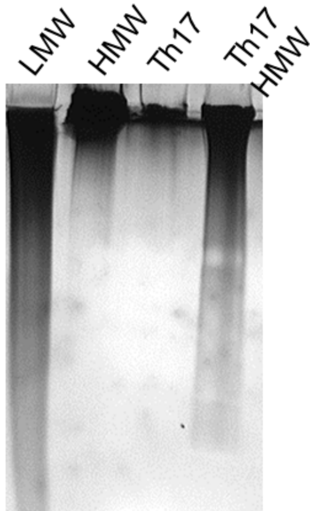


Figure 8. SDS-PAGE analysis of hyaluronan in the supernatant following the differentiation experiment. Control samples of low and high molecular hyaluronan, that have never been incubated with cells, are to the left. Supernatant from WT Th17 and Th17 +1000 HMW hyaluronan are to the right. High molecular weight hyaluronan treated cellular supernatant contained lower weight fragments.

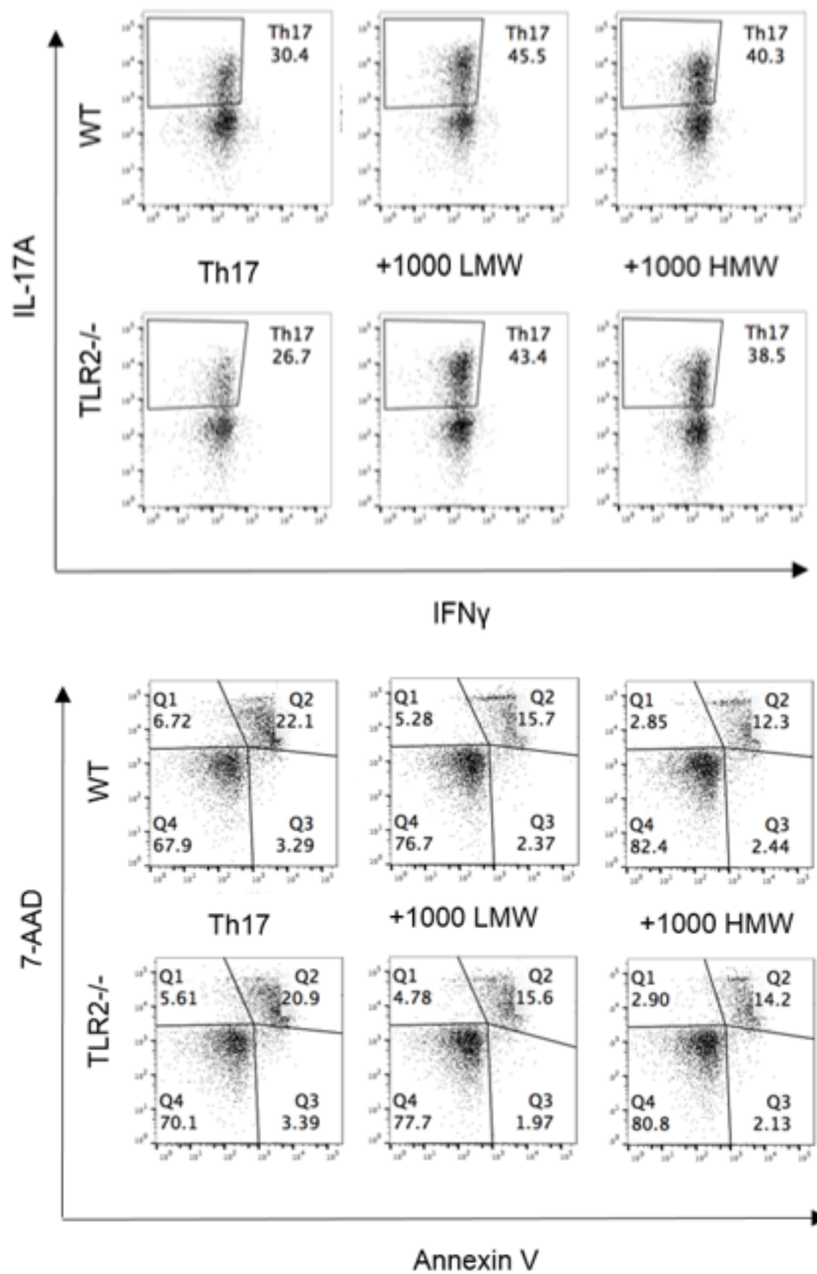


Figure 9. Flow cytometry analysis of cytokine expression and staining following the viability assay. There was a decrease in 7-AAD+AnnexinV+ cells with the hyaluronan treatment, indicating that there was a decrease in the number of dying cells, and thus an increase in the number of proliferating cells.

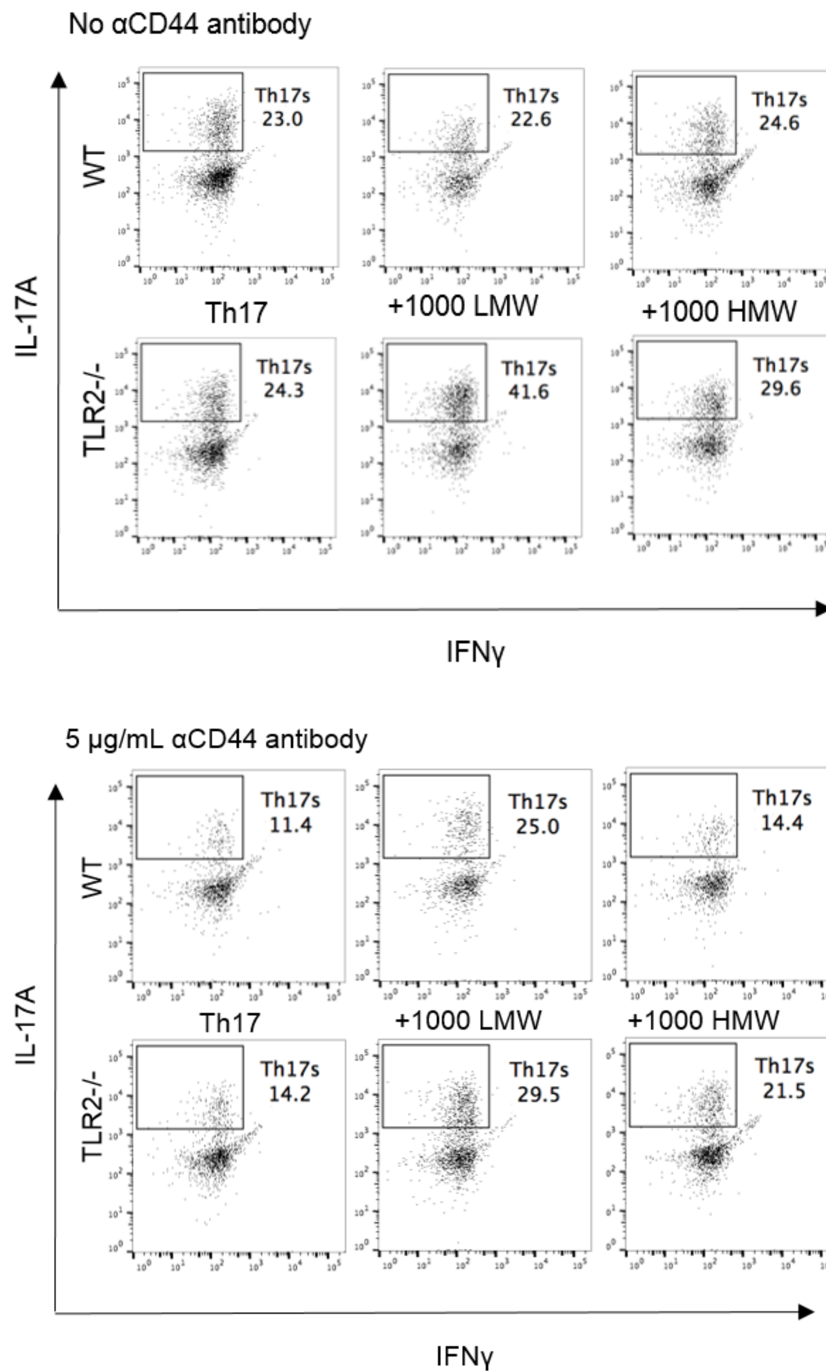


Figure 10. Flow cytometry analysis of cytokine expression following the αCD44 treatment. The untreated WT cells do not follow the previously observed trend, possibly caused by an issue with the naïve cell sorting. There was no difference in trends between the untreated and αCD44 treated TLR2-/- cells.

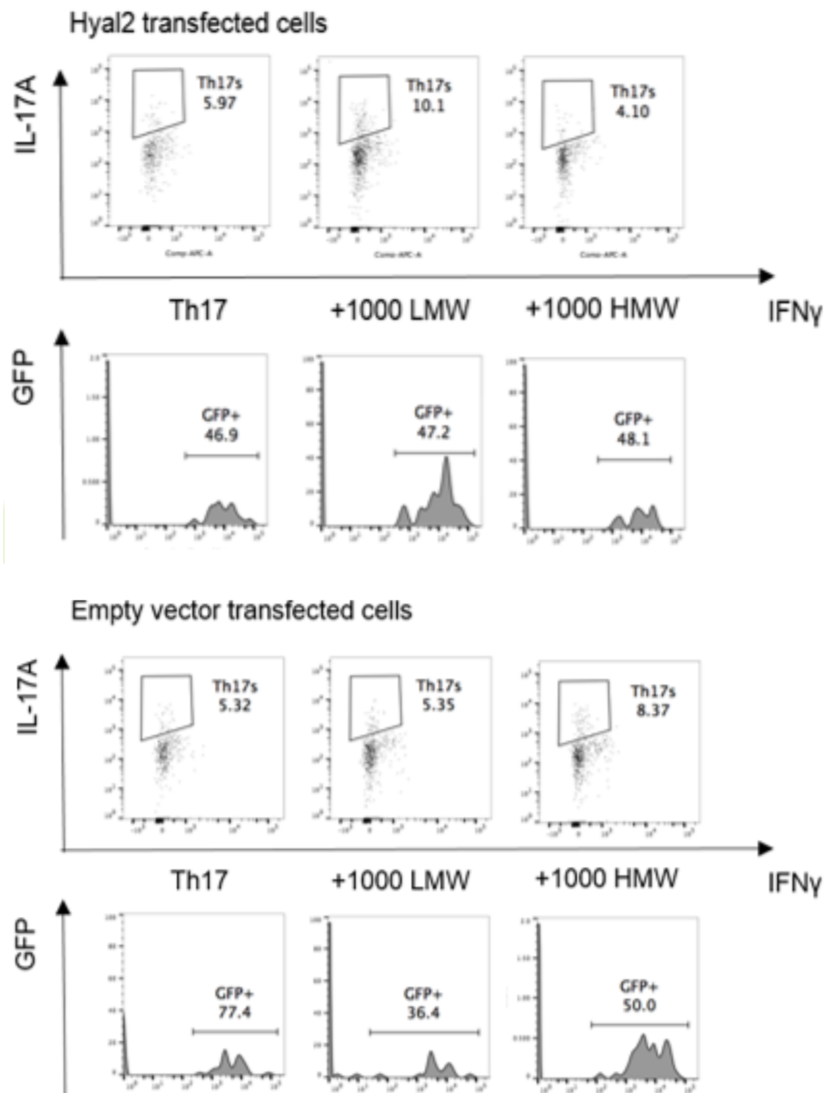


Figure 11. Flow cytometry analysis of cytokine expression following retroviral transfection. GFP expression confirmed the percentage of cells transfected. No discernable trend was observed with these results.

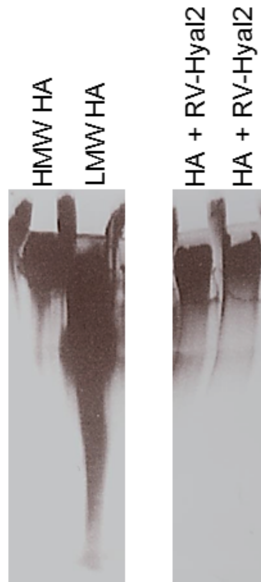


Figure 12. SDS-PAGE analysis of supernatants following retroviral transfection. Cellular supernatant from the Hyal2 overexpressing high molecular weight hyaluronan treated samples showed greater digestion of hyaluronan, as indicated by the lower molecular weight fragments.

Discussion

Our main hypothesis was that hyaluronan signals through TLR2 to upregulate Th17 differentiation. More specifically, we initially thought that low molecular weight hyaluronan, which is an indication of a wound or injury, would upregulate Th17 differentiation. However, our initial results of the differentiation assay were not consistent with this hypothesis. Surprisingly, high molecular weight hyaluronan, which is the normal state of hyaluronan in the body, also upregulated Th17 differentiation. Upon further examination, we found that the high molecular weight hyaluronan sample cellular supernatant also contained low molecular weight hyaluronan.

As such, we revised our hypothesis: Th17 cells express Hyal2 which cleaves high molecular weight hyaluronan into molecular weight hyaluronan, which then upregulates Th17 differentiation by signaling through TLR2. Our results provide evidence that supports this hypothesis.

Differentiation Assay

The flow cytometry results support our hypothesis that low molecular weight hyaluronan upregulates Th17 differentiation. However, it also shows that there was an upregulation of Th17 differentiation by high molecular weight hyaluronan. This was unexpected, as high molecular weight hyaluronan is common within the body and is not indicative of injury, as is the case with low molecular weight hyaluronan (Jiang, 2005). Furthermore, high molecular weight hyaluronan travels through the lymph system to the liver where it is then degraded (Banerji et al., 1999). Naïve T-cells travel in the lymph as well where

they likely interact with hyaluronan, however there is no evidence to suggest it upregulates Th17 differentiation in the lymph. Previous research has shown that naïve T-cells require exposure to polarizing cytokines to initiate Th17 differentiation, so signaling by hyaluronan alone is likely unable to induce differentiation (Reynolds & Dong, 2013).

The upregulation of Th17 differentiation by high molecular weight hyaluronan may be explained by examining the results from the SDS-PAGE analysis. As shown earlier, the supernatant of high molecular weight hyaluronan treated samples contained hyaluronan of differing weights ranging from the normal high weight to smaller fragments. Thus, it is possible that the Th17 cells were able to cleave the hyaluronan using a hyaluronidase. There are several hyaluronidases known to exist in the body: hyaluronidase 1, 2, and 3 (Lepperdinger et al., 1998). Previous research in oligodendrocyte progenitor (OPC) cells has revealed a similar mechanism in which hyaluronidases are secreted to cleave hyaluronan, which then signals through TLR2 (Miranda-Hernandez & Baxter, 2013). However, the researchers found that hyaluronan signaling in OPCs downregulated cell differentiation. Other research hypothesized that TLR2 signaling produces Th17 specific factors, so those same factors may downregulate OPC differentiation (Reynolds & Dong, 2013). We considered it was hyaluronidase 2 (Hyal2) specifically because we knew it was likely expressed in T-cells and it showed high specificity for high molecular weight hyaluronan (Lepperdinger et al., 1998; Jiang et al., 2011). Thus, our new hypothesis was that high molecular weight hyaluronan is cleaved by Th17

secreted Hyal2 into low molecular weight hyaluronan which then signals through TLR2 to upregulate Th17 differentiation.

If this hypothesis is correct, Th17 cells could form a positive-feedback loop: greater concentrations of low molecular weight hyaluronan would lead to greater Th17 differentiation, which in turn would increase hyaluronidase 2 expression, thereby resulting in higher concentrations of low molecular weight hyaluronan. This process would have the potential to be useful in cases of injury, or even infection in tissues containing hyaluronan by increasing a Th17 response. However, this reaction could also be harmful in cases where there is an autoimmune inflammatory response. This is similar to IL-23 signaling in Th17 cells; Il-23, expressed by dendritic cells, stimulates pathogenic Th17 differentiation (Rostami & Ciric, 2013). Pathogenic Th17 cells then express GMCSF, thereby inducing dendritic cells or other APCs to release more IL-23 and stimulate more Th17 differentiation (El-Behi et al., 2011). It is important to note that an antigen, a costimulatory molecule such as low molecular weight hyaluronan, and polarizing cytokines are needed to initiate Th17 differentiation (Jin et al., 2012). Only after Th17 cells are present will Hyal2 be secreted, and hyaluronan digestion occur. Thus, this process will not occur without some initial injury, infection, or other antigen presentation.

Further evidence to support the above explanations comes from results obtained with qPCR and ELISA. Both show some of the same trends as was seen with the flow cytometry data. Essentially we saw higher expression of IL-17A and RORγt mRNA, as well as IL-17A protein in our hyaluronan samples.

There are some differences between some of the samples, however; for example, the low concentration high molecular weight hyaluronan showed the least amount of upregulation with flow cytometry, but the highest IL-17A mRNA expression.

Some of the differences in the trends we observed could be attributable to the different forms of cytokine expression we measured. We used flow cytometry to analyze intracellular cytokines protein expression, whereas qPCR was used to measure cytokine mRNA relative expression and ELISA to measure extracellular cytokine protein concentration. Flow cytometry and qPCR are both measured on a per cell basis, while ELISA measures total protein concentration, so differences in cell proliferation across conditions could impact the ELISA results. Different factors such as mRNA transcription time and protein stability also lead to discrepancies between mRNA and protein expression trends (Vogel & Marcotte, 2013).

However, we also should consider that cells were taken for analysis when the intracellular cytokine expression was optimal, or had the largest differences between conditions, for our Th17 control. Given that we have to wait for mRNA to be translated into protein to be analyzed by flow cytometry, and then wait for the protein to be secreted into the supernatant to be analyzed by ELISA, the optimal mRNA or extracellular cytokine expression would not have been at the same time as optimal intracellular cytokine expression. Since, as discussed above, Hyal2 will not be expressed until some naïve cells have differentiated into Th17, there will be an additional time delay between the high molecular weight samples

and the control/low molecular weight samples. This could be one possible explanation for the discrepancies in results between the high molecular weight qPCR and ELISA data: the high molecular weight samples were at the point where IL-17A mRNA expression was optimal, but not protein expression.

More evidence to support this time delay phenomenon, and thus our hypothesis, can be seen with the RORγt qPCR results. As explained previously, RORγt is one of several transcription factors necessary to induce Th17 differentiation (Louten et al., 2009). The highest RORγt expression was in the high molecular weight samples, indicating that the cells were actively differentiating. In contrast, the control and low molecular samples had lower levels of expression, indicating that the bulk of their differentiation was complete.

We also saw a similar trend of Th17 differentiation upregulation in the TLR2-/- cells, as was seen in the WT, which we did not expect given our hypothesis. If hyaluronan only signaled through TLR2 for differentiation and proliferation, we would not have expected to have seen Th17 differentiation upregulation in TLR2-/- cells. Given that the removal of TLR2 resulted in a reduction of Th17 differentiation, there is evidence that hyaluronan signals through TLR2. Numerous other studies also found that hyaluronan signals through TLR2 in various other cell types (Sloane et al., 2010; Shimada et al., 2008). For example, Scheiber et al. (2006) found that low molecular weight hyaluronan signals through TLR2 to induce inflammatory cytokine production in macrophages and dendritic cells. However, the researchers also found that high molecular weight hyaluronan can inhibit TLR2 signaling. They determined this by

looking at expression of TNFα, a cytokine not produced by naïve T-cells, so it is unknown whether signaling in naïve T-cells would be affected in the same way.

It is most likely that hyaluronan signals through multiple receptors, which is why we still see some upregulation in TLR2-/- T-cells. Hyaluronan is unique, because most TLR2 ligands are bacterial lipoproteins that do not signal through multiple receptors (Parham, 2009). However, hyaluronan signals through a variety of receptors such as CD44 and LYVE-1 (Hernandez-Pedro et al., 2013). The study by Scheiber et al. (2006) also found that low molecular weight hyaluronan stimulation on dendritic cells was CD44 independent; that may or may not be true in T-cells as well. Th17 cells do express CD44, so that is the most likely receptor for hyaluronan to signal through in addition to TLR2.

We did not see a difference in the flow cytometry results between low and high molecular weight hyaluronan conditions in the TLR2-/- cells. This may indicate that the other receptor, CD44, binds to both low and high molecular weight hyaluronan and promotes upregulation, and so the Hyal2 does not play a significant role here like it does with TLR2. However, the qPCR data show substantial differences between the high concentration high and low molecular weight conditions. Thus, the time delay discussed above may still have been playing a role, offering evidence that Hyal2 is involved with this process. Furthermore, it has been discussed previously that while both molecular weights may bind CD44, there is a difference in avidity as well as other factors that may alter downstream signaling. Cyphert et al. (2015) review the topic, noting that CD44-hyaluronan binding in particular is dependent on the ability of CD44

receptors to cluster, which is in turn dependent on hyaluronan size, cell activation, and cell type. As interesting as this is, the effects of hyaluronan signaling through CD44 to upregulate Th17 differentiation were outside the scope of this study. To exclude the effects of CD44 signaling, and determine the role of TLR2 signaling alone, we used the neutralizing αCD44 antibody treatment (discussed later).

Overall, figure 13 shows the revised hypothesis for hyaluronan signaling in Th17 cells, including the role of Hyal2 and CD44. In summary, low molecular weight hyaluronan signals through both CD44 and TLR2 to upregulate Th17 differentiation. The mature Th17 cell produces Hyal2, which digests high molecular weight hyaluronan to low molecular weights, which then cycle back to upregulate Th17 differentiation again.

Viability Assay

The viability assay supports the assertion that hyaluronan signaling upregulated Th17 differentiation and proliferation. Interestingly, the highest number of living cells was in the high molecular weight hyaluronan samples, and not in the low molecular weight samples which had the highest differentiation. This may also be explained by the time delay we observed above: if the high molecular weight samples were delayed, there would be greater differentiation at the time at which the cells were taken for analysis, and actively differentiating cells would be less likely to die.

However, this assay does not provide evidence for the role of TLR2 in hyaluronan signaling. WT and TLR2-/- conditions showed nearly identical

percentages of Th17 cells and apoptotic (7-AAD+AnnexinV+) cells. One possible explanation is that this assay measured the number of dying cells, and the number of proliferating cells and dying cells might not be proportionate in this system. The proliferating cells may be proliferating at different rates in the different samples, given that we have a time delay between our high and low molecular weight hyaluronan samples. To determine if this is the case, we could use a proliferation assay and compare to the viability assay results. Other studies, such as the one reported by Liu et al. (2012), used a tritium proliferation assay to measure cell proliferation directly. In this assay, tritium atoms are incorporated into DNA of developing cells, and the end cell DNA radioactivity is measured to determine the number of cells divisions, which could show differences between WT and TLR2-/- groups. Alternatively, we had not excluded CD44 signaling, which could also be driving cell proliferation (Baaten et al., 2010). Either way, the results of this assay were consistent with the results of the differentiation assay.

Anti-CD44 Treatment

As discussed above, the differentiation assay shows evidence of hyaluronan signaling in the TLR2-/- cells, likely through CD44. To determine if the upregulation of Th17 differentiation was caused by hyaluronan signaling through TLR2, we used αCD44 antibodies to block all CD44 signaling which could be producing a similar effect. Although the αCD44 treated cells experienced heavy cell death, they still displayed an upregulation of Th17 differentiation with hyaluronan. It is most likely that the concentration of αCD44

antibody was insufficient to completely block CD44 signaling, but an increase in the antibody concentration may have been toxic to the cells. This is not entirely surprising; CD44 signaling is not essential for the survival of Th17 cells, but it is known to participate in the control of cell expansion (Baaten et al., 2010). Also, sodium azide or other chemicals in the antibody solution could have damaged the cells. To the best of our knowledge, no other studies attempted to use this antibody on living cells, so it cannot be determined if this is an issue with the antibody or with the lack of CD44 signaling.

Furthermore, it appears that the WT cells used for this experiment were compromised, as indicated by our WT no antibody conditions which did not follow previously observed trends. Multiple experiments conducted with the same pool of mice at the same time showed irregular results and increased cell death. We believe there may have been health issues with the WT mice we used. We have not confirmed any health issues with our original mice, but because switching to commercially available WT mice corrected the irregularities in other experiments, we are reasonably certain this was the problem. However, the TLR2-/- mice did not appear to have the same health issues, and thus the TLR2-/- data were valid. This may also be why there was less cell death in the TLR2-/- antibody treated conditions; the WT cells were already unhealthy and so the antibody stressed them more and increased cell death. However, there was still significant cell death in the TLR2-/- cells, indicating that the antibody concentration was indeed too high.

As it stands presently, we cannot provide a definitive role for TLR2 signaling in hyaluronan mediated Th17 differentiation upregulation. We need to repeat this experiment with a higher antibody concentration and healthier cells. If, as we suspect, these conditions are still too stressful, we would need to then find another route to block CD44 signaling. One possibility would be crossing TLR2-/- mice with CD44 knockout mice, which do not express CD44, and isolate naïve cells. These cells would not possess CD44 or TLR2, and thus could be compared to CD44KO cells in a differentiation assay to examine the effect of hyaluronan signaling through TLR2 exclusively.

Retroviral Transfection

Based on our hypothesis, Hyal2 digests high molecular weight hyaluronan into lower molecular weight fragments, which then signal through TLR2 to upregulate Th17 differentiation. If we overexpress Hyal2, as we did in our retroviral transfection, we would then expect to see increased Th17 differentiation compared to control, non-overexpressed samples. Despite the successful transfection, indicated by the high number of GFP+ cells, most of the cells died. As such, the cells were unable to produce significant amounts of Hyal2 and proliferate before dying. Consequently, there were no observable trends. The SDS-PAGE analysis showed no more digestion in the retroviral transfection than it did in the initial SDS-PAGE analysis of the differentiation assay, confirming that most of the cells died before greater amounts of Hyal2 protein could be produced.

Presumably, the high cell death was caused by the stressful conditions of the retroviral transfection: to determine the role of Hyal2, we would need to alter the conditions of the transfection such that the cells would be able to survive and proliferate. The control transfection, using the empty vector, shows the same high cell death, indicating this is not a result of the upregulation of Hyal2. This is difficult, because there are toxic compounds such as polybrene, and also mechanical stresses during centrifugation that are essential to the transfection that may injure the cells. One factor we could change would be to allow for more time between naïve T-cell plating and transfection; if the cells are not activated before exposure to the virus, they would be much more likely to die (Berggren, 2012).

Should we be unable to adjust the retroviral transfection sufficiently to enable cell survival, we could add additional Hyal2 directly to the cells. However, there is no murine Hyal2 commercially available, so the enzyme may have unforeseen interactions in murine cells. Alternatively, if we find that the overexpression of Hyal2, and not the transfection itself, was causing cell death, we could try using another vector to suppress Hyal2 expression. This vector would encode short hairpin RNA (shRNA) that would attach to the Hyal2 gene and prevent translation of mRNA. Then, we could examine whether the upregulation of Th17 differentiation by high molecular weight hyaluronan is dispelled when Hyal2 is no longer present.

Future Studies

As discussed previously, one clinical aspect of this research is to identify a self-molecule that could aggravate the sterile inflammation encountered in autoimmune diseases such as MS. Previous research has shown that TLR2 ligands such as peptidoglycan can upregulate Th17 differentiation, and Th17 cells are heavily implicated in MS (Reynolds et al., 2010). Hyaluronan is unique because it is an abundant self-molecule. Furthermore, if the positive feedback loop we speculated about is possible, then hyaluronan would be more potent than other TLR2 ligands because it is self-replenishing. The results of this study provide preliminary evidence for this role of hyaluronan, but require greater work to confirm the trends are significant. We need to repeat the unreliable assays with the suggested revisions to verify the results, as well as confirm the presence of Hyal2; the retroviral transfection would provide evidence for its presence. To explicitly show Hyal2's presence, we could perform a western blot on sample supernatant; this procedure would allow us to visualize specific proteins such as Hyal2, allowing us to confirm its presence. Assuming we verify everything and our results are as expected, we should continue our work by confirming the same trends hold true with *in vivo* experiments.

One avenue of future research could be to confirm if hyaluronan will upregulate Th17 differentiation in MS specifically. To do this, we could use hyaluronan in a murine model of MS, EAE. In an EAE experiment, mice are injected with myelin oligodendrocyte glycoprotein (MOG), which is a component of the nerve sheath, to foster the development of MOG reactive T-cells (Reynolds

et al., 2010). The CD4+ T-cells are then removed from these mice, allowed to differentiate and proliferate, then injected into Rag1 mice, which lack any T-cells. These Rag1 mice then develop EAE, which is scored by the extent of paralysis. To determine the effect of hyaluronan, we could incubate the CD4+ cells with hyaluronan before injection into the Rag1 mice. We would expect to see higher Th17 differentiation and proliferation, which would lead to greater disease progression as measured by a higher clinical score, in the hyaluronan treated samples.

More interestingly, we could examine the role of Hyal2 to determine whether the digestion of hyaluronan is physiologically relevant. To do this, we would essentially repeat the EAE experiment detailed above, but instead of incubating the cells with hyaluronan during expansion, we would conduct a retroviral transfection with the Hyal2 vector, then add hyaluronan before injecting into Rag1 mice. This assumes that we will be able to revise the retroviral transfection conditions sufficiently to allow the cells to survive, but that is likely to be possible. The results of this experiment would help determine if Hyal2 does play a role in MS development; for the *in vitro* experiments we conducted, we had comparatively high hyaluronan levels and no known hyaluronidase inhibitors, which may not mimic conditions in the body.

Once we have confirmed that we still can observe these effects *in vivo*, we could then examine the mechanism behind TLR2 signaling that leads to Th17 differentiation. We have conducted some research on this topic, focusing on the role of runt-related transcription factor 3 and chromodomain-helicase-DNA-

binding protein 7 (ChD7) in the process. Once the mechanism is fully elucidated, clinical research can begin looking for target molecules to inhibit so as to stop MS development and progression. Finally, research has shown a role for TLR2 activation in other Th17 driven autoimmune disorders, such as rheumatoid arthritis, so we could also examine if hyaluronan plays a role there as well (McGarry et al., 2015).

Conclusion

Overall our study was conducted to show that low molecular weight hyaluronan signals through TLR2 to upregulate Th17 differentiation and proliferation. After reviewing the differentiation assay results, we adjusted our hypothesis to include the role of Hyal2, which digests high molecular weight hyaluronan into a lower molecular weight. Figure 13 shows our adjusted hypothesis containing roles for both Hyal2 and CD44 in hyaluronan signaling for Th17 differentiation. Our results provide evidence for the role of hyaluronan and TLR2, but more experimental work must be conducted to confirm the role of Hyal2. Both low and high molecular weight hyaluronan were shown to upregulate Th17 differentiation, and the removal of TLR2 impaired Th17 differentiation slightly, indicating TLR2 is but one of multiple receptors that hyaluronan signals through. However, experimental difficulties prevented us from obtaining sufficient evidence for the role of Hyal2. This work illuminates a possible self-molecule that can aggravate sterile inflammation in autoimmune diseases such as MS.

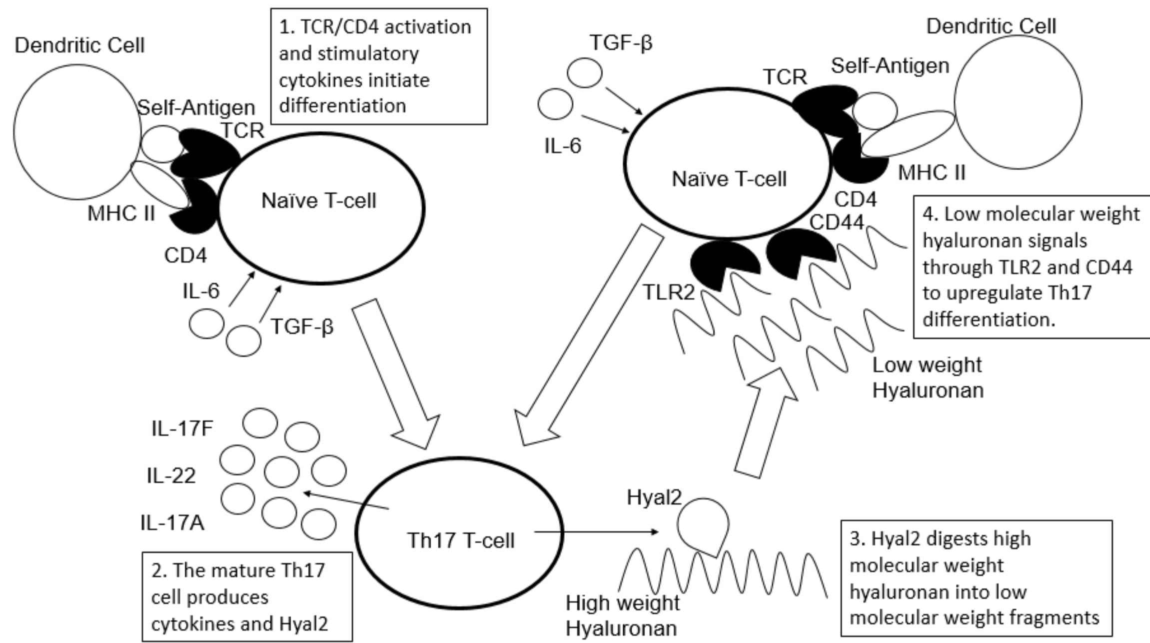


Figure 13. A diagram of the revised hypothesis. 1. A self-antigen-MHC II complex signals through the TCR and CD4, and stimulatory cytokines activate the naïve T-cell. 2. The naïve T-cell differentiates into a mature Th17, which releases pro-inflammatory cytokines and Hyal2. 3. Hyal2 digests high molecular weight hyaluronan in the supernatant. 4. The low molecular weight hyaluronan signals through TLR2 and CD44 on an activated naïve T-cell to upregulate Th17 differentiation, forming a positive feedback loop.

Literature Cited

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2015). *Molecular Biology of the Cell*. New York, NY: Garland Science.

Baaten, B.J.G., Li, C.R., Deiro, M.F., Lin, M.M., Linton, P.J., & Bradley, L.M. 2010. CD44 regulates survival and memory development in Th1 cells. *Immunity, 32*. 104-115.

Back, S.A., Tuohy, T.M.F., Chen, H., Wallingford, N., Craig, A., Struve, J., Luo, N.L., Banine, G., Liu, Y., Chang, A., Trapp, B.D., Bebo, B.F., Rao, M.S., Sherman, L.S. 2005. Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. *Nature Medicine, 11* (9). 966-972.

Banerji, S., Ni, J., Wang, S.X., Clasper, S., Su, J., Tammi, R., Jones, M., & Jackson, D.G. 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *Journal of Cell Biology, 144* (4), 789-801.

Berggren, T., Lutz, M., Modesto, V. (2012). *General Spinfection Protocol*. Retrieved from NIH website: https://commonfund.nih.gov/sites/default/files/Salk_GeneralSpinfection_ESC_iPSC.pdf

Bhaskaran, N., Cohen, S., Zhang, Y., Weinberg, A., & Pandiyan, P. 2015. TLR-2 signaling promotes IL-17A production in CD4+CD25+Foxp3+ regulatory cells during oropharyngeal candidiasis. *Pathogens, 4,* 90-110.

Burkett, P.R., Horste, G.M., & Kuchroo, V.K. 2015. Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. *Journal of Clinical Investigation, 125* (6), 2211-2219.

Carpenter, A.C., & Bosselut, R. 2010. Decision checkpoints in the thymus. *Nature Immunology, 11* (8), 666-674.

Chawla, A. 2010. Control of Macrophage Activation and Function by PPARs. *Circulation Research, 106* (10), 1559-1569.

Cyphert, J.M., Trempus, C.S., & Garantziotis, S. 2015. Size matters: Molecular weight specificity of hyaluronan effects in cell biology. *International Journal of Cell Biology, 2015,* 1-8.

El-Behi, M., Ciric, B., Dai, H., Yan, Y., Cullimore, M., Safavi, F., Zhang, G.X., Dittel, B.N., & Rostami, A. 2011. The encephalitogenicity of Th17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nature Immunology, 12* (6), 568-576.

Esser, P.R., Wolfle, U., Durr, C., von Loewenich, F.D., Schempp, C.M., Freudenberg, M.A., Jakob, T., & Martin, S.F. 2012. Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS One, 7* (7), 1-16.

Gi, M., Im, W., & Hong, S. 2009. Dendritic Cells as Danger-Recognizing Biosensors. *Sensors, 9* (9), 6730-6751.

Gomez-Artistizabal, A., Kim, K.P., & Viswanathan, S. 2016. A systematic study of the effect of different molecular weights of hyaluronic acid on

mesenchymal stromal cell-mediated immunomodulation. *PLoS One, 11,* (1). 1-18.

Goverman, J. 2009. Autoimmune T cell responses in the central nervous system. *Nature Reviews Immunology, 9* (6), 1-28.

He, C., Lai, P., Wang, J., Zhou, T., Huang, Z., Zhau, L., & Liu, X. 2016. TLR2/4 deficiency prevents oxygen-induced vascular degeneration and promotes revascularization by downregulating IL-17 in the retina. *Scientific Reports, 6,* 1-12.

Hernandez-Pedro, N.Y., Espinosa-Ramirez, G., de la Cruz, V.P., Pineda, B., & Sotelo, J. 2013. Initial immunopathogenesis of multiple sclerosis: innate immune response. *Clinical and Developmental Immunology, 2013,* 1-15.

Humphrey, J.H. 1943. Antigenic properties of hyaluronic acid. *Biochemistry Journal, 37* (4), 460-463.

Jadidi-Niaragh, F., & Mirshafiey, A. 2011. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. *Scandinavian Journal of Immunology, 74,* 1-13.

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., Prestwich, G.D., Mascarenhas, M.M., Garg, H.G., Quinn, D.A., Homer, R.J., Goldstein, D.R., Bucala, R., Lee, P.J., Medzhitov, R., & Noble, P.W. 2005. Regulation of lung injury and repair by toll-like receptors and hyaluronan. *Nature Medicine, 11* (11), 1173-1179.

Jiang, D., Liang, J., & Noble, P.W. 2011. Hyaluronan as an immune regulator in human diseases. *Physiology Review, 91*, 221-264.

Jin, B., Sun, T., Yu, X.H., Yang, Y.X., & Yeo, A.E.T. 2012. The effects of TLR activation on T-cell development and differentiation. *Clinical and Developmental Immunology, 2012*, 1-32.

Kuipers, H.F., Rieck, M., Gurevich, I., Nagy, N., Butte, M.J., Negrin, R.S., Wight, T.N., Steinman, L., Bollyky, P.L. 2015. Hyaluronan synthesis is necessary for autoreactive T-cell trafficking, activation, and Th1 polarization. *Proceedings of the National Academy of Sciences, 113* (5), 1339-1344.

Laurent, T.C. & Fraser, J.R.E. 1992. Hyaluronan. *Federation of American Societies for Experimental Biology Journal, 6* (7), 2397-2404.

Lee, J.H., Cho, M.L., Kim, J.I., Moon, Y.M., Oh, H.J., Kim, G.T., Ryu, S., Baek, S.H., Lee, S.H., Kim, H.Y., & Kim, S.I. 2009. Interleukin 17 (IL-17) increases the expression of toll-like receptor-2, 4, and 9 by increasing IL-1 β and IL-6 production in autoimmune arthritis. *Journal of Rheumatology, 36* (4), 684-692.

Lee-Sayer, S.S.M., Dong, Y., Arif, A.A., Olsson, M., Brown, K.L., & Johnson, P. 2015. The where, when, how, and why of hyaluronan binding by immune cells*. Frontiers in Immunology, 6,* 1-12.

Lepperdinger, G., Strobl, B., & Kreil, G. 1998. HYAL2, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. *Journal of Biological Chemistry, 273* (35), 22466-22470.

Liu, J.Q., Liu, Z., Zhang, X., Shi, Y., Talebian, F., Carl Jr., J.W., Yu, C., Shi, F.D., Whitacre, C.C., Trgovcich, J., & Bai, X.F. 2012. Increased Th17 and regulatory T cell responses in EBV-induced gene 3-deficient mice lead to marginally enhanced development of autoimmune encephalomyelitis. *Journal of Immunology, 188* (7), 3099-3106.

Louten, J., Boniface, K., & de Waal Malefyt R. 2009. Development and function of th17 cells in health and disease. *Journal of Allergy and Clinical Immunology, 123* (5), 1004-1011.

Lubberts, E. 2015. The IL-23-IL-17 axis in inflammatory arthritis*. Nature Reviews Rheumatology, 11,* 415-429.

McGarry, T., Veale, D.J., Gao, W., Orr, C., Fearon, U., & Connolly, M. 2015. Toll-like receptor 2 (TLR2) induces migration and invasive mechanisms in rheumatoid arthritis. *Arthritis Research & Therapy, 17,* 1-14.

Miranda-Hernandez, S., & Baxter, A.G. 2013. Role of toll-like receptors in multiple sclerosis. *American Journal of Clinical Experimental Immunology, 2* (1), 75-93.

Nishimori, J.H., Newman, T.N., Oppong, G.O., Rapsinski, G.J., Yen, J.H., Biesecker, S.G., Wilson, R.P., Butler, B.P., Winter, M.G., Tsolis, R.M., Ganea, D., & Tukel, C. 2012. Microbial amyloids induce interleukin 17A (IL-17A) and IL-22 responses via toll-like receptor 2 activation in the intestinal mucosa. *Infection and Immunity, 80* (12), 4398-4408.

Nyirenda, M.H., Morandi, E., Vinkemeier, U., Constantin-Teodosiu, D., Drinkwater, S., Mee, M., King, L., Podda, G., Zhang, G.X., Ghaemmaghami, A., Constantinescu, C.S., Bar-Or, A., & Gran, B. 2015. TLR2 stimulation regulates the balance between regulatory T cell and Th17 function: a novel mechanism of reduced regulatory T cell function in multiple sclerosis. *Journal of Immunology, 194* (12), 5761-5774.

Nyirenda, M.H., Sanvito, L., Darlington, P.J., O'Brien, K., Zhang, G.X., Constantinescu, C.S., Bar-Or, A., & Gran, B. 2011. TLR2 stimulation drives human naïve and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function. *Journal of Immunology, 187* (5), 2278-2200.

Patel, D.D., & Kuchroo, V.K. 2015. Th17 cell pathway in human immunity: lessons from genetics and therapeutic interventions. *Immunity, 43*, 1040-1051.

Parham, P. (2009). *The Immune System*. New York, NY: Garland Science.

Reynolds, J.M., & Dong, C. 2013. Toll-like receptor regulation of effector T lymphocyte function. *Trends in Immunology, 2013,* 1-8.

Reynolds, J.M., Pappu, B.P., Peng, J., Martinez, G.J., Zhang, Y., Chung, Y., Ma, L., Yang, X.O., Nurieva, R.I., Tian, Q., & Dong, C. 2010. Toll-like receptor 2 signaling in CD4+ T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity, 32,* 692-702.

Romagnani, S., Maggi, E., Liotta, F., Cosmi, L., & Annunziato, F. 2009. Properties and origin of human Th17 cells. *Molecular Immunology, 47,* 3-7.

Rostami, A., & Ciric, B. 2013. Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. *Journal of the Neurological Sciences, 333* (1-2), 76-87.

Russi, A.E., & Brown, M.A. 2015. The Meninges: New therapeutic targets for multiple sclerosis. *Translational Research, 165* (2), 255-269.

Scheibner, K.A., Lutz, M.A., Boodoo, S., Fenton, M.J., Powell, J.D., & Horton, M.R. 2006. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *Journal of Immunology, 177,* 1272-1281.

Shah, D.K., & Zuniga-Pflucker, J.C. 2014. An overview of the intrathymic intricacies of T cell development. *Journal of Immunology, 192,* 4017-4023.

Shimada, M., Yanai, Y., Okazaki, T., Noma, N., Kawashima, I., Mori, T., & Richards, J.S. 2008. Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. *Development, 135,* 2001-2011.

Sigma-Aldrich. Hyaluronan. Retrieved from http://www.sigmaaldrich.com/ technical-documents/articles/biology /glycobiology/glycosaminoglycans-and-proteoglycans.html

Sloane, J.A., Batt, C., Ma, Y., Harris, Z.M., Trapp, B., & Vartanian, T. 2010. Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. *Proceedings of the National Academy of Science, 107* (25), 11555-11560.

Spinelli, F.M., Vitale, D.L., Demarchi, G., Cristina, C., & Alaniz, L. 2015. The immunological effect of hyaluronan in tumor angiogenesis. *Clinical & Translational Immunology, 4,* 1-9.

Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., & Lotze, M.T. 2012. PAMPs and DAMPs: Signal 0s that spur autophagy and immunity. *Immunology Review, 249* (1), 158-175.

Vogel, C., & Marcotte, E.M. 2013. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics, 13* (4), 227-232.

Zhao, R.R., Yang, X.F., Dong, J., Zhao, Y.Y., Wei, X., Huang, C.X., Liah, J.Q., & Zhang, Y. 2015. Toll-like receptor 2 promotes T helper 17 cells response in hepatitis B virus infection. *International Journal of Clinical Experimental Medicine, 8* (5), 7315-7323.