Identification and Analysis of Omentum Derived Suppressor Cells in Regards to Th17 Inhibition

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LOYOLA UNIVERSITY CHICAGO

IDENTIFICATION AND ANALYSIS OF OMENTUM DERIVED SUPPRESSOR CELLS IN REGARDS TO TH17 INHIBITION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
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ABSTRACT

Omentum has been harnessed by surgeons for hundreds of years, providing an ideal environment for graft tissue regeneration and acceptance. However, little is known about the cellular mechanisms that promote the tolergenic environment of the omentum. We examined the cellular composition and role of activated omentum in regards to T-cell immunomodulation. We then tested activated omentum as a cellular therapy in a mouse allogenic lung transplantation model.

Our findings demonstrated activated omentum is mostly comprised of non-hematopoietic cells resembling mesenchymal stem-like cells (MSCs) and myeloid derived suppressor cells (MDSCs). Activated omentum exhibited anti-inflammatory properties through suppression of Th1 and Th17, while promoting tolerance through expansion and survival of Tregs. The mechanism of Th17 inhibition relied on IFNγ-mediated upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), leading to generation of nitric oxide (NO) and prostaglandin E2 (PGE2). These mediators inhibited differentiated Th17 cells in vitro, resulting in cell loss, and blockade of cytokine production.

Omentum also caused Th17 cells to become anergic, and unable to flux calcium under TCR-activating conditions. T-cell size and endoplasmic reticulum (ER) were found to be expanded after co-culture suggesting ER stress may be induced by omentum.
Th17 is associated with the development of bronchiolitis obliterans following lung transplantation, a syndrome involving chronic inflammation of airways and development of mucus plugs. We tested the role of omental cells as cellular therapy in our lung transplant model, under the hypothesis these cells could promote a tolerogenic response. Transplanted mice treated with omental cells by intraperitoneal administration had reduced airway inflammation. Using a T-cell imaging mouse model, we also observed qualitative decrease in T-cell infiltration within the lung following omental injection. Further studies will be needed to determine administration and dose for optimal therapeutic outcomes.

In conclusion, our lab has demonstrated NO and PGE2 release by activated omentum promoted an anti-inflammatory environment. Furthermore, we are the first lab to test activated omentum as cellular therapy in a rigorous transplant model with preliminary success. The findings of these results suggest that future research should focus on the development of an activated omentum model in human studies, and its experimental use in other inflammatory diseases.
CHAPTER ONE
AN OVERVIEW OF THE OMENTUM

Described as “the policeman of the abdomen” by the British surgeon, Rutherford Morrison in the 20th century, the omentum has had a long history in clinical medicine, but a short one in the basic sciences (Alagumuthu et al., 2006). This chapter provides a detailed overview of the omentum; describing the anatomy, function, clinical uses and cellular composition of omental tissue.

Anatomy of the Omentum

Anatomically, the omentum is comprised of folds of visceral peritoneum that divide into two parts. The greater omentum starts from the greater curvature of the stomach, stretching over the intestines down to the pelvis. A lesser omentum starts from the lesser curvature of the stomach, bordering the liver. It is comprised of two mesothelial layers, with connective tissue in the middle consisting of adipocytes, fibroblasts, endothelium, and Milky spots that contain immune cells.

Function of the Omentum

Speculation over the function of the omentum has been ongoing since the 19th century, when early anatomists described the tissue as a “protector of the intestines against chill”. While the omentum does contain a significant source of visceral fat, this
description has long been discarded. In 1908, a surgeon, Gordan K. Dickinson summarized the omentum to possess four basic functions: circulation, absorption, cohesive and adhesive properties, and a protective role (Dickinson G. K., 1908). Dickinson’s conjectures proved to be mostly correct, as these attributes, particularly the function of the omentum playing a protective role has become the basis of many studies over the 20th century.

Starting with his first function, circulation, the omentum has been shown to promote angiogenesis in many clinical contexts. It is now well known that omentum is potent producer of vascular endothelial growth factor (VEGF), a promoter of neovascularization (Litbarg et al., 2007). This property has been exploited by many surgeons and will be reviewed in the next section.

One function the omentum does not play as much of a role in is absorption. It was originally thought to be an access point to the lymphatic system and function in removal of toxins (Dickinson G. K., 1908). A study by Batchelder and colleagues in 1931, examined the ability for omentum in dog to absorb particulate matter. They concluded that matter was most likely absorbed by the capillaries to the liver, downplaying the role of lymphatics. The role of omentum in absorption is likely due to its high vascularity and has otherwise not received much attention.

One key property that Dickinson described for the omentum was the ability to form a cohesive barrier against infection. Multiple studies have demonstrated that wrapping infected aortic graft with omentum “walled off” the infection (Jamieson et al., 2012, Oderich et al., 2011). These studies highlight the ability of omentum to rapidly induce cellular proliferation and form a physical barrier. In addition, the omentum plays
a role in innate immunity, by production of cytokines and anti-microbial peptides from adipocytes (Chandra et al., 2011).

In regards to its protective function, there has been much speculation on if omentum plays an important role in protecting the peritoneum against infection. Early observations suggested that removal of the omentum (omentectomy) made the subject more prone to peritonitis (Dickinson G.K., 1908). However, whether the omentum has a positive or negative role has not always been straightforward, as some surgeons speculated omentectomy in conjunction with gastrostomy reduced post-operative symptoms and allowed for a better recovery (Kirschner et al., 1954). A study in which omentectomy was performed in rats undergoing experimental peritonitis, supported a role for protection as rats with intact omentum had lower rates of culture positivity (Uzunköy et al., 2009). However, even with current studies, controversy remains, as other studies testing omentectomy in an E. Coli-model of peritonitis showed that the animals were still protected even without their omentum. This suggested that peritoneal macrophages could respond to the pathogen in a compensatory mechanism and that the omentum was not an essential tissue for peritoneal immune responses (Agca et al., 2003). However, these studies importantly do not exclude the omentum in having any role, as they most certainly contain many immune cells that can be involved depending on the context of the infection.

Overall, the omentum is a multifunctional organ that while unessential for life, appears to play varying roles in promoting regeneration and immunity. It importantly acts as a stimulator of angiogenesis, and itself possesses many capillaries. These qualities have been harnessed by clinicians for centuries in medicine.
Clinical Uses for Omentum

During the 1960s, increasing numbers of reports regarding omental use during surgery were published. Over the course of several decades, omentum was widely championed by Dr. Harry S. Goldsmith for use in numerous surgeries from peripheral vascular insufficiency, chronic lymphedema, spinal cord injury to Alzheimer’s disease. Importantly, Goldsmith laid groundwork for the technique, omental transposition, a surgical method of elongating omentum from the peritoneum to form a pedicle, and then transpositioning it to sites of injury so that the healing property of the tissue could be harnessed. An early paper by Goldsmith utilizing a spinal injury cord model in cats, showed that omental transposition gave a 50% chance of some nerve function recovery, compared to untreated, which had 0% recovery. In a follow up paper by Goldsmith in 1986, omental transposition was performed on 4 tetraplegic patients with 3 of 4 having some nerve recovery and alleviation of symptoms (Goldsmith et al.). Unfortunately, those early studies were bereft with controversy, as follow-up studies had either difficulty achieving success or found only limited success (Baskov et al., 1998). Omental transposition was also unfortunately associated with complications and morbidity (Sgouros et al., 1996, Duffill et al., 2001). As a result, omental transposition’s use for spinal cord injury has been limited, and these findings highlight the need for basic science studies of the omentum. Currently a few studies utilizing omentum for nerve transection in animal models have been published with mixed success. One study showed that omental transposition has only an equivalent effect in a peripheral nerve
repair model compared to collagen use (Cemil et al., 2009). In a model of rat sciatic nerve transection, however, omentum was used as an adjuvant with nerve grafts, with increased improvement in the group receiving omental adjuvant (Mohammadi et al., 2012). These recent studies suggest omentum may possess nerve regenerating properties. However, the authors continue to highlight the need for an in-depth examination as the mechanism of action remains unknown.

Despite the limited success of omentum transposition on spinal cord injury patients, it has found success for a variety of clinical uses. Many of these studies take advantage of the omentum’s cohesive and adhesive properties and its ability to promote angiogenesis. For example, omental transposition does provide an alternative option for patients with Moyamoya disease, a disease in which arteries in the brain are constricted, through promotion of neovascularization to prevent ischemia (Navarro et al., 2014). Omentum transposition has found use in breast reconstructive surgery with success (Romanini et al., 2013, Costa et al., 2010). Omental flaps can be utilized to cover deep sternal wounds and deep perforations (Vyas et al., 2013, Husain et al., 2011, Stump et al. 2010, Van Wingerden et al., 2010). For these purposes, omentum has proven superior to muscle flaps (Van Wingerden et al., 2011). Omental wrap on the site of esophagogastric stapled anastomosis following gastric cancer removal resulted in fewer complications (Dai et al., 2011). Many studies are still ongoing but ultimately support the omentum’s ability to promote angiogenesis and healing.

Omental wrapping surgeries have proven unnecessary in some instances. In the case of pancreatic-duodenectomy, omentum wrapping did not decrease complications following the surgery (Tani et al., 2012).
Overall, omentum has had numerous successes in the clinical field, and recent studies have found it of particular help in surgeries requiring bulk tissue to facilitate wound healing. A few studies have indicated non-use in specific clinical instances, but the wide variety of contexts that omentum does provide benefit in, warrants further investigation.

**Omentum can be used to Promote Graft Survival**

One unique use of omentum is that it has been used as a site to for graft implantation. The earliest published study was from 1948, where embedding skin grafts within the greater omentum was thought to provide a vascular environment that promoted healing and protected from infection (McCorriston et al., 1948).

Since then, studies have looked into utilization of the omentum as a site for graft implantation. Due to proximity of the pancreas, several studies have looked into whether islet graft implantation can be performed in the omentum. The clinical significance of these studies was to determine if the omentum could be used therapeutically to help patients with diabetes. When comparing the omentum to the kidney or liver as a site for islet graft implantation, the omentum was determined to be a superior site for viability (Bartholomeus et al., 2013, Jacobs-Tulleneers-Thevissen et al., 2010). Artificial devices fared equally well. Implantation of insulin delivering devices into the omentum was found to be capable of reversing diabetes in a rat model (Kriz et al., 2012)

Graft models with omental transposition have been successful, where a procedure introducing another tissue appears to heal better when omentum is placed around it. For example, implanted induced-pluripotent stem cell-derived cardiomyocytes given with
omental tranposition in a pig model, had better angiogenesis and cell survival than without (Kawamura et al., 2013). These same benefits were achieved by combining omental transposition with skeletal myoblasts sheets in a porcine myocardial infarct model with increased angiogenesis (Shudo et al., 2011). Similar findings were also achieved when myocardial cell sheets were given with omental wrap in a rat myocardial infarct model (Suzuki et al., 2009). The mechanism behind better graft uptake is generally thought to be due to increased blood supply induced by the omentum. This property has been solely harnessed to prevascularize cardiac tissue prior to transplantation in a rat infarct model (Dvir et al., 2011).

How does the omentum improve regeneration and healing? The omentum can either directly contribute progenitor cells, or stimulate regeneration. In a blood vessel injury model, omentum was shown to directly contribute cells to revascularization (Shelton et al., 2013). Alternatively, fusion of omental tissue to a liver-injury model, demonstrated they promoted generation of liver-stem cells (Pancholi et al., 2010). It is also thought that omentum can produce factors key to promote cellular remodeling. In a study by De Siena and colleagues in 2010, human omentum was expanded and the stromal cells were given as cell therapy in a porcine myocardial infarction model. They detected the production of broad angiogenic promoting factors including VEGF, hepatocyte growth factor (HGF), IL-6 and IL-8, as well as immunomodulatory cytokines including IL-10 and TGFβ.

**Cellular Understanding of Omentum**
The cellular composition of the omentum is complex and the understanding of its composition has changed over the decades. Currently, it is understood that omentum is comprised of mesothelial cells, adipocytes, adipocyte-derived stem cells, and fibroblasts. In focal areas within the tissue, milky spots have been described from which immune cells can be isolated. The initial studies that looked into the composition of milky spots in humans identified them as predominantly macrophage (67%), with T-cells and B-cells comprising of 10% accordingly (Krist et al., 1995). Mast cells make a smaller percentage of the omentum, consisting of roughly 6%. These percentages have varied slightly but have been largely consistent among follow-up immunohistological studies (Shimotsuma et al., 1991, Yildirim et al., 2010).

**Macrophages.** The largest cell group within the omentum, macrophages in the omentum can be found within the adipose tissue or within milky spots. Macrophages within the adipose tissue have been identified as lipid-laden macrophages or foam cells in obese patients, which have been implicated in atherosclerosis (Shapiro et al., 2013). Within the milky spots, immature monocytes develop into monocyte and macrophages from the 20th to 40th week of gestation (Krist et al., 1997). The macrophages in the adult omentum, have been postulated to have some tumorcidial effect, but the level of cytotoxicity appears limited (Krist et al., 1995). Depletion of omental macrophages led to proliferation and replenishment of new macrophages from cells within the milky spots, supporting the presence of a self-renewing macrophage population (Zhu et al., 1997).
**B-cells.** B-cells are the next largest population of cells in the omental milky spots. In an infectious state, a gammaherpesvirus mouse model has identified formation of B-cell germinal centers in the omentum, demonstrating that antigen presentation can occur within the tissue (Gray et al., 2012). In an experimental model of intraperitoneal antigen injection, antigen-specific B-cells undergoing somatic hypermutation and affinity maturation were observed (Rangel-Moreno et al., 2009). In addition, by using confocal microscopy to track intravenously injected B-cells, B-cells were found to migrate from the blood stream and to the peritoneal cavity through the omentum (Berberich et al., 2008, Ito et al., 2004). Early B-cell lineage cells could be identified in mouse resting omentum, and these cells were capable of differentiating into mature B-cells in vitro, demonstrating omentum may be a site for new B-cell development (Pinho et al., 2005). Collectively, these studies support an active role for the omentum as a site for B-cell immunity directed against peritoneal antigens.

**T-cells.** T-cells make up the third largest population of cells within the milky spots of the omentum. Like B-cells, they are capable of immunological response within the omental tissue. Carlow and colleagues demonstrated T-cells can traffic to the omentum from the peritoneum and be stimulated by a small fraction of dendritic cells in the omentum (Carlow et al., 2009). Antigen experienced CD4 and CD8s could be identified in the omentum under two infection models, supporting a role for T-cell antigen presentation (Rangel-Moreno et al., 2009). Taken with the studies on B-cells, these findings support the role of the omentum as a secondary lymphoid organ.
**Dendritic Cells (DCs).** Dendritic cells are an important antigen-presenting cell in the immune system. A study of human and mouse omentum has characterized the presence of DCs (CD11c+) within the omentum by phenotype, structural and functional assays (Bedford et al., 2006, Maroof et al., 2005).

**Invariant Natural Killer T-cells (iNKT).** iNKT cells are a minority population of cells that can recognize glycolipid antigens by CD1d presentation and provide help for B-cell proliferation and antibody production (Vomhof-DeKrey et al., 2014). Omental tissue possesses the highest fraction (30%) of invariant natural killer T-cells as identified by the expression of Vα24Jα18 invariant t-cell receptor (TCR). For comparative purposes, splenic tissue only has 1%. CD1d was also found expressed on a variety of omental cells, suggesting omentum might be an important site for glycolipid presentation (Lynch et al., 2009). Further studies are needed to establish the role for the high presence of iNKT cells in omental tissue.

**Adipose-derived Mesenchymal Stem Cells (ADMSCs).** The omentum has been used as a source of adipose-derived mesenchymal stem cells (ADMSCs) (Ahmed et al., 2014, Nowicka et al., 2013, Marappagounder et al., 2012, Klopp et al., 2012). These cells by definition have the ability to transdifferentiate into multiple cell lineages, including adipocytes, and osteoclasts. In addition to these groups, they have also demonstrated the ability to transdifferentiate into pancreatic insulin-secreting islet cells in vitro (Dhanasekaran et al., 2013). Phenotypically, they have been characterized as CD31−CD34+CD45−CD90+CD105+CD146− from human omentum (Toyoda et al., 2009). The
presence of these cells may explain some of the regenerative capacity of omentum, as mesenchymal stem cells have multi-lineage capabilities, as well as the ability to enter sites of injury and promote tissue regeneration (Caplan & Bruder, 2001).

**Adipocytes.** Adipocytes derived from omentum possess potent immunomodulatory effects. Cytokine expression of IL-1β, TNFα, IL-10, IL-2, IL-8 and a variety of anti-microbial peptides (AMP) were found in cultured adipocytes in response to LPS (Chandra et al., 2011). These data corroborated earlier studies demonstrating increased secretion of proinflammatory cytokines, IL-6, IL-8, GRO and MCP-1 in a similar study looking at omental-derived adipocytes in response to LPS (Bassols et al., 2009). Taken together, adipocytes play an important role in immune response within the omentum through release of cytokines and AMPs.

**Mesothelial Cells.** The mesothelium is a layer of squamous-like epithelial cells that line the peritoneum. Their role is multi-functional: capable of antigen-presentation, cytokine production, growth factors, and generation of extracellular matrix. Mesothelial cells have been isolated from the omentum (Darimont et al., 2007, Chen & Chen, 2012). These mesothelial cells can express MHC Class II and ICAM-1 in response to IFNγ suggesting they play a role in T-cell activation (Valle et al., 1995). It is still unclear however, what their direct role in immune modulation is, as an in vitro culture of mesothelial cells with activated T-cells was found to be suppressive in another model (Lin et al., 2013)
Activated Omentum

Activated omentum is a term used to describe changes in omentum composition due to either a foreign body insult, or due to responses in cytokine production due to infection. It is important this is distinguished from resting omentum, as our studies have demonstrated the two can be dramatically different in cellular composition. Depending on the stimuli, specific cells may expand and the phenotype of activated omentum can vary in disease models. There are also important differences in omentum composition from organism to organism, as omentum in humans is generally larger and consisting of more adipocytes, where omentum in mice is small and does not cover the peritoneum as it does in humans.

Stem cells. Litbarg and colleagues begin an important study on activated omentum. In their model, they inject polydextran slurry into rats to cause omental expansion and activation. When omentum is activated this way, non-adipose cells within the omentum are expanded, and neo-angiogenesis is induced. Progenitor cells (WT-1\(^*\)CXCR-4\(^*\)SDF-1a\(^*\)) expressing stem cell markers (Nanog, Oct–4, and SSEA–1) are found increased in activated omentum (Singh et al., 2008).

Summary

The omentum is a tissue found in the body containing a myriad of cell types. It has functions to promote angiogenesis, can self-proliferate or induce proliferation in other organs, and plays various roles in the immune system, acting as both a secondary lymphoid organ as well as a depot for innate immune cells.
Clinically it has some function as a reservoir for myeloid lineage cells within the peritoneum and contributes to innate immune responses for the peritoneum. Surgeons have harnessed the omentum as a tissue to promote regeneration, angiogenesis and graft acceptance. For these same purposes, it has found use in many reconstructive surgeries, where bulk tissue is often needed.

We hypothesize that the omentum consists of immunomodulatory cells capable of promoting healing and regeneration. Based on these characteristics, we hypothesize these cells can be used as cellular therapy to improve lung transplantation outcomes. We begin our investigations into the omentum by: (1) examining the cellular composition of activated omentum, (2) understanding the broad role of activated omentum in regards to T-cell immunomodulation, (3) investigation into the mechanism of immunomodulation, and (4) the application of activated omentum as cellular therapy in an in vivo lung transplantation model.
CHAPTER TWO
MATERIALS AND METHODS

Mice.
8-10 week old C57BL/6J females (Harlan Laboratories), 8-10 week old CB6F1 males (Harlan Laboratories), 8-10 week old BALB/cJ females (Jackson Laboratories), and 8-10 week old B10.A-H2a H2-T18a/SgSnJ males (Jackson Laboratories) were used for this study.

Reagents.
The following antibodies were purchased from Biolegend and used for cell culture: LEAF Purified anti-IL-4 (11B11), LEAF Purified anti-mouse IL-12/IL-23 p40 (C17.8) and LEAF-purified anti-mouse IFN-γ. The following reagents were purchased for cell culture: recombinant human TGF-β1 (R&D Systems), and recombinant mouse IL-2, IL-6, IL-12, IL-23, IFN-γ (Biolegend). The following mouse antibodies were purchased from BioLegend (San Diego, CA) and used for surface or intracellularstaining: CD4 (GK1.5), IFNγ (XMG1.2), and IL-2 (JES6-5H4). Anti-Mouse/Rat IL-17A FITC (eBio17B7) and anti-mouse IL-22 PE (1H8PWSR) was purchased from eBioscience. For NO donors, DETA-NONOate was purchased from Sigma-Aldrich and S-Nitroso-N-
Acetyl-D,L-Penicillamine (SNAP) was obtained from Cayman Chemical (Ann Arbor, Michigan). NS-398 and ODQ was purchased from Cayman Chemical. Stocks of 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt monohydrate (8-Br-cGMP), concanavalin A (5 mg/ml), thapsigargin, ionomycin and Ng-Methyl-L-Arginine Acetate (L-NMMA acetate) was obtained from Sigma-Aldrich (St. Louis, MO). Western blot antibodies used were: Mouse anti-β-actin (AC-15) from Sigma-Aldrich, rabbit anti-PLCγ1 (#2822) and Mouse anti-Itk (2F12) from Cell Signaling Technology (Beverly, MA), mouse anti-NFATc1 (7A6) from Biolegend, rabbit anti-NOS2 (sc-651), mouse anti-CD3ζ (6B10.2) from Santa Cruz Biotechnology.

**Isolation of Omental Cells.**

Expansion of omentum was performed by intraperitoneal injection of 1 ml of Bio-Gel P-60 polyacrylamide beads (BioRad, Hercules, CA) as previously described (Shah et al., 2012). Five days after injection, the mouse was sacrificed, and the activated omentum was carefully dissected from the pancreas by pulling the tissue outward and trimming the borders. The tissue was then cut up finely and digested in 50 mg/ml collagenase IA for 30 min at 37°C (Sigma-Aldrich). The digest tissue was strained through nylon mesh and resuspended in RPMI. The resuspension was underlain with 2 ml of lympholyte-M (Cedarlane Labs, Cedarlane, Canada) and spun at 2500 rpm for 15 min with no brake to remove erythrocytes and dead cells. Viable cells were then removed by pipetting out the lymphocyte layer in between the lymphocyte and media. This fraction was washed in PBS twice and counted and resuspended in RPMI media. For isolation of CD45+ and CD45− fractions, magnetic column separation by CD45+
marker was followed according to manufacturer’s directions (Miltenyi Biotec, San Diego, CA).

**Isolation of Peritoneal Cells.**

For isolation of peritoneal cells from the peritoneal cavity, C57BL/6J mice were injected with 1 ml of saline by intraperitoneal injection. This fluid was then pulled back into the syringe to isolate peritoneal cells.

**Isolation and Generation of Th17 cells.**

Spleen was harvested and homogenized using frosted glass slides. Afterwards, the cell pellet was resuspended in ammonium chloride potassium (ACK) lysis buffer (Life Technologies) to remove red blood cells. T-cells were isolated by positive selection using CD4 microbeads (L3T4) by magnet separation following manufacturer’s protocol (Miltenyi Biotec). Typically, 30-40% of cells isolated were CD4+ with a purity of >90%, consistent with manufacturer’s reported purity. T-cells were cultured by combining 1 x 10⁶ cells with 5 x 10⁶ irradiated APCs (3000 rads) and activated using soluble anti-CD3 (145-2C11 clone, Biolegend, San Diego, CA) at a final concentration of 1 ug /ml in 2 ml of RPMI in a 24-well culture. Th17 differentiating conditions: anti-IFNγ (2 ug/ml), anti-IL-4 (2 ug/ml), IL-6 (20 ng/ml), IL-23 (10 ng/ml), TGFβ (2.5 ng/ml) for 5 days. Cells were harvested, counted and resuspended in media at 2 x 10⁶ cells / ml for experimental use.
Th17 and Omental Cell Co-culture.

5-day generated Th17 cells were co-cultured in a 24-well plate with omental cells at a ratio of 1:1. Neutralizing antibodies or inhibiting drugs were added at the time of co-culture unless otherwise indicated. Cells were co-cultured for 24 hrs for calcium flux, western blot and nitric oxide measurement experiments. All other experiments were performed after 48-hrs co-culture. A schematic of general co-culture procedure is provided for reference (Figure 1).
Figure 1. Basic schematic of co-culture conditions. CD4+ cells isolated from spleen of untreated C57BL/6J mouse are activated with anti-CD3 and irradiated APCs (5:1 ratio) in the presence of Th17 polarizing conditions: IL-6, IL-23, TGFβ and neutralizing antibodies against IFNγ and IL-4. Cells were split once at day 3, and IL-6, IL-23, TGFβ were replenished. After 5 days of differentiation, cells were counted and cultured in the presence and absence of omental cells at a ratio of 1:1. Chemical inhibitors or neutralizing antibody were given at the time of initial co-culture. The cultures were allowed to proceed to the appropriate time point and harvested for analysis.
Intracellular Staining of Cells

Samples were harvested, washed twice with PBS and stimulated with PMA/Ionomycin for 4 hrs. After the 1st hour of stimulation, 1000X monensin (BioLegend, San Diego, CA) was added for the remaining 3 hrs. The sample was washed 2x with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature. Samples were washed, permeabilized with 1% Triton X for 10 min at 4°C. For transcription factor staining, FOXP3 Fixation/Permeabilization buffer (eBioscience, San Diego, CA) was used. Afterwards, cells were blocked for 15 min in 3% BSA in 1X PBS. Cells were then washed twice with PBS and stained with antibodies against respective cytokines for 1 hr. After washing, samples were run on BD LSRFortessa. Software analysis was performed using FlowJo 7.

Cell Lysis and Western Blot

Cells were lysed using Laemmli buffer (4% SDS, 10% beta-mercaptoethanol, 20% glycerol, 125 mM Tris, pH 6.8), boiled and frozen at -20°C until use. Cell lysate was adjusted by cell number. Mini-Protean Precast Gels 4-20% or 12% gels were used depending on target (Bio-Rad). For running gel and gel transfer onto PDVF membrane, Bio-Rad Mini-PROTEAN protocol was used. Following transfer, membrane was blocked with 5% dry milk fat for 1 hr, washed twice in 1X TBST and probed with antibody specific for iNOS (BD Biosciences, San Jose, CA) for 2 hrs. Following incubation of horse anti-mouse IgG HRP (#7076) or goat anti-rabbit IgG (#7074) (Cell signaling, Danver, MA) for 30 min, signal was detected using SuperSignal West Pico Substrate (Pierce, Rockford, IL).
Detection of Intracellular NO

Differentiated Th17 cells were co-cultured with and without omental cells were harvested on day 1. Cells were loaded with resuspended in HBSS and loaded with 5 uM of DAF-FM (Life Technologies) for 20 min. The probe was washed out and the cells were permitted to rest for 30 min at RT. During this time cells were surface stained for CD4. Afterwards, the samples were rinsed twice in HBSS and evaluated by flow cytometry.

Annexin V Staining

Differentiated Th17 cells with and without co-culture with omental cells were harvested on day 1 and day 2. Cells were surface-stained with APC-CD4. Annexin V Staining was performed using the Annexin V-FITC Apoptosis detection kit (88-8005) as indicated by manufacturer’s directions (eBioscience).

Isolation of mRNA and qRT-PCR

For isolation of mRNA from samples, the cells were FACS sorted for CD4+ marker to re-isolate T-cells. RNA was isolated using manufacturer’s protocol for ReliaPrep RNA Cell and Tissue System (Promega, Madison, Wisconsin). cDNA was generated using Superscript III First-Strand Synthesis System for RT-PCR according to manufacturer’s protocol (Invitrogen). Amplification of target genes was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) on CFX96 Touch Real-Time PCR Detection System (Bio-Rad). OAZ1 was used as housekeeping gene (ribosomal protein).
with the forward and reverse sequence of 5’-TGGACTAACCCAGGAGAGGG-3’ and 5’-ATCCCCCTTGCCCCAACAAAGAGA-3’ respectively. Analysis was performed using CFX manager software (Bio-Rad) using normalized expression ΔΔCq of reference gene (OAZ1).

**Measurement of Intracellular Calcium.**

Cells were harvested, washed twice in PBS and then stained with Fluo-3 at a final concentration of 1 μM for 20 min room temperature (Life Technologies, Carlsbad, CA). Cells were washed and allowed to rest for 20 min and stained with azide-free Alexa Fluor 700 anti-mouse CD4 (BioLegend). Cells were run on BD LSRFortessa (San Jose, CA) as follows: The cells were first run on the machine to establish baseline fluorescence values. The voltage was set to accommodate the maximum flux achieved by ionomycin (Sigma-Aldrich). Then, samples were briefly removed and treated with thapsigargin or 5 mg / ml concanvalin A. The samples were quickly replaced onto the machine for 5 min to record flux in response to the stimulus. The sample was then removed again and treated with ionomycin to record maximum Ca^{2+} flux. Data was collected and analyzed using FlowJo 7.

**Imaging of Endoplasmic Reticulum**

Cells were harvested, washed once in HBSS and cultured with ER Tracker Green (Life Technologies) at a final concentration of 1 μM for 20 min at room temperature. Surface staining for CD4 was performed. Cells were washed twice in HBSS. Analysis of cells was performed by running sample through Amnis Imagestream or by
immobilizing cells onto CelTak coated coverslide and viewing under fluorescent microscope.

**Mouse Lung Transplantation Method**

Left lungs from B10.A-H2a or C57BL/6J mice were orthotopically transplanted into F1 mice (C57BL/6J x BALB/cJ) using previously described methodology (Jungraithmayr et al., 2009, Krupnick et al., 2009). Recipient mice did not receive immunosuppressive drugs or antibiotics following transplantation. Mice treated with omentum were given an intraperitoneal injection of $1 \times 10^6$ omentum cells suspended in 0.1 ml saline 4 hrs following operation. An equal volume of saline without cells was given in untreated mice as a negative control. 14 days following transplantation, lungs were harvested for histology.

**Histology of Lung Tissue.**

Mice were sacrificed. The thoracic cavity was opened and the heart and lungs were removed en bloc by cutting the trachea and gently dissecting the entire bloc. Lungs were fixed through the trachea at a pressure of 30 cm H2O in 1% formaldehyde for 1 hr. Lobes were cut in transverse slabs and embedded in OCT. Embedded samples were frozen on dry ice or snap-frozen with liquid nitrogen depending on availability of liquid nitrogen or dry ice. H&E staining was performed by the Loyola University Medical Center Pathology Core. Grading of slides was performed by a Loyola pathologist, Razan Massarani-Wafai MD, according to the 2007 International Society for Heart and Lung Transplantation (ISHLT) classification (Stewart et al., 2007).
Live Imaging of Activated T-cells in Transplantation

The left lung from Balb/C / B10A (allogenic) or FVB / B6 (syngenic) was transplanted into a Rosa26GFP/IL-2Cre mouse (B6 background). For in vivo imaging experiments, mice were given $1 \times 10^7$ omental cells on day 1 and day 7 following operation. An equal volume of saline was given in untreated mice as a negative control. On day 21 following transplantation, mice were anesthetized, and shaven on the chest and sides. Nair was used to remove hair completely. D-luciferin (Caliper Lifesciences, Hopkinton, MA) was given by intraperitoneal injection at 3.3 ul/g of body weight from a 15 mg/ml stock in DPBS. Mice were allowed to rest for 10 min to allow for stabilization of signal. Mice were then imaged on Xenogen IVIS for 2 minute bioluminescence exposure on ventral and lateral sides (PerkinElmer, Waltham, MA).

Mixed Lymphocyte Reactions

Mixed lymphocyte reactions were performed by isolating CD4$^+$ cells from the spleen and then incubating them with irradiated CD90.2 depleted APCs derived from a B10.A-H2a or C57BL/6J mouse to evoke allogenic or syngenic responses respectively. They were co-cultured at a ratio or 1:1 for CD4 to APC for 3-days and evaluated for cytokine production in CD4$^+$ cells using Ready-Set-Go! ELISPOT following manufacturer’s protocol (eBioscience).
CHAPTER THREE
THE ROLE OF OMENTUM IN IMMUNOMODULATION

Introduction

Activation of omentum by foreign bodies causes rapid expansion of cellular subsets within the omentum. Previous examination of activated omentum has demonstrated that a subset of non-hematopoietic cells with stem cell markers are present (Litbarg et al., 2007). The goal of our study was to further examine the cellular composition of activated omentum to determine if we could identify cells that contribute to omentum’s function as a promoter of cellular regeneration and graft tolerance. Like the Litbarg study, we were able to identify a stem cell population. In addition, we discovered the presence of a population of myeloid derived suppressor cells (MDSCs) that comprise a majority of the cells within activated omentum. We then examined the effects of activated omentum on differentiated T-helper subsets to determine what role it played in immunomodulation of T-cells. For the purposes of this study and to emphasize the heterogeneity of omental cells, we have dubbed them omental derived suppressor cells (ODSCs) since they are comprised of many cell types.
Results

Stem cells

Our lab has confirmed the presence of stem cells in activated omentum as we were able to independently identify a CD45^-CD34^+ subset with the ability to differentiate into lung epithelium and osteoclasts (Shah et al., 2012). These phenotypically were similar to a mesenchymal stem-cell-like (MSC-like) fraction although conditions to differentiate them to adipocytes have not yet been established. Because of that, we call them MSC-like since they cannot be considered true MSCs until they have been demonstrated to have full-multilineage potential.

Myeloid Derived Suppressor Cells

In our model of activated omentum, we have taken steps to characterize the myeloid cells, which prior to this point have not been described. We harvested omentum from mice five days after intraperitoneal injection with polyacrylamide and compared them to peritoneal cells (harvest procedure in Methods and Materials). Our phenotypical analysis by FACS staining showed that myeloid cells within the activated omentum were not peritoneal macrophages (CD11b^+F4/80^+) but fit the markers of myeloid derived suppressor cells (MDSCs) (CD11b^+Ly6C^+F4/80^-) (Figure 1B). Peritoneal cells were F4/80^+Ly6C^-Ly6G^{low} matching the description of peritoneal macrophages. Notably they were not expressing Ly6C, which distinguishes them from MDSCs. We were able to further stratify the omental-derived MDSCs into two previously identified sub-populations, granulocytic MDSCs (Ly6G^{low}) and monocytic MDSCs (Ly6G^{hi}) (Ribechini et al., 2010, Fig. 1A). These results were supported by Song and colleagues’ work on an
IL-1b model of induced peritonitis, where they observed neutrophil (CD11b\(^+\)Ly6G\(^+\)) accumulation in the activated omentum and not in the peritoneum or mesenterium. In that study, they did not characterize these cells by the Ly6C marker, and it is possible the neutrophils they described were in fact MDSCs, as they share the same overlapping markers.

We also examined the CD11b\(^+\) population for the presence of major histocompatibility (MHC) Class II molecules. MHC Class II has been reported to be found in varying expression on MDSCs depending on the tumor model. On MDSCs, MHC-class II has been reported to play a role in inducing CD4\(^+\) tolerance although the mechanism is unclear (Nagaraj et al., 2012). Within our omental cells, we identified 31% of CD11b\(^+\) cells express MHC Class II (Fig. 1C).
Figure 2. A. Cell profile of activated omentum by FACS staining. A single cell suspension of omental cells was stained with CD11b, Ly6G and Ly6C. Gated CD11b+ cells were then analyzed for Ly6G and Ly6C. B. Comparison of OC and peritoneal cells (PeCs) stained for F4/80, Ly6G and Ly6C. C. Expression of MHC-Class II in activated omental cells. Representative histograms are shown of two or more independent experiments.
Overall, in the activated omentum, MDSCs were found in very high abundance, ranging from ~50-70%. For comparison, MDSCs are naturally found as high as 20-30% in bone marrow, and 2-4% in splenocytes (Ribechini et al., 2010). It is unclear if these MDSCs are locally generated or traffic into the omentum in response to injection of foreign matter. However, given the abundance of MDSCs in activated omentum, combined with prior observations that the omentum acts as a tissue for macrophage development, these data collectively suggest that omentum is a significant tissue reservoir for MDSCs.

**Omentum Inhibition of T-cells**

CD4^+ T-cells play a fundamental role in the adaptive immunity by recognizing of antigen and mounting appropriate responses to the foreign threat. Since the discovery of Th1 and Th2 cells in 1986 by Coffman and Mossman, we now know the T-helper field is increasingly diverse, consisting of Th17, Th22, Th9, Treg and T follicular helper cells, each with their distinct cytokine profile and key transcription factors (Bluestone et al., 2009). Regulation of T-cells is a critical component of the immune system. Just as key as it is for the host to amount responses to antigen, the ability to shut them off is essential to prevent chronic inflammation. T-regulatory cells highlight the importance of this, as deficiency of FOXP3, leads to auto-immune disease (Hori et al., 2003). However, Tregs are not the only cell capable of affecting T-cells, various other cell types such as macrophages and MDSCs can interact with T-cells and promote or suppress them.
Little is known about the capacity of omentum in immunomodulation of T-cells. While it is known that the omentum is capable of pro-inflammatory and anti-microbial responses, there are no data showing the specific responses of omentum with T-cells (Chandra et al., 2011). We examined the role of omentum in an in vitro co-culture with differentiated T-cells to determine their effect. We expanded CD4+ cells from the spleen using anti-CD3 and irradiated APCs, for five days under Th1, Th2, Th17 and iTreg polarizing conditions (Detail provided in Materials and Methods). Omentum was reconstituted into a single-cell resuspension following collagen digestion and Ficoll separation. After T-helper expansion, we co-cultured T-helper cells with activated omental cells for 2-days and measured cytokine production by FACS analysis, looking for the intracellular presence of IFNγ for Th1, IL-4 for Th2, and IL-17A for Th17. For the examination of iTregs we looked for the presence of the transcription factor FOXP3.

We observed omental cells to be strong inhibitors of the primary cytokines for Th1 and Th17 (Fig. 3A). We observed a milder decrease in production of IL-4 when omental cells were cultured with Th2 cells, and little to no decrease of iTregs (Fig. 3A-B). Since iTregs are a generally unstable form of FOXP3, we further analyzed the effects of omentum on naturally arising Tregs (nTregs), which stably express FOXP3.

To examine the effects of omentum on the nTreg phenotype, we isolated CD4+CD25+ cells from the thymus and expanded them with IL-2 for 2-weeks to generate natural Tregs. Upon co-culture with omentum, nTregs partially expanded (Fig. 4). This suggests that unlike Th1 or Th17, nTregs are not inhibited by omental cells.

We hypothesized the lack of inhibition of nTregs may be due to the lack of IFNγ in the system. Both Th1 and Th17 cultures generate IFNγ and therefore, nTregs may not
be inhibited, since no IFNγ is present to activate omental cells. Interestingly, addition of IFNγ to nTreg cultures with omental cells did not result in inhibition (Fig. 4). This suggests two possibilities: (1) additional cytokines or signaling events from nTregs block omental cells from becoming inhibitory or (2) nTregs are more resistant to omentum-mediated inhibition than other T-helper subsets.
Figure 3. Effects of ODSC on differentiated T-helper subsets. A. CD4+ cells were isolated and cultured under differentiating conditions for 5-days. ODSCs were added at a 1:1 ratio on day 5 for 2 days. Cells were harvested, stimulated with PMA/ionomycin for 4 hrs, permeabilized, blocked and stained. Representative FACS plots are shown. n = 4. C. Cumulative data is shown for Thelper subsets. OC designates ODSC added samples. * p < 0.05. ** p < 0.005. *** p < 0.0005 by student’s t-test.
Figure 4. Effects of ODSCs on nTreg cells. nTregs were isolated by CD4^+CD25^+ FACS sort from thymus and expanded with IL-2 for 3 weeks. Afterwards, expanded cells were co-cultured with ODSCs for 2-days in the presence and absence of IFNg. Representative FACS data is shown.

Taken together, the phenotype of activated omentum in regards to T-cell modulation generally promotes an anti-inflammatory, pro-tolerance environment. The immunomodulatory properties of omentum are similar to alternatively activated macrophages (M2) which produce potent anti-inflammatory responses to promote tissue healing (Novak & Koh, 2013). We will describe in later chapters how omentum does this mechanistically.
Discussion

The omentum is a complex tissue made up of multiple cell types. Here we have examined the phenotype of activated omentum. Activated omentum surprisingly differs substantially from resting omentum in that it mostly devoid of mature macrophages, T-cells, and B-cells. Instead, due to the foreign particle injected (in this case, polyacrylamide gel), there is rapid recruitment or expansion of MDSCs, and to a lesser extent, MSC-like cells.

Investigations into the immunomodulatory properties of activated omentum revealed that in vitro these cells promote an anti-inflammatory environment. ODSCs notably inhibit Th1 and Th17 subsets, both pro-inflammatory subsets. The inhibition appears specific, as Th2 cells are not as inhibited as strongly and iTregs and nTregs are preserved.

MDSCs have been shown to preferentially induce expansion of nTregs via an arginase-dependent pathway (Serafini et al., 2008). While the mechanism here is unknown, MDSCs within the omentum may be responsible for the similar expansion we observe with nTregs in vitro. Interestingly, despite addition of IFNγ, an activator of ODSC-inhibition, omental cells had little effect on nTreg numbers. These suggest that intrinsically nTregs may be better poised to survive and proliferate under these stressful conditions where arginase is active. Further studies should be conducted to determine how nTregs appear to be resistant to specific immunosuppressive pathways.

Our study of activated omentum demonstrates that the omentum is a dynamic tissue capable of responding to foreign matter by expanding specific cell populations. Its unique composition contains cells capable of acting as a secondary lymphoid organ, but
also upon activation possesses the property to be anti-inflammatory, a state that is
iimportant to promote tissue healing and regeneration. The orchestrated responses of
MDSCs and MSC-like cells likely contribute as an immunoregulatory response to curb T-
cell responses when appropriate.
CHAPTER FOUR
OMENTUM
 AND
TH17 INHIBITION

Introduction

Th17

First identified in mice in 2005, Th17 cells represent a distinct T-helper subset important for the clearance of fungi and extracellular bacteria (Park et al., 2005). Th17 cells can be generated in vitro by addition of TGFβ, and IL-6, and are maintained by addition of IL-23. These cells are characterized by expression of the cytokine, IL-17A and stable expression of the transcription factor, RORγt (Noack & Miossek, 2014). IL-17A plays a crucial role in innate immunity by recruiting neutrophils during infection (Way et al., 2013). Th17 cells are of particular interest in clinical research, as they have been linked to a variety of chronic inflammatory and auto-immune diseases including, but not limited to, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis, and inflammatory bowel disease.

We have demonstrated ODSCs have potent immunomodulatory effects and are particularly inhibitory of Th1 and Th17 subsets. In this study, we investigated the mechanism behind ODSC-mediated inhibition of Th17 cells. Understanding this mechanism may provide insights into how cell types within the peritoneum can respond to control Th17 responses.
Results

**ODSCs inhibit Cytokine Production in Th17 cells**

Previously, we have shown expression of IL-17A expressing cells are reduced after Th17 cells are co-cultured with ODSCs (Fig. 3). Using this same analysis, we further examined the cytokine profile of Th17 cells after co-culture by looking at expression of IL-2 and IFNγ. IL-2 is a key cytokine for T-cell growth. Although the presence of IL-2 in T-cells cells undergoing differentiation to Th17 impairs differentiation, IL-2 was found capable of inducing proliferation in T-cells that are already differentiated to Th17 (Liao et al., 2011). Therefore, if ODSCs impair IL-2 production this could potentially contribute to Th17 arrest by blocking the cells ability to produce a key proliferative cytokine.

We examined IFNγ expression since a subset of Th17 cells are known to have plasticity for Th1 and are capable of producing IFNγ in addition to IL-17A. These Th1-deviated Th17 cells are required for disease development in experimental autoimmune encephalomyelitis and thereby considered a pathogenic subset as IL-17A only producing Th17 cells are insufficient to cause disease (Basu et al., 2013). We were interested if these subsets existed in our *in vitro* system and whether they were also being inhibited by ODSCs.

To test cytokine expression, Th17 cells were co-cultured with ODSCs for 2-days as previously described and stained for FACS analysis. Under non-co-culture conditions our Th17 culture consists of a mixture of Th1-Th17 cells producing both IFNγ and IL-17A, and single positive IL-17A producers (Fig. 5). In addition, we observed combinations of IL-2 expression, with some populations of IL-17A+IL-2+, IL-17A+IL-2−
and IL-17A$^+$ IL-2$^-$. When Th17 cells are co-cultured with ODSCs, IFN$\gamma$ and IL-17A were potently inhibited (Fig. 5). IL-2 production was partly inhibited, with some cells expressing only IL-2 but not IL-17A surviving (Fig. 5). These data demonstrated that ODSCs are capable of broadly inhibiting cytokine responses in Th17 cells.
Figure 5. Effects of ODSC on cytokine expression in Th17 cells. CD4+ cells were isolated from spleen and differentiated under Th17 conditions for 5-days. ODSCs were added at a ratio of 1:1 to Th17 cells for 2-days. Cells were harvested and stimulated with PMA/ionomycin for 4 hrs. Cells were fixed, permeabilized, blocked and stained for FACS analysis. Cytokines measured included IL-17A, IL-2 and IFNg. n = 4-5. ** denotes p < 0.005. **** denotes p < 0.0001. Representative FACS plots shown.
Multiple Subpopulations of Cells within ODSCs Inhibit Th17 Cells

Omentum consists predominantly of MDSCs of hematopoietic origin and MSC-like cells that lack hematopoietic markers. To determine which subset of omental cells was responsible for ODSC inhibition, we separated omentum based on the CD45+ marker, a glycoprotein exclusively found on hematopoietic-derived cells. We isolated fractions of these cells using magnetic bead separation based on positive selection of the CD45 marker. The unbound fraction was kept as the CD45- fraction. We then co-cultured these fractions with Th17 cells to determine if a specific subset of omentum was responsible for inhibition.

For this experiment, we examined two hallmark Th17 cytokines, IL-17A and IL-22. Through cytokine analysis, we determined that both fractions suppress IL-17A+ cells with similar efficacy, suggesting both hematopoietic and non-hematopoietic populations of the omentum can inhibit T-cells (Fig. 6). Interestingly, when we analyzed cells for expression of IL-22, we observed specific increases of IL-22 by ODSCs, and the CD45+ fraction (Fig. 6). These data demonstrate that multiple populations within the ODSCs, from both MSC-like and MDSC phenotypes can inhibit Th17 cells. Upregulation of IL-22 however, is CD45+ specific, suggesting these cells have a unique response to Th17 cells that is not shared with CD45- cells.
Figure 6. Effects of CD45 fractions from omentum on IL17A and IL-22 expression in Th17 cells. Omentum was separated into CD45+ and CD45- fractions using magnetic bead isolation (Miltenyi-Biotec). Th17 cells were generated as previously described over 5-days. Th17 cells were co-cultured with whole ODSC or fractionated ODSCs at a ratio of 1:1. Expression of IL-17A and IL-22 was evaluated. n = 3. * p < 0.05. ** p < 0.005. **** p < 0.0001.
We attempted to expand our findings by examining more specific markers, such as Ly6G to isolate myeloid lineage cells or CD34 to isolate MSC-like cells. To do this we employed various approaches, including separation of cells by FACS sort, cell density and magnetic bead isolation. Our data determined these markers and methods to be unreliable as we obtained inconsistent levels of inhibition among fractions (data not shown). We attribute the difficulty in separating populations of omentum to several potential reasons: (1) the markers are not reliable in order to separate cells populations adequately (2) multiple cell populations within the omentum use overlapping mechanisms of inhibition (3) cellular interaction among omental cells is necessary for T-cell inhibition. Of these possibilities, the last seems most likely, as our findings showed that unseparated omentum most consistently inhibits Th17 cells with the greatest potency. It is possible removal or alteration of cellular subsets leads to an impaired response.

**ODSC Mechanism of Th17 inhibition**

We investigated the mechanism of omental inhibition based on our knowledge of its cellular composition. Omentum is comprised largely of MDSCs, so we hypothesized these cells within the omentum play the largest role in Th17 inhibition. It has been previously described that MDSCs can inhibit T-cell proliferation and differentiation a variety of ways. MDSCs upregulate indoleamine 2, 3 dioxygenase (IDO) production to deplete the environment of tryptophan needed for proliferation (Yu et al., 2013). Arginase expression has been demonstrated to deplete L-arg, and T-cells in a depleted L-arg environment lose expression of CD3ζ, a critical component for T-cell activation (Rodriguez et al., 2004). Inducible nitric oxide synthase (NO) has been classically
reported to be upregulated in macrophages in response to T-cells, and this mechanism has also utilized by MDSCs (Kusmartsev et al., 2005). Generation of NO by iNOS is thought to cause cell cycle arrest through free radical generation, although the exact mechanism is unknown. MDSCs also express high levels of programmed cell death protein 1 ligand (PDL1), the ligand to programmed cell death protein 1 (PD1), found on T-cells. When this receptor ligand interaction occurs, T-cell proliferation and cytokine production are blocked (Pardoll, D. M., 2012). Prostaglandin E2 (PGE2), a downstream byproduct catalyzed by cyclooxygenase-2 (COX-2), has also been demonstrated as a T-cell inhibitor secreted by MDSCs (Mao et al., 2013).

Through use of chemical inhibitors or neutralizing antibodies, we tested if these mechanisms were active in our co-culture assay. Blockade of these immunomodulators should alleviate Th17 inhibition if the pathway is active in our in vitro system. We screened through numerous inhibitory pathways, testing the role of iNOS, arginase, IDO, PGE2 and PD1. Notably, addition of L-NG-monomethyl Arginine (L-NMMA), an iNOS inhibitor, at the start of omental co-culture with Th17 cells completely blocked inhibition. When iNOS was inhibited, we observed restoration of IL-17A+ cells by both percentage and number (Fig. 7). This suggested nitric oxide (NO) was required for inhibition.
Figure 7. The effect of L-NMMA on ODSC-mediated Th17 inhibition. L-NMMA was added at the beginning of 2-day co-culture ODSC and Th17 cells. L-NMMA was added to Th17 alone as a control. Cells were evaluated for IL-17A production by flow cytometry. ** denotes p < 0.005. * denotes p < 0.05. n = 3.
iNOS is Dependent on IFNγ

Classically, iNOS was found upregulated by IFNγ in models where macrophages were exposed to splenocytes in the presence and absence of neutralizing antibody against IFNγ. These experiments showed the accumulation of an inert product of nitric oxide, nitrate (NO3−), to be dependent on IFNγ (Albina et al., 1991). We set out to test the role of IFNγ in our system. To do this, we utilized the same approach, adding neutralizing antibody against IFNγ during initial co-culture of the two populations. Neutralization of IFNγ was sufficient to restore both cell number and percentage of IL-17A+ cells (Fig. 8). This demonstrated IFNγ within our culture system is required for inhibition.
Figure 8. The effect of neutralizing IFNg on ODSC-mediated Th17 inhibition. Anti-IFNg was added at the beginning of 2-day co-culture ODSC and Th17 cells. Anti-IFNg was added to Th17 alone as a control. Cells were evaluated for IL-17A production by flow cytometry. *** denotes $p < 0.001$. * denotes $p < 0.05$. n = 3.
In our culture system, Th17 cells are maintained in the same starting differentiating media and are co-cultured with ODSCs in that same media. As a result, we hypothesized that carry over of IFNγ from the start of T-cell differentiation may be activating our ODSCs. To test if this is the case, we washed out Th17 differentiating media, and resuspended the Th17 cells in fresh media after two washes in PBS. This was done to remove most cytokine production up to that point, although Th17 cells can continue to secrete new cytokines once they are resuspended into fresh media. Under these conditions where fresh media was replaced (wash), we observed that ODSCs were incapable of inhibition (Fig. 7). To determine if IFNγ alone could be the sole initiator of inhibition, we added 10 ng/ml IFNγ to the fresh media, to determine if inhibition could be restored. Under these conditions, potent inhibition was restored, suggesting that IFNγ is sufficient to activate ODSC-mediated Th17 inhibition (Fig. 7).
Figure 9. The effect of addition of IFNγ to the culture after washing out culture supernatant. Prior to co-culture Th17 cells were given a 2X PBS wash and resuspended in fresh media (wash). Cells were then co-cultured in the presence or absence of 10 ng/ml IFNγ for 2 days and evaluated for IL-17A production. n = 4.
iNOS is Upregulated in ODSCs

IFNγ is a multi-functional cytokine, capable of many effects. It was originally demonstrated that IFNγ exposure to macrophages was linked to upregulation of iNOS mRNA (Deng et al., 1993). IFNγ can also synergize with IL-17A to further enhance iNOS expression (Zhang et al., 2012). To determine if IL-17A could play a role in our system, we examined if neutralization of IL-17A could block ODSC inhibition. Addition of neutralizing antibody to IL-17A did not restore IL-17A+ cells, indicating IFNγ in our system was sufficient to upregulate iNOS (data not shown).

While iNOS expression is generally observed in myeloid-lineage cells in response to IFNγ, a study by Yang and colleagues in 2013 demonstrated the presence of iNOS in stimulated Th17 cells, suggesting the possibility that Th17 can also upregulate iNOS. As a result, iNOS could be upregulated in either Th17 or ODSCs. To determine which cell type was upregulating iNOS, we treated Th17 cells and ODSCs with and without IFNγ and performed western blot analysis for the expression of iNOS. Th17 co-cultured with ODSCs were used as a positive control. We only detected expression of iNOS in ODSC cells given IFNγ or in the sample where, Th17 was co-cultured with ODSCs, demonstrating that upregulation of iNOS occurs specifically in ODSCs (Fig. 10).
Figure 10. Western blot for iNOS expression in Th17 or ODSC in the presence and absence of IFNγ. Cells were cultured in the presence and absence of 10 ng IFNγ. Whole cell lysates were made after 1-day. Protein loading was adjusted by cell number. Th17 co-cultured with ODSCs was used as a positive control.
**ODSCs Increase NO levels in Th17 cells**

Since we have demonstrated iNOS was upregulated specifically in ODSCs, we set out to show that the direct downstream mediator, NO, was increased in Th17 cells. In order to measure NO within cells, we utilized a diaminofluorescein known as DAF-FM. This dye is permeable to live cells and can produce a specific fluorescent product in the presence of NO. With this dye, we can detect relative levels of NO in live cells by flow cytometry. Initial experiments to determine peak NO increase showed that T-cells had relatively highest NO levels at approximately 1-day after co-culture. When we performed this assay, we found the MFI of DAF-FM increased by 2.5-fold in CD4+ cells when cells were cultured with ODSCs compared to cells that were not (Fig 11A). We developed a standard curve to estimate the level of NO, and found the increase in MFI corresponded to a 20 min exposure of 500 uM of DETA-NO, an NO donor (not shown). Given the 20 min incubation time, we estimated concentrations of ~4.1 uM of NO within the cell using the half-life of DETA-NO. These data demonstrate that NO levels are increased in an ODSC-dependent manner within the T-cells during co-culture.

We also examined the NO levels of CD11b+ populations of ODSCs with and without Th17 co-culture. Relative to Th17 cells, NO levels were high within the myeloid population of the cell, at a similar level to Th17 cells (not shown). These data suggest accumulation of NO occurs in ODSCs as well. Given that ODSCs are relatively short-lived within T-cell media *in vitro*, it is possible that ODSCs may undergo self-induced NO arrest.
**NO is sufficient for inhibition**

We hypothesized that NO was the primary driver of ODSC-mediated Th17 inhibition. To test this, we examined if NO-donors were sufficient to inhibit Th17 cells. We tested two NO-donors, S-nitrosothiol (SNAP) and diethylenetriamine NONOate (DETA-NO). Varying concentrations of SNAP induced a similar percentage of IL-17A+ loss at concentrations of 1000 uM when compared to ODSCs (Fig. 1B). We also tested the addition of 500 uM DETA-NO on our differentiated Th17 culture over 2-days. Addition of 500 uM DETA-NO inhibited IL-17A+ production with similar efficacy as ODSC (Fig. 1C). These experiments demonstrate that NO is sufficient to inhibit Th17 cells at high concentrations.

These findings were supported by previous work by Niedbala and colleagues in 2011, who investigated the role of nitric oxide donor, NOC-18 on differentiated Th17 cells. In a similar system, they demonstrated 3-day exposure to 200 uM NOC-18 arrested Th17 cells and blocked expression of IL-17A.
Figure 11. A. Nitric oxide levels measured in Th17 cells co-cultured with OC. After 1-day of co-culture cells are loaded with DAF-FM. Remaining DAF-FM is removed by media wash and the cells are given another 20 min of incubation. DAF-FM is then read by FITC channel. Shown is median fluorescent intensity of gated CD4+ cells. N = 3. B. Addition SNAP (NO donor) to Th17 cells is compared with OC (ODSC) addition. N = 3. C. The effects of 500 uM of DETA-NO (NO donor) on IL-17A expression in Th17 cells compared with ODSCs. Representative histogram shown, gated on CD4+ cells.
Soluble Guanylyl Cyclase (sGC) is not activated by NO

One specific effect of nitric oxide is activation of soluble guanylyl cyclase (sGC), an enzyme that converts GTP to cGMP. We examined if NO in our system was activating sGC leading to cGMP generation to inhibit Th17 cells. If NO produced by ODSCs was acting to stimulate sGC, then inhibiting sGC should block inhibition. To test this, we performed a dose titration of methylene blue, a well-described sGC inhibitor, from 1 uM to 10 uM. While methylene blue had a partial restorative effect particularly of IL-17A expression at 5 uM, it also had direct cytotoxicity on Th17 cells as demonstrated by loss of Th17 cells under non-co-cultured conditions (Fig. 12). To validate our findings, we also tested ODQ, an irreversible, highly potent sGC inhibitor. Administration of ODQ had no effect on restoration of expression of IL-17A in the Th17 cells by number or percentage (not shown).
Figure 12. Effects of increasing concentrations of methylene blue on omental inhibition of Th17 cells by both percentage and number. Th17 cells are co-cultured with OC cells in the presence and absence of increasing concentrations of methylene blue. n = 3. * denotes statistically significant differences (p < 0.05).
To clarify the discrepancy in our methylene blue and ODQ data, we took the alternative approach, and asked if activation of sGC was sufficient to inhibit Th17 cells. To do this we tested sGC activators that had been originally devised as therapeutic drugs. Bay41 and Bay58 (Cinaciguat) are known to mimic NO effect by enhancing the ability of sGC to produce cGMP. A dose titration of these drugs had no inhibitory effect on Th17 cells (data not shown). To confirm these findings, we also tested if addition of 8-bromo-cGMP, a soluble cGMP analog, could inhibit Th17 cells. Th17 cells given 8-bromo-cGMP had no loss of IL-17A expression, demonstrating sGC activation is not sufficient to inhibit Th17 cells (data not shown).

To reconcile the effects of our methylene blue data, a literature review suggested that methylene blue may be acting in part as an iNOS inhibitor and is not as potent of a sGC inhibitor as it is classically described. In addition, some reports have suggested that methylene blue may inhibit the sGC pathway indirectly through generation of superoxide radicals (Mayer et al., 1993, Hwang et al., 1998). If methylene blue was acting partially as an iNOS inhibitor, it would explain our findings since it would mimic the effect of L-NMMA, which we had demonstrated earlier.

COX-2 Is Required for Th17 Inhibition

The interplay between NO and COX enzymes has been well established. While the mechanism is not completely understood, it is known that iNOS potentiates the effect of COX-2 in generation of prostaglandins. This occurs through S-nitrosylation of a specific cysteine residue by NO (Salvemini et al., 1993). Given these functions, we have also analyzed the role of COX-2 in our system. We utilized COX-2 inhibitor, NS-398, to
block COX-2 from converting arachadonic acid to PGH2 (a PGE2 precursor).

Interestingly, addition of NS-398 restored percentages of IL-17A+ cells in our co-culture model, suggesting inhibition was PGE2 dependent (Fig. 13). However, it was unable to restore the absolute number of IL-17A+ cells, suggesting that loss of T-cell number may be an independent effect of IL-17A expression (Fig. 13). Since blocking production of PGE2 downstream alleviates only IL-17A expression but does not restore cell number, it suggests that the upstream effect of NO is acting to reduce cell number as a potentially independent effect. This suggests that PGE2 might have a specific effect on IL-17A expression without affecting cell survival.
Figure 13. Effects of COX-2 inhibitor (NS-398) on omental inhibition of Th17 cells by both percentage and number. Th17 cells are co-cultured with OC cells in the presence and absence of 10 uM NS-398. Graph bars show expression of IL-17A by percent and by cell number. n = 4. ** denotes statistically significant differences (p < 0.005), *** p < 0.0005.
**ODSCs Utilize TGFβ to Affect IL-2 Production**

TGFβ is a protein involved in differentiation of Th17 and Tregs. Human MDSCs have been reported to utilize TGFβ to promote Treg induction from naïve T-cells and transdifferentiation of Th17 cells to Tregs (Hoechst et al., 2011). We therefore examined the role of TGFβ in our system to determine if ODSCs utilize TGFβ as a mechanism of inhibiting Th17 cells. We added SB-431542, a potent inhibitor of TGFβ Type I receptor kinases to our Th17-ODSC co-culture and examined cytokine expression of IL-2 and IL-17A (Inman, 2002). Interestingly, addition of SB-431542 restored IL-2 single positive cells, but did not restore IL-17A expression (Fig. 14). This suggests that TGFβ is secreted by ODSCs, to specifically block expression of IL-2 in T-cells in our culture system.
Figure 14. Effects of TGFβ Type I receptor kinase inhibitor (SB-431542) on omental inhibition of Th17 cells by both percentage and number. Th17 cells are co-cultured with OC cells for 2-days in the presence and absence of 10 uM SB-431542. Cells were restimulated with PMA/Ionomycin, fixed, permeablized and stained as described in methods. IL-2 and IL-17A expression was evaluated. Representative FACS profiles are shown. Controls shown here were part of the same experiment in Figure 5.
This corroborates with previous findings that have looked at TGFβ signaling. TGFβ exposure to activated T-cells is known to inhibit IL-2 expression in a SMAD3-dependent manner (McKarns & Schwartz, 2005). These support our findings that TGFβ release by ODSCs can inhibit specific IL-2 expression in our co-culture system.

**ODSCs are Not Causing Th17 Cells to Undergo Apoptosis**

NO has been linked to apoptosis by multiple mechanisms. Depending on the cell type following exposure to NO, some cells will undergo an early accumulation of p53, indicating the presence of DNA damage, due to the generation of nitric oxide free radicals (Beck et al., 1999). Cells that are exposed to NO also undergo endoplasmic reticulum stress (ERS), a condition where unfolded or misfolded proteins in the ER lead to caspase activation and subsequently, apoptosis (Takada et al., 2013). However, this is known to be dependent on levels of nitric oxide, as well as cell type, as certain cells, including B-cells, eosinophils, hepatocytes, plasma cells and endothelial cells are resistant to NO and may even have prolonged survival due to exposure (Dimmeler and Zeiher, 1997, Saini et al, 2014).

To address whether our cells were undergoing apoptosis or not after co-culture with ODSCs, we examined cells for the expression of 7-AAD positivity and annexin V. Cells undergoing apoptosis express phosphatidylserine (PS) on the outer leaflet of the plasma membrane instead of the inner, and the detection of PS by annexin V has been well-established as a marker of apoptosis (Brumatti et al., 2008). Comparison of Th17 cells with and without co-culture with ODSCs for 2 day co-culture revealed that CD4+ cells had few necrotic cells (7AAD+AnnexinV+), and possessed similar percentages of
live cells (7AAD-AnnexinV-) (Fig. 15). No cells detected undergoing apoptosis (annexinV+7AAD-) were detected (Fig. 15). We examined both day 1 and day 2 time points as ODSC-mediated cell loss occurs between day 1 and day 2. Even at the earlier time point, day 1, which is prior to cell loss, we were unable to detect AnnexinV+ cells, suggesting that ODSC-mediated Th17 inhibition does not involve NO-mediated apoptosis.

Figure 15. Expression of Annexin V and 7-AAD of Th17 cells co-cultured for two days with and without ODSCs. Cells were harvested, and stained according to manufacturer's protocol (eBioscience). Day 2 data is shown here. n = 2.
Omentum causes T-cell anergy by blocking TCR-mediated Ca$^{2+}$ flux

T-cells are considered anergic when they do not respond to stimulation by their T-cell receptor (TCR). Stimulation of T-cells is critical to proliferation and cytokine expression. Broadly, T-cell stimulation involves TCR pathway and involves two key signaling events: activation of MAP kinases, and calcium flux from the ER. These events are required for effective Nuclear factor of activated T-cells (NFAT) signaling, a transcription factor coordinating cytokine production and proliferation in T-cells.

We hypothesized that ODSC inhibition of Th17 involves induction of T-cell anergy. Establishing a state of anergy could explain the cytokine defect we observe in our Th17 cells after ODSC-co-culture. To test this, we determined if Th17 cells after co-culture with ODSCs could calcium flux in response to concanavalin A (ConA). ConA is a classic T-cell mitogen that is TCR-dependent. Upon addition of ConA, TCR-activation leads to a well-described signal transduction pathway where phosphorylation of immunoreceptor tyrosine-based activation motif (ITAMS) on the TCR-CD3 complex by Src family kinases, Lck and Fyn enable recruitment and activation of Zap70. Zap70 can then phosphorylate Lat, which acts as an adaptor protein to recruit other proteins including Itk to phosphorylate and activate PLCγ1. PLCγ1 then plays the key function of hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP3) to generate intracellular messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 binds to IP3 receptors on the ER, which in turn results in calcium efflux from the ER into the cytosolic space. This flux leads to a low calcium environment in the ER which is sensed by a protein called STIM1. STIM1 will promote oligomerization of ORA1, which causes CRAC pores to form on the plasma membrane, permitting extracellular calcium flux into
the cell. The coordination of these processes leads to calcineurin activation, dephosphorylating NFAT and permitting its translocation into the nucleus (Joseph et al., 2014).

To test the effects of ODSCs on calcium signaling, we co-cultured ODSCs with Th17 cells as previously described. After 1-day co-culture, we harvested and loaded cells with Fluo-3, a calcium indicator. Fluo-3 will increase in fluorescent intensity when bound to calcium. Since Fluo-3 can only measure bound calcium, we also tested our model using BTC/AM (Invitrogen), a ratiometric calcium indicator that measured unbound and bound calcium. To induce TCR-mediated calcium flux, we treated cells with ConA. Th17 cells fluxed when ConA was added (Fig. 16A). Interestingly, Th17 cells co-cultured with ODSCs were unresponsive to ConA suggesting that TCR-dependent calcium flux was impaired (Fig. 16A). Generally, we observed that our baseline levels of calcium were higher in ODSC treated cells, suggesting more Fluo-3 was loaded into these cells. Our data obtained with BTC/AM confirmed these findings, as ratiometric analysis corrected the differences in loading (not shown). BTC/AM staining, however, was not sensitive enough to pick up primary cell Ca^{2+} flux in T-cells, and could not be used for testing ConA.

We further characterized our calcium defect by testing to see if ODSC-treated Th17 cells could respond to thapsigardin, a sarco-endoplasmic reticulum calcium ATPase (SERCA) inhibitor. Thapsigardin prevents calcium from being pumped into the ER, leading to low calcium stores, and ultimately extracellular calcium flux by CRAC. If ODSC-cultured Th17 cells can respond to thapsigardin, then it suggests that the Ca^{2+} defect is not due to impairment of the mechanism of Ca^{2+} release from the ER. Addition
of thapsigardin, demonstrated that ODSC-cultured Th17 cells can still respond, demonstrating that Ca\(^{2+}\) efflux from the ER and STIM1 detection of low Ca\(^{2+}\) stores were still intact (Fig. 16B). This suggests there could be a defect upstream from TCR-activation to the activation of IP3 channels on the ER.
Figure 16. Evaluation of calcium flux in Th17 cells. A. Representative plot showing intensity of fluo-3 as measured on FACS Fortessa over time. Th17 cells (black) were given concanavalin A (black arrow). Th17 cells co-cultured with ODSCs (blue) were given concanavalin A (black arrow). n = 4. B. Representative plot showing intensity of fluo-3 as measured on FACS Fortessa over time. Th17 cells (black) were treated with thapsigardin followed by ionomycin (black arrow). Th17 cells co-cultured with ODSCs (blue) were treated with thapsigardin followed by ionomycin (black arrow). n = 3.
Based on these findings, we investigated the pathway of TCR-signaling up to the point of IP3 activation of IP3 channels to determine how the pathway was impaired. We hypothesized in particular, that CD3ζ would be disrupted, as CD3ζ downregulation or disassociation has been demonstrated in MDSC induction of anergy (Nagaraj et al., 2011). Downregulation of CD3ζ disrupts the TCR-signaling pathway as it is required for the assembly and surface expression of the TCR. To do this, we looked at protein expression of key signaling molecules, CD3ζ, Itk, Lck, Lat, and PLCγ, to determine if they were changed in expression. We examined CD3ζ by FACS and Amnis one-day after co-culture. Compared with Th17 only samples, we did not observe a decrease in the intensity of CD3ζ (Fig. 17B). Qualitatively, CD3ζ’s staining pattern seems similar, suggesting that CD3ζ localization in Th17 cells may not be affected (Fig. 17A)
Figure 17. Evaluation of CD3ζ in Th17 cells co-cultured with and without ODSCs. Cells were co-cultured for 1-day, fixed, permeablized, and stained for CD3ζ and CD4. A. Representative images taken with Amnis Imagestream shown for n = 2. B. Histograms shown for CD3ζ staining gated on CD4⁺ cells.
We examined other members of the TCR-pathway by protein expression. To examine Itk, Lat, Lck and PLCγ we generated whole cell lysates at day 1 and performed western blots to determine protein expression. Examination of expression of Lat and Lck suggested they may be slightly downregulated under co-culture conditions, while PLCγ levels are unchanged (Fig. 18). Itk and PLCγ appears higher in expression in Th17 +OC samples compared to Th17 samples. Collectively, these suggest that major proteins of the TCR-pathway appear to be mostly intact. Downregulation of Lat and Lck was observed and more significantly in Lat, making Lat downregulation a possible target.

We also examined NFATc1, one of the members of the NFAT family that is downstream of the Ca^{2+} signaling pathway. NFATc1 protein expression appeared relatively higher in Th17 +ODSC conditions (Fig. 18). This demonstrated that NFAT protein levels were intact and present for signaling. Collectively, these data suggest that ODSCs does not generally affect protein expression as a means of induction of anergy in Th17 cells with the exception of Lat, which had a more noticeable decrease. Further evaluation of Lat is needed to determine if that decrease could lead to an anergic phenotype.
Figure 18. Western blot analysis of TCR-pathway proteins. Whole cell lysate was generated from 1-day co-culture and non-co-culture samples. Western blot was performed as described in methods. Lanes were loaded according to cell number.
Since we have previously demonstrated NO is a mediator of Th17 inhibition, we determined if induction of anergy was dependent on NO. To do this, we added iNOS inhibitor to determine if addition of iNOS inhibitor to Th17 co-culture with ODSC would rescue cells from anergy. Blockade of iNOS did not rescue anergy, suggesting that this is a mechanism independent of the NO pathway (data not shown). Given that induction of anergy occurs before Th17 cytokine inhibition can be observed, anergy may still play a critical role in Th17 inhibition.

There are other mediators that could be potentially secreted by ODSCs to induce anergy. For example, TGFβ addition to CD4+ cells in vitro can reduce responsiveness to TCR-dependent calcium flux (Chen et al., 2003). Since we have demonstrated that TGFβ contributes specifically to inhibition of IL-2 expression in Th17 cells, TGFβ secretion could be a possible cause for anergy.

**Omentum affects the forward and side scatter of Th17 cells**

Since protein expression of the TCR-pathway did not seem to be drastically altered, we examined broader changes in cells following ODSC-co-culture that could possibly provide us explanations for Th17 anergy. One observation we made was that co-culture of Th17 cells with omentum affects the forward and side-scatter of cells.

Classically, forward and side scatter refer to the deflection of laser light that occurs when they hit the cell. Side-scatter (SSC) is a measurement of how much light deflects at a 90° angle when it is shone through a cell. Changes in side scatter can correspond to changes in cells, including decondensing of chromatin, or correlations to
protein levels within cells (Shapiro, H. M., 2005). It is important however, to acknowledge SSC changes are non-specific and can reflect a variety of processes occurring within the cell. Our initial observations were made by FACS cytometry, as we noticed Th17 cells specifically exhibited higher side scatter after co-culture with ODSCs (Fig. A, blue depicts cells co-cultured with ODSCs, grey without). This increase was specific to Th17 cells as activated T-cells under neutral conditions did not significantly increase in SSC after co-culture with ODSCs (Fig. 19A). We further examined this phenotype by running cells through the Amnis Imagestream. The Amnis Imagestream is a flow cytometry capable of visually capturing SSC changes. Images of Th17 cells without co-culture exhibited fewer punctate structures, whereas Th17 cells co-cultured with ODSCs, had more punctate structures (Fig. 19B). Visually, we speculate these alterations could reflect changes in membrane or granular structures, and will be discussed further in the next subsection.

We also examined the size of Th17 cells with and without co-culture with ODSCs. While forward scatter (FSC) is typically referred to as changes in size, they cannot be used to predict size accurately because the absorption of light through a larger object prevents there being a linear relationship for FSC and area (Shapiro, H. M., 2005). In addition, fixed cells do not represent the size of live cells, as osmotic changes after swelling can affect FSC. To avoid these issues, we examined cell size on Amnis Imagestream, which can observe cell size as a function of area on brightfield. Qualitatively, live CD4^+ cells captured at 40X appeared larger consistently after co-culture with ODSCs (Fig. 19B).
Figure 19. Evaluation of side scatter in co-cultured Th17 cells. A. Increase in side scatter of neutral and Th17 cells co-cultured with ODSCs for 2-days. Cells were restimulated with PMA/Ionomycin and evaluated by flow cytometry. Representative FACS plot of n = 5 is shown. Graph shows cumulative data demonstrating increase in SSC of Th17 cells with and without ODSCs. ***denotes p < 0.0005. B. Three sample
brightfield and SSC images (pink) shown of cell images taken on Amnis Imagestream of CD4+ gated cells with and without ODSC co-culture.
**Omentum Affects ER Homeostasis in T-cells**

Calcium flux changes can be due to alterations in ER homeostasis (Jia et al., 2011). Alterations in ER homeostasis can be due to ER stress, which can result from an accumulation of misfolded proteins within the ER. Since the endoplasmic reticulum acts as the primary storage for calcium in T-cells, we examined if omentum could affect the endoplasmic reticulum and calcium signaling. To test this, we examined ER staining patterns in Th17 cells using ER Tracker Green (Invitrogen). Live cells were harvested with and without omentum, stained for ER Tracker Green and CD4 or CD90.2, and analyzed by fluorescent microscopy or Amnis Imagestream to determine levels of ER Tracker Green.

Amnis Imagestream takes unifocal images of bulk cells. Analysis of images captured revealed that the ER pattern of staining looked similar, forming a circular crescent structure within the cell in both Th17 cells co-cultured with and without omentum (Fig. 20B). These were confirmed by fluorescent microscopy, where we observed a similar distribution of ER Tracker in CD90.2+ cells under both conditions (Fig. 20C). However, measurement of ER tracker intensity demonstrated that the cells ER intensity was increased in cells co-cultured with ODSCs (Fig. 20A).

The increase in ER tracker shows that ER homeostasis is increased in T-cells by ODSCs. While we do not know the direct cause of the ER stress, an alteration in ER homeostasis can lead to an increase in Ca\(^{2+}\) storage or potentially ineffective storage of Ca\(^{2+}\). Changes in Ca\(^{2+}\) storage can affect NFAT activation profoundly, as effective NFAT phosphorylation requires prolonged concentrations of intracellular calcium (Joseph et al., 2014). If NFAT signaling is impaired, IL-17A expression can be impaired.
as NFAT is known to bind and promote IL-17A expression (Gomez-Rodriguez et al., 2009). NFAT has also been demonstrated to be important for IL-2 expression (Chow et al., 1999). Ineffective NFAT signaling could explain the phenotype we observe in ODSC-treated cells, as expression of IL-17A and IL-2 is impaired in these cells.
Figure 20. Visualization and measurement of ER in Th17 cells co-cultured with ODSCs.

A. Representative histogram showing changes in expression of ER Tracker following co-culture with ODSCs after 2-days. Cells are gated on CD4+ marker. n = 2. B. Representative images showing ER Tracker staining from Amnis Imagestream. Two cells shown for either condition. Cells are gated on CD4+ marker. C. Representative images taken using fluorescent microscopy shown. Cells were immobilized onto poly-lysine coated coverslip, and stained with ER tracker. Representative image of two experiments shown.
ODSCs Causes Upregulation and Alterations in Localization of IRF4

Interferon regulatory factor 4 (IRF4) – formerly known as lymphocyte-specific interferon regulatory factor (LSIRF) was found to be critical for mature B-cell / T-cell development in 1997. Mittrucker and colleagues characterized IRF4 knockout mice, they found the T-cells had impaired proliferation and cytokine in response to TCR stimulation. Further examination of Th-subsets revealed defects in both Th1 and Th2 differentiation under IRF4 deficient conditions. Analysis of Th17 differentiation in IRF4 deficient cells also demonstrated defective IL-17A and IL-22 expression. Investigators found that lack of IRF4 resulted in a skewing toward FOXP3 expressing cells in lieu of RORγt expressing cells under Th17 conditions (Brüstle et al.). The mechanism for this skew may be due to impaired IL-6 and IL-21 expression in IRF4 deficient T-cells (Mudter et al., 2008, Huber et al., 2008). Interestingly, PGE2 also inhibits DNA binding and expression of IRF4, which results in specific IL-17 blockade (Valdez et al., 2012).

Due to the important role of IRF4 for the differentiation of Th17, and the use of PGE2 by ODSCs in our system, we examined the localization and expression of IRF4 to determine if ODSC impaired IRF4 in Th17 cells. Using FACS analysis, we examined expression of IRF4. IRF4 expression was found consistently increased (Fig. 21A). To validate our increase in expression, we also examined upregulation of mRNA by qRT-PCR of RNA isolated from FACS-sorted CD4⁺ cells for co-cultured and non-co-cultured Th17 cells. Our findings coincided with FACS data, as IRF4 transcript was upregulated in Th17 cells co-cultured with ODSCs (Fig. 21B). To determine the localization of IRF4, we utilized the Amnis Imagestream X to take unifocal images of individual cells. Gating
on CD4^+ cells with nuclear stain, we found that IRF4 was regularly localized in a perinuclear area in discrete punctate staining (Fig. 22). When Th17 cells are exposed to ODSCs, IRF4 was no longer present in punctate staining but appeared as bright diffuse staining throughout the cell. Concurrent with our FACS and qRT-PCR data, IRF4 appears upregulated by qualitative analysis.

These data are consistent with dysregulation of IRF4 being a potential mechanism for dysfunctional Th17 phenotype. Abnormal upregulation and localization at an inappropriate time may impact Th17 cells detrimentally.

We tested to determine if upregulation of IRF4 in our model is NO dependent. Addition of DETA-NONATE, an NO donor, was insufficient to upregulate IRF4. These observations are supported by Niedbala and colleagues, who also did not see any changes in IRF4 expression upon exposure to NO donor. Inhibition of iNOS also did not decrease IRF4 expression in ODSC-treated Th17 as expected, if the process were NO-dependent.

The mechanism behind ODSC upregulation of IRF4 in Th17 cells has not been identified. Further studies to detail the mechanism of this dysregulation and tie it to function are needed to determine the role of this unique observation.
Figure 21. Upregulation of IRF4 in Th17 cells following co-culture with ODSCs. A. Representative histogram showing changes in expression of IRF4 following co-culture with ODSCs after 2-days. Th17 cells alone shown in red, Th17 co-culture with ODSCs shown in blue. Cells are gated on CD4+ marker. Chart shows average of MFI. * designates p < 0.05. n = 6. B. qRT-PCR results on mRNA from Th17 cells and Th17 cells co-cultured with ODSCs. After co-culture CD4+ cells were FACS sorted to ensure
T-cell specificity. mRNA was isolated and converted to cDNA. IRF4 data is presented as relative change normalized to housekeeping gene OAZ1. Representative data from 2 experiments performed with triplicate values is shown.
**Figure 22.** Visualization of IRF4 localization in Th17 cells cultured with ODSCs.

Representative images of cells from Amnis Imagestream X showing changes in expression and localization of IRF4 following co-culture with ODSCs after 2-days. Cells are gated on CD4+. n = 2.
Discussion

We have identified two models of ODSC-mediated Th17 inhibition. The predominant mechanism is by nitric oxide. In our model, a subset of Th17 cells produce IFNγ in addition to IL-17A. The secretion of IFNγ causes specific upregulation of iNOS in ODSCs. Close contact of these ODSCs and Th17 cells then permits passive transfer of NO into Th17 cells. The generation of NO arrests cells and contributes towards cell loss (Fig. 23). NO then potentiates COX-2 activation resulting in PGE2 secretion. PGE2 acts to block IL-17A expression.
Figure 23. Proposed Model of Th17 Inhibition. IFNγ produced by Th1-Th17 cells leads to expression of iNOS. Release of NO leads to cell cycle arrest. NO also potentiates COX-2, resulting in PGE2 release. PGE2 release specifically affects IL-17A expression in Th17 cells.
The role of NO in inhibition of T-cells has been a long-standing concept, but recent studies have also demonstrated that NO can be important in induction and stability of Th17 cells (Obermajer et al., 2013). The key difference in behavior is explained by the dose of NO received by cells. Obermajer and colleagues demonstrated that NO promotes Th17 conditions at around 25-50 uM of NO donor, DETA-NONate, and inhibits Th17 when produced at higher ranges 100-200 uM of NO donor. Our co-culture model certainly points to a fairly high dose of NO donor, as upwards of 500 uM is needed to replicate the effect seen by ODSC. However, given the presence of other inhibitory mediators, we speculate that ODSCs release a lower dose of NO with inhibitory effects amplified by other immunomodulators like PGE2 and TGFβ which have both been shown to be involved in our system.

Although nitric oxide has been demonstrated to be cytostatic and cause apoptosis, it does not appear apoptosis is the mechanism of cellular death following ODSC inhibition. We speculate that NO is likely causing cell cycle arrest, leading to cellular death, but the mechanism involved is not apoptosis.

Besides loss of T-cells and blockade of cytokine production, the other model of impairment we identified was induction of anergy of Th17 cells. In our model, anergy can be observed as early as 16 hrs after co-culture. Blockade of iNOS does not restore Ca²⁺ responsiveness, demonstrating the induction of anergy is NO-independent. ODSCs likely through other mediators such as TGFβ, may generate this anergic phenotype. It is possible that this early state of anergy coupled with additional insult by NO and PGE2 cause the Th17 cells to be particularly susceptible to cell death.
While we have not identified a sole mechanism involved in induction of anergy, our data supports several possibilities. Since we have demonstrated that TGFβ plays a role in our system, studies have demonstrated TGFβ to be capable of inhibiting phosphorylation of itk, resulting in Ca\(^{2+}\) flux impairment, and lack of NFATc activation (Chen et al. 2003). TGFβ also affects this pathway transcriptionally, as it is capable of upregulated miR-155, which has been demonstrated to downregulate IL-2 and itk mRNA (Das et al., 2013). While we did not observe changes in protein levels of itk, changes in phosphorylation of itk could still be a possible explanation.

We propose that altered ER homeostasis is the cause of anergy in our system. We have demonstrated surviving T-cells possess enlarged ER space. This points to possible stress induced expansion of ER. Alterations of ER have an important impact on Ca\(^{2+}\) signaling, as a larger ER could result in potentially a larger calcium storage. Another possibility is that if ER is enlarged, preventing STIM1, the ER sensor for low calcium, from oligomerizing, then the threshold activation required for CRAC channel formation could be higher. This would be prohibitive to flux, and subsequently impair NFAT translocation (Joseph et al. 2014).

Dysregulation of IRF4 is another phenotype observed in surviving cells. The increased expression combined with changes in localization suggest IRF4 is no longer signaling effectively. We originally analyzed IRF4 expression because it is known to be necessary for Th17 differentiation and can work in conjunction with RORγt to promote IL-17A expression. These changes suggest that this dysregulation could also generate a possible Th17 defect. However, one key observation we made was that inhibition of nitric oxide pathway by addition of iNOS inhibitor does not reverse the upregulation of
IRF4 observed in Th17 cells. Therefore, cells that are co-cultured with ODSCs will upregulate IRF4, even if inhibition of IL-17A cytokine production and cell loss does not occur. This suggests that IRF4 upregulation is not involved in Th17 inhibition.

So how can we reconcile IRF4 upregulation? What is known about IRF4 in Th17 is that it is upregulated following TCR-activation and IRF4 binds directly with BATF, a part of the AP-1 family of transcription factors, to permit access to other important transcription factors such as STAT3, c-Maf and RORgt (Ciofani et al., 2012). It is possible ODSC provides signals that aberrantly activate IRF4 upregulation, but the pro-Th17 inducing effects are constrained by immunomodulators, NO and PGE2. Further studies are needed to determine what factor(s) cause IRF4 upregulation in T-cells that survive ODSC co-culture, and the biological function of this IRF4 upregulation.

In summary, ODSC co-culture has multiple effects on Th17 cells. Early events involve deactivation of Ca\(^{2+}\) signaling, a key component of T-cell proliferation and cytokine production. Then, later events involve generation of NO and PGE2 which results in cell loss and blockade of cytokine expression. The combination of these effects results in potent Th17 inhibition. We speculate omentum may act biologically as a regulatory organ within the gut to reign in overactive responses. Since Th17 cells are implicated in many diseases of the gut such as Crohn’s disease and inflammatory bowel disease, it may be of interest to see if omentum plays a role in reigning in these cells in vivo. Basic science studies should explore further the role of activation of omentum and its relationship with pathogenic Th17 cells within the gut.
CHAPTER THREE
OMENTUM AS A CELLULAR THERAPY
FOR
ALLOGENIC LUNG TRANSPLANTATION

Introduction

The Clinical Problem

The first successful single-lung transplantation was performed in 1983 by the Toronto Lung Transplant Group in patients with end-stage pulmonary fibrosis (Grossman et al., 1990). This was followed by the first successful bilateral lung transplantation in 1985 (Patterson et al., 1988). Since then, the use of lung transplantation continues to grow, with 3,747 lung transplants documented by the International Society of Heart and Lung Transplant (ISHLT) Registry for the year of 2013 (ISHLT Registries - Heart/Lung Registries, 2014). Lung transplantation has an important role in clinical medicine, oftentimes the only treatment for end-stage diseases. Currently, the five top indications for lung transplantation include chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), cystic fibrosis, alpha1-antitrypsin deficiency emphysema and idiopathic pulmonary arterial hypertension (Christie et al., 2010).

For the first decade of lung transplantation from 1988-1994, the median survival was roughly 5 years. In the current era, 1994-2011, the median survival for all lung transplants remains around 5.6 years, only a marginal benefit compared to the last decade, with most improvements to survival primarily due to refinement in surgical
technique (ISHLT, 2013). Despite advances in short-term outcomes, methods to improve long-term outcomes for patients have only had limited success.

The primary cause of long-term graft failure is bronchiolitis obliterans syndrome (BOS), a progressive airway obstruction that results from airway fibrosis. While BOS is generally thought to be immune-mediated, the pathogenesis has not yet been identified (Todd & Palmer, 2011). Currently, long-term treatment of patients involves induction therapy, a strategy of employing immunosuppressive drugs to minimize allograft rejection. Studies involving induction agents have only limited effects on patients, and long-term survival remains the same when comparing patients who receive induction agents and patients that do not (Sweet S.C., 2013). The inability for induction agents to prevent rejection in the lung suggests the lung rejects differently from other organs, as induction therapy successfully prevents organ rejection and prolong survival in other organ transplants. As it stands, there are currently no strong recommendations for induction therapy (Saldanha et al., 2013).

The main clinical problem of lung transplantation continues to be the question of how to improve long-term outcomes. Two broad directions in the research field have been actively pursued to address this: (1) identification of pathogenesis and (2) the development of an animal research model to test therapies upon.

**Mouse model**

The mouse model of allogenic lung transplantation was developed to fulfill the need in the lung transplantation research field for a low-cost, reproducible animal model system. Advantages of this model include a much lower cost than primate or rat models,
and also access to the array of developed genetic approaches that are available in the mouse model (Okazaki et al., 2007). Two groups, Jungraithmayr and colleagues, and Krupnick and colleagues have made considerable improvements to the original mouse model since its inception, reducing surgical time and mortality which has allowed for more consistent and reproducible results.

In brief, our lab employs a modified version of the transplant protocol from both groups. We perform a left lung transplant that simplifies the complexity that comes from transplanting a right lung in the mouse, which consists of multiple lobes and is constrained by access. We intubate the donor to ventilate the lung during the procedure. The left ventricle is cut to allow injection of heparin to prevent blood clotting and drain blood from the organ prior to transplant. The heart-lung bloc is then dissected from the chest cavity and removed. The vasculature is dissected out. To permit anastomoses, vessel cuffs are made, and vasculature is secured over the cuffs, to facilitate insertion and suturing. The recipient mouse undergoes anesthesia by injection of ketamine and xylazine with minimal amounts of isofluorane only used as needed to maintain anesthesia. To prepare the recipient mouse, an incision is made between the 3rd and 4th intercostal space. We employ rib spreaders to gain access to the chest cavity, gently removing the native left lung, which when extracted from the chest cavity permits surgical access to the vasculature. Separation of the blood vessels is a critical step, as any mishap will result in a failed transplant. By using blunt-forceps, vessels can be separated to allow suture placement around the vessels to then restrict blood flow. Air flow through the bronchus can be blocked using a micro-clamp. At this point, incisions can be made into the vasculature and bronchus to permit insertion of prepared cuffs. This allows
attachment of the donor vessels. Then using 10-0 nylon suture (Ethicon), cuffs from the
donor vessels can be tied to the recipient vasculature and bronchus to permit blood and
air flow from recipient to donor. After this, native vessels can be unclamped and untied
to permit reperfusion of the transplanted lung. The native lung can be excised and
discarded and the donor lung is gently placed into the chest cavity, and allowed to return
to its natural orientation. The chest wall can be closed, and sutured. Afterwards, the
mouse is given pain medication to manage discomfort, and additional fluids if needed.

As described in the Krupnick lab, the lung transplantation procedure requires
basic microsurgical skills that can be acquired with months of training. For our lab,
approximate time for transplant can be completed in roughly 1.5 hrs for preparation of
donor lung, with 2 hrs allocated to the transplantation process accommodating for
anesthesia induction as well as post-surgical monitoring. Complications can arise if
vasculature is blocked or torn. Acute rejection occurs rapidly in this model under full
histocompatibility mismatch and can be observed as soon as 14-days after transplant.

COMPLICATIONS + TROUBLESHOOTING

Use of Omentum in Transplantation

Since activated omentum is predominantly made up of cells with a mesenchymal
stem-cell like phenotype and myeloid derived suppressor cells, we hypothesized that
omentum could potentially be used as cellular therapy in the mouse model of allogenic
lung transplantation.

Both populations within omentum have been demonstrated to have positive
effects in transplantation. Mesenchymal stem cells have the capability to home into
injured tissues and provide regenerative factors. They are considered naturally immuno-
privileged cells that do not illicit immune response due to low expression of MHC Class
II and co-stimulatory ligands CD40, CD80, CD86 (Chamberlain et al., 2007).

MDSCs are immunosuppressive by definition, and were originally discovered in
the context of cancer. Cancers were thought to enhance their survival by promoting local
accumulation of MDSCs to mask the tumor from adaptive immune responses by the host.
MDSCs. The potential for use of MDSCs as therapy particularly against T-cell mediated
rejection in transplantation is a novel concept that has received some attention only
recently. Performing pancreatic islet transplantation model into diabetic mice with bone-
marrow derived MDSCs given as cellular adjuvant significantly extended graft survival
in an NO-dependent manner (Arakawa et al., 2014). The potential benefits of nitric oxide
have been demonstrated in vivo in a rat renal transplant model as inhibitors of inducible
nitric oxide synthase aggravate alloreactive immune-mediated injury (Stojanovic et al.,
1996).

We hypothesized the combination of MSCs and MDSCs provided by the
omentum may promote a tolerance-promoting environment that can provide therapeutic
benefit to mice undergoing allogenic lung transplantation.

The route of administration we chose was to provide cells by intraperitoneal
injection. Ortiz and colleagues have utilized intravenous injection of MSCs into a
bleomycin lung injury model and found donor cells within the lung (Ortiz et al., 2003)
Likewise, our lab has transferred GFP+ omentum from a mouse expressing GFP in non-
hematopoietic tissues in a bleomycin lung injury model, and was able to detect presence
of GFP\(^+\) cells within the lung, demonstrating that trafficking of omental cells can occur in vivo (Shah et al., 2012).

**Th17 and Transplant Rejection**

Th17 cytokines have been associated with acute rejection and bronchiolitis obliterans syndrome (BOS) following lung transplantation in human patients (Vanaudenaerde et al., 2008). Acute rejection of the lung can occur when there is an auto-immune response against formerly cryptic antigens. In one model, collagen V-sensitized lymphocytes were transferred into a mouse inducing a similar pattern of acute rejection seen in lung transplantation (Braun et al., 2009). Data from human transplant patients have also demonstrated the presence of collagen V reactive lymphocytes expressing IL-17A (Yoshida et al., 2006). Other models have examined a different auto-antigen, K-\(\alpha\)1 tubulin (K-\(\alpha\)1T) in human patients, and determined in similar fashion that PBMCs responding to this antigen proliferated more and produced IL-17A in patients with BOS, than those without (Saini et al., 2011). Neutralization of IL-17A in the murine model of allogenic lung transplant, reduced rejection scores (Fan et al., 2011). Together these studies implicate a role for IL-17A\(^+\) lymphocytes in the process of acute and chronic rejection (Burlingham et al., 2007, Braun et al., 2009).

**Results**

**ODSCs reduce Airway Inflammation**

To test the effects of ODSC as a form of cellular therapy we employed the model of mouse allogenic left lung transplantation as previously described (Okazaki et al.,
2007). In this model, we performed syngenic transplants (H2b -> H2bd) and allogenic transplants (H2a -> H2bd) B10A (Table 1). Allogenic transplants had a full-mismatch of MHC Class I and II. In the human literature, MHC Class I and II are referred to as human leukocyte antigen (HLA) when describing the protein. Major mismatches of HLA in human studies have demonstrated a reduction in survival and increased incidence of BOS (Peltz et al.). Lung selection criteria currently are based on matching HLA to reduce hyperacute rejection, where preexisting antibodies against HLA, result in rapid rejection of the lung. In our model, we intentionally exploiting MHC mismatch to produce acute rejection. In contrast to other mouse models, we do not provide immunosuppressive drug regimen post-transplantation.

<table>
<thead>
<tr>
<th>Response</th>
<th>Strain</th>
<th>Degree of histoincompatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngenic</td>
<td>C57BL/6 (H2b) → CB6F1/J (H2d/b)</td>
<td>Low**</td>
</tr>
<tr>
<td>Allogenic</td>
<td>B10.A-H2a H2-T18a/SgSnJ (H2a) → CB6F1/J (H2d/b)</td>
<td>High</td>
</tr>
</tbody>
</table>

Table 1. Transplantation strategy based on major histocompatibility mismatch.

**Potential for graft vs host against H2d.

We administered 1 x 10^6 of ODSCs on day 1 and day 7 following transplant by intraperitoneal injection. Non-treated groups were given an equal volume of saline as a control. At day 14, when acute rejection was underway, we sacrificed the mice, inflating the lungs with OCT. We dissected the lung bloc and snap freeze the specimen for further histopathology. Lungs were graded by the 2007 International Society for Heart
and Lung Transplantation (ISHLT) classification, the current standard for human lung transplant (Stewart et al., 2007, Table 2)
<table>
<thead>
<tr>
<th>A: Acute Rejection</th>
<th>B: Airway Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0 None</td>
<td>B0 None</td>
</tr>
<tr>
<td>A1 Minimal</td>
<td>B1 Low grade</td>
</tr>
<tr>
<td>A2 Mild</td>
<td>B2 High grade</td>
</tr>
<tr>
<td>A3 Moderate</td>
<td>BX Ungradeable</td>
</tr>
<tr>
<td>A4 Severe</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Classification and Grading of Pulmonary Allograft Rejection. The criteria here demonstrates how classification is defined as acute rejection (A), generally based on the extent and degree of mononuclear infiltrate. Airway inflammation (B), is generally based on mononuclear infiltration to the submucosa of the bronchioles (Stewart et al., 2007)

Syngenic transplants showed a base level of mild to moderate acute rejection (A2-A3) and none to low grade airway inflammation (B0-B1) (Fig. 24-26). Mice that received allogenic transplantation with saline had moderate to severe acute rejection (A3-A4) with low to high grade airway inflammation (B1-B2). When given ODSC treatment, ODSC-treated mice had a broader range of acute rejection ranging from minimal to severe rejection (A1-A4), although not statistically different when compared to the syngenic or the allogenic group (n = 14, N.S.). Airway inflammation was significantly reduced in the ODSC-treated condition ranging from none to mild (B0-B1) with only one mouse graded at high (B2) (Fig. 25)
Figure 24. Acute rejection (A) histological grading of left lung samples. Left lungs were isolated, formalin fixed, and H&E stained. Grading was according to ISHLT classification. * denotes significant difference $p = 0.0300$ for syngenec and allogenic pairs. $p = 0.3557$ for allogenic + OC and syngenec and $p = 0.1221$ for allogenic and allogenic +OC pairs. n = 5-13.
Figure 25. Airway inflammation (B) histological grading of left lung samples. Left lungs were isolated, formalin fixed, and H&E stained. Grading was according to ISHLT classification. * denotes significant difference, p = 0.0122. ** denotes highly significant difference, p = 0.0034. p = 0.5850 for syngenic and allogenic +OC pairs. n = 5-13.
Figure 26. Representative histology of left lung samples (1 from each group).
While administration of omentum does not significantly block acute rejection (n.s), these findings demonstrate that omentum has an effect on airway inflammation, possibly through the anti-inflammatory effects of the cellular subsets.

**ODSC administration decreases T-cell Infiltration of Lung in vivo**

Having demonstrated the efficacy of ODSCs on inhibition of Th17 cells in vitro, we set out to determine if ODSCs had the ability to inhibit T-cells in an in vivo model of allogenic lung transplantation. To do this, we utilized a knock-in mouse to monitor T cell activation in live animals (Yamamoto et al., 2013). In these mice, T-cells that have produced IL-2 produce Cre recombinase. The production of cre results in an irreversible deletion of an early stop codon in front of the luciferase gene, permitting luciferase expression in these IL-2 producing cells. As a result, cells that have produced IL-2, will express luciferase. These cells can then be captured in a live mouse by injection of the substrate luciferin, and visualized using a high sensitivity CCD camera (Xenogen 200).

We used this system (IL-2Cre x Rosa26Luciferase mouse) to track activated T-cell aggregation in transplanted mice. We determined basal levels of luminescence in mice by mock surgery in which the thoracic cavity of the mice was opened but no transplant was performed. These mice may have some residual activation within the axillary lymph node, but it generally does not spread to the lung. To test the effect of ODSCs in the transplant model, we then performed allogenic transplants in mice treated with saline or ODSCs are previously described, imaging them over the course if several weeks. In mice treated with omentum, the luminescence qualitatively was lower compared to mice without omentum administration (Fig. 27). In the left thoracic region
we can see diminished signal over the chest cavity, likely reflecting activated T-cells within the left lung. The distinct circular hotspot (red) seen when the mouse is turned on its side, likely represents cells aggregating in the axillary lymph node. The aggregation of activated T-cells to the lymph node does not seem diminished with omental cell treatment. These data demonstrate it is possible for ODSCs to inhibit T-cell activation in vivo. There still remains many questions however, regarding how long these ODSCs can survive in the lung, if they are capable of trafficking and retaining in lung tissue, and whether they have specific inhibitory properties in vivo. Further studies are needed to address these questions.
Figure 27. Bioluminescence of IL-2 expressing T-cells in mice that received saline or ODSC therapy on day 1 and day 7. Mice were imaged on day 14. Representative images shown here for a 2 min exposure, after intraperitoneal injection of D-luciferin.
ODSCs do not Affect Secondary T-cell responses

In our allograft model, T-cells will respond to the alloantigen presented by the donor lung due to MHC mismatch (H2a in H2b/d host). As a consequence of activation, T-cells will traffic to secondary lymph organs like the spleen and lymph node where populations are maintained for secondary response. We analyzed if ODSCs had any effect on secondary T-cell responses. We hypothesized that if ODSCs had the potential to reduce levels of T-cell activation in the lung, then secondary T-cell responses would likely be reduced as well.

To test this, we isolated CD4$^+$ cells from mice that had undergone allogenic transplantation on day 14 that were either treated with saline or ODSC cells at day 1 and day 7 post transplantation. A mouse that had not undergone transplantation was used as a control. To test memory responses, we performed mixed lymphocyte reactions (MLR), where we either cultured them with irradiated APCs from a syngenic B6 host (H2b) or an allogenic B10A host (H2a). Co-culture with syngenic APCs should elicit no response as they should have tolerance to self-antigen, where as co-culture with allogenic APCs should elicit a memory response as these mice have already been sensitized from the allogenic left lung. We performed ELISPOT on CD4 cells for IL-2, IFNγ, and IL-17A, with our prediction being that ODSC treated mice would have reduced memory responses to the respective cytokines based on our in vitro data.

Analyzed data revealed little differences between saline vs ODSC treated in all groups. In general, measured IL-17A response was similar for B6 or B10A, suggesting no memory response was being invoked (Fig. 28). Furthermore, the control mouse (non-
transplanted) had similar responses to the lung-transplanted mice. These data suggest that memory Th17 cells are not stimulated by alloantigen in our mouse model. IL-2 expression could be detected in control mice exposed to B10A APCs, suggesting a primary response. IL-2 expression was further elevated in lung transplanted mice, demonstrating the presence of a memory response. However, no differences in the allogenic response could be detected between saline treated (LT) or ODSC-treated (LTO) groups (Fig. 28). IFNγ expression was not observed in the control mice to B6 or B10A APCs, suggesting that primary response did not evoke IFNγ expression. Memory responses to B10A APCs were observed in the saline treated and ODSC-treated mice. However, no differences were detected between the two groups (Fig. 28).

ODSCs do not affect memory T-cell responses in our mouse lung transplantation model. We conjecture that the failure to inhibit T-cells within the secondary lymph organs may due to the limited efficacy of ODSC cells in vivo. Since we provide only two doses by intratracheal administration, it is likely these cells only have limited action within the lung tissue as they are dependent on cell-to-cell contact, and use nitric oxide release as their primary mechanism. Secondary T-cell responses may thereby remain intact due to sensitization and accumulation of cells outside the lung.
Figure 28. Mixed lymphocyte reactions of splenic CD4+ cells following 3-day co-culture with irradiated APCs from a B6 mouse (syngenic) or B10A mouse (allogenic). Response was evaluated by ELISPOT for IL-17A, IL-2, and IFNγ. Ctrl denotes mouse that has not undergone transplantation, LT denotes mouse that has undergone transplantation with saline treatment, and LTO denotes mouse that has undergone transplantation and received ODSC treatment.
Discussion

We have demonstrated in a robust model of mouse allogenic lung transplantation in which infusion of ODSCs attenuates airway inflammation. ODSCs may also have a mild effect on acute rejection although it was not found to be statistically significant. Observation of T-cell responses in out knock-in mouse model, demonstrated that T-cells accumulate rapidly following transplantation to the lung, with peak intensity observed from day 9 to 11. ODSC treatment given directly to the lung in this model reduced T-cell accumulation, matching our in vitro data that ODSCs are capable of inhibiting T-cells. This suggests that the mechanism of ODSC on airway inflammation may be directly due to suppression of T-cells and possibly other immune cells.

Based on this, we set out to analyze the effects of ODSC on memory T-cell responses. ODSCs however, had no effect on memory T-cell responses (Fig. 28) and did not reduce T-cell infiltration to the axillary lymph node qualitatively (Fig. 27) demonstrating the effects of ODSCs in our model may be potentially constrained to the lung.

Research on cellular therapies is still in its infancy and there are many obstacles that need to be overcome. This represents the first experimental study of ODSCs as a cellular therapy in a lung injury model. Our model highlights that ODSCs may have potential use in disease models but require further studies into understanding cellular homing, and retention in injured tissues to optimize their tissue regenerative and anti-inflammatory responses. One may speculate that if the beneficial properties are further delineated by experimental studies, omental cell extraction from transplant patients where the cells are expanded and activated in vitro may have therapeutic use. The autologous
nature and abundance of omentum residing in patients may be highly exploitable in this context.

Further studies are needed to provide evidence for the practicality and feasibility of this for clinical usage. We propose future research should be done to establish an activated model of omentum in humans, further expand the use of cellular therapy to other experimental models and importantly assess potential adverse events of ODSC use.
Dickinson’s early descriptions on the function of omentum have in a large part shaped its usage for the century that followed it. Many surgeons harnessed the functions of the omentum in promoting angiogenesis and in tissue protection in a variety of surgical situations with success. This led to its use in the procedure, omental transposition, where the omentum is elongated to form a pedicle such that it can be wrapped over the injured organ or tissue to promote healing presumably by stimulating regeneration, promoting neovascularization, and contributing regenerative cells. A major shift in thinking regarding the omentum occurred in 2009, when Rangel-Moreno and colleagues demonstrated that T-cell and B-cell antigen presentation and proliferation could occur within the milky spots of the omentum, validating the role of the omentum as a secondary lymph organ. Follow-up studies supported this viewpoint, suggesting it acts as another layer of defense for the peritoneum (Gray et al., 2012).

However, the puzzling aspect of this is that despite its role, the omentum is a non-essential tissue, and is removed at times in treatment of gastric cancer, leading back to the question- what is the role of omentum biologically? The experiments on animals receiving omentectomy in peritonitis models demonstrated that the omentum may help in some instances, but still shows that often times, peritoneal immune responses are more than adequate to control infection. If the omentum is not a required contributor for the immune responses in the peritoneum, what other role could it be playing?
Our studies into omentum may shed some light on this question. Importantly, we have a model of activated omentum which we have followed up from Litbarg and colleagues, where polyacrylamide bead injected into the omentum rapidly expands the tissue. Our studies have shown that activated omentum differs dramatically from resting omentum. This key point suggests that the omentum acts as a reservoir for rapid cellular proliferation or recruitment of cells to peritoneal responses. Interestingly, our model demonstrates that the cellular composition of omentum can rapidly shift toward that of an immature cell composition, comprising of MDSC and MSC-like composition. This is a composition that is very different from the normal distribution of mature macrophages, T-cells and B-cells that other investigators have identified in resting omentum. In our animal model, injection of these beads rapidly expands the omentum in as little as 5-days from a relatively thin tissue, to a fleshy mass that measures roughly 3-5 mm in size. In some instances, we have observed omental expansion from the stomach to nearly cover the entire colon.

What is the function of this expansion? We have identified these omental cells to be predominantly immunosuppressive against inflammatory subsets like Th1 and Th17, and tolerance promoting through expansion of Tregs. This largely suggests to us that omental tissue may act under an activated state to supply myeloid cells in the peritoneum, and that these replenished cells have a large role in immunoregulation. This immunoregulatory role is supported by much of the clinical observations, particularly the applications where omentum has been used as a site of graft placement. Presumably, recruitment of immunosuppressive MDSCs and MSC-like cells in those scenarios may underlie the success of those models in lieu of the simple explanation of
neovascularization that is often attributed to their success. In a bigger picture, what could be happening is that the omentum are receiving “activating” conditions and signals from the peritoneum, may act to replenish cells to the gut and adipose tissue and importantly, may even expand under circumstances of inflammation as a regulatory tissue to curb excessive inflammation in the peritoneum.

In a clinical context, the idea of omentum as an immunomodulatory depot for cells could have important consequences. To this day, inflammatory bowel disease has an unknown etiology, although it is generally thought that environmental factors set off an ongoing inflammatory process. Clinical evidence has associated the presence of Th17 cells in bulk within the colon, although it is still unclear if they play a protective or pathogenic role (Wallace et al., 2014). The omentum has a very close relationship with the bowel in humans as it covers the entirety of the organ and shares vasculature with the mesentery. So far it has not been studied if there exists a relationship between the omentum and chronic inflammation in the gut. A shunting of omentum composition or state may be associated to a lack of immunoregulation of the peritoneum. If omentum is somehow altered, say by obesity or some underlying infection, it is possible that a lack of regulatory myeloid cell replenishment occurs, leading to a shunting of inflammatory responses in the gut.

In addition, one might propose that the MDSC population is not necessarily a stable one within the omentum. It is possible these cells mature and help replenish macrophage populations within the peritoneum and adipose tissue. M2 macrophages play an important role for Treg promotion as well as promoting healing and regenerative responses in adipose tissue and the migration of these cells in clinical cases of omental
transposition certainly could be the possible mechanism (Harford et al., 2011). In our model, we propose M2 macrophages may be partly replenished by the omentum. Future experiments where omental cells are tagged and then tracked should be able to better delineate if these cells eventually play an alternative role in the peritoneum.

Ultimately, the capacity to curb such inflammatory responses to the gut is critical, as when inflammatory processes are overwhelming, such as in the case of peritoneal sepsis, they are often fatal. Our model of the omentum as a depot for regulatory myeloid cells would fit the notion of omentum as non-essential tissue. Removal of omentum under this model would not have any short-term consequences as presumably immune cells could be replenished by other sources. However, in long-term scenarios, coupled with other environmental insults, the lack of omentum could presumably lead to then pathology that would be more difficult to identify, where inflammatory processes then become more difficult to reign in and lead to chronic inflammation. It would be of interest to examine the omental composition of patients undergoing disease processes, to determine if there is a signature response of the omentum to specific diseases, given the diversity of cells within the tissue.

Therapeutically, the omentum could represent a fairly lucrative target for autologous cell therapy. Expansion of omental cells in a patient dealing with inflammatory bowel disease, and reintroduction into the host could be an interesting way of attempting to reign in imbalance that has occurred in the course of the disease. The challenges to this of course, reside in that omentum within humans contains more adipocytes. In addition, we do not know the phenotype of activated omentum for human models, which would need to be established. Ways to begin this investigation would
likely be fundamentally to look at omentum of patients with feeding tubes and compare them to those without.

The presence of a feeding tube in the peritoneum, could act as a crude human proxy for “activated” omentum, as clinical observations have described omentum growth around those objects. Studies on human omentum under this state would answer the question if the findings in mice were indeed translatable.

**Omentum Cells as Cellular Therapy**

Cellular therapy is still very much in its infancy, and currently studies are still laying the groundwork in regards to how to expand cells and appropriately target them to injured organs or tissues. Our *in vivo* studies on activated omentum have some insights into the pros and cons of autogulous cellular therapy. To begin, the omentum in the mouse model, dose appear to be a significant source of cells that can be rapidly expanded *in vivo*. A complication is that the cellular composition of omentum needs to further phenotyped, and there also remains questions of whether specific cell types should be separated from the omentum for cellular therapy.

From a treatment standpoint, the effect we managed to observe was limited as it produced no long-term effects in having minimal effect on secondary T-cell responses. However, in some ways this is telling of how transient the effects of omentum can be when given as a cellular therapy. Clinically, omental transposition involves harvesting a pedicle of the whole tissue, and guiding it to the site of injury, where it remains joined with the vasculature, and can thereby continually secrete factors or supply cells. In our omental model, we resuspend omentum into single cell suspension. These cells when
used, may no longer continue to expand, differentiate to mature macrophages, or traffic back to other tissues. These questions should be addressed by future studies.

In our model, we speculate that injection of immature myeloid cells for example, could lead to maturity, and integration into the host, whereas differentiation of MSC-like cells may provide some contribution to the tissue as well. The long-term effects of this type of injection likely are limited, as these cells, may not reside in the tissue long or may not be able to retain their phenotype.

The problems we face are the same problems the MSC therapy field runs into today. While therapeutic effects have been reported in some limited trials, the long-term effects of these cells is not well known and the mechanisms they utilize in vivo are still unknown (Ng et al., 2014). While use of cellular therapy is still being explored, many questions to address efficacy, dosing, and mechanism are needed before further examination of omentum therapy would be appropriate.

The exciting angle however, is that the concept of using MDSC cells in cellular therapy is novel, and only a few studies have explored using MDSCs expressing iNOS from a therapeutic standpoint. Interestingly, they have been shown to be immunoprotective in a kidney graft model, in a mechanism that is dependent on production of NO (Arakawa et al., 2014). This raises the question if MDSCs should be considered as an adjuvant in other cellular therapies. These cells, for example, may be worth injecting with Treg therapy, as they have been demonstrated to enhance proliferation of Tregs. This action in conjunction with the broader inhibition provided by NO production, could have a more potent regulatory effect than just transfer of cells alone.
**ODSCs and T-cell Inhibition**

The immune system has multiple methods of regulation, and it is not surprising that we have identified several mediators released by ODSCs that affect T-cell proliferation and cytokine expression. NO, PGE2, and TGFβ all are involved in inhibition in some facet, with NO contributing to a block in proliferation, PGE2 affecting IL-17A expression and TGFβ affecting IL-2 expression. The novelty of our model seems to be in the earlier events that ODSCs have on Th17 cells.

The early observation of anergy in our Th17 cells leads to a question of how these cells end up in this state and what causes it. Classically, anergy is defined as a T-cell that is unable to proliferate to stimulation and does not produce IL-2. We indeed observe this phenotype in our cells as early as day 1. It has been suggested that anergy is an intermediate stage for T-cells into either becoming Treg or acting in a preapoptotic state (Kuklina E. M., 2013). Our model suggests neither of these possibilities, as we have not detected any Th17 cells transdifferentiating into Treg (not shown) and have not been able to detect any significant increases in annexin V staining as a marker for apoptosis on either day 1 or day 2 after co-culture. As a consequence, it still raises the question of what cell phenotype we are observing after our co-culture.

There are several suggestions that direct engagement of ODSCs with Th17 cells occurs that may lead to this early phenotype. We have observed these cells form clusters with ODSCs quite readily. In addition, we have observed polarization of surface molecules like CD90.2, which we used to stain the T-cells. Normally, CD90.2 is
observed as evenly distributed around the cell membrane, under ODSC-co-culture conditions, it is observed polarized to one end of the cell. Interestingly this is not translatable to all markers, as CD4 is still found in an even distribution around the cell surface. This suggests that ODSCs may form some kind of synapse with Th17 cells early on, and this direct contact signaling might be where anergy is induced early on. CTLA4 expressed on Tregs can block co-stimulatory signals and lead to IDO upregulation but neither of them seemed to be involved in our system.

We have explored the hypothesis if ODSCs were involved in inducing autophagy in our Th17 cells. This model would be consistent with the increases in side scatter and the expansion of ER within our cells that would suggest aberrant activation of autophagy could produce an anergic state. However, we were not able to detect LC3, a hallmark marker of autophagy membranes, excluding this possibility.

Speculation into what is happening to our Th17 cells early on suggest that they may be “stimulated” somehow in a way that aberrantly activates TCR-signaling. We do know that a fraction of ODSCs are MHC Class II positive, suggesting they can present antigen to CD4+ T-cells. As mentioned previously, the cell-to-cell dependent contact coupled with staining data suggest the formation of a synapse. Our IRF4 upregulation coupled with redistribution into the nucleus also points towards a TCR-activating event, as IRF4 upregulation has been shown to occur with TCR-activation. It is generally thought that IRF4 is upregulated with BATF early on in Th17 cells to alter chromatin accessibility to allow for then Th17 specific transcription factors to transcribe their target genes (Ciofani et al., 2012). Our images do reveal IRF4 upregulated, and broadly distributed throughout the nucleus, suggesting that it may be acting in this manner
following ODSC co-culture (Fig. 22). The aberrant expression of IRF4 by ODSCs, may suggest the possibility of some kind of “reset” mechanism, where Th17 cells are reactivated in an environment where they are no longer poised to proliferate and differentiate appropriately. Upregulation of IRF4 also occurs when neutrally activated T-cells are co-cultured with ODSCs, demonstrating T-cell this activation is possibly broadly applicable to other T-cell subsets.

To address further questions of the biological relevance of IRF4 upregulation, further experiments should be performed to look at whether ODSCs can play a role in early co-inhibition/stimulation of T-cells. We have briefly explored whether ODSCs can act as antigen-presenting cells by co-culturing them with Th17 cells in the presence of TCR-activating anti-CD3. In this model they proved to be still inhibitory. However, this was in the presence of IFNγ and activation of the NO/PGE2 pathway. A clean experiment to test the effects of ODSCs on T-cell activation would be to take T-cells in fresh media with activating anti-CD3 and co-culture them with irradiated ODSCs and examine T-cells for activating markers. We have only performed ODSC-co-culture under activated T-cell conditions that is T-cells activated by TCR engagement with irradiated APCs from the spleen. It would be interesting to determine from this model (a) if ODSCs have any co-stimulatory or inhibitory effect on T-cell activation (b) if IRF4 is upregulated in these cells (c) determine the phenotype of these cells following co-culture. One might expect that ODSCs are still predominantly inhibitory, but it is possible that we may observe activation and differentiation toward Tregs if the NO/PGE2 pathway is not activated. Alternatively, another way to possibly get at this question would be to dilute the ratio of ODSC to T-cells down from the 1:1 ratio they are currently used to determine
the minimal threshold for IRF4 activation. Currently our assay is optimized for Th17 inhibition which is NO/PGE2 dependent, but an assay that is optimized for IRF4 upregulation may involve a lower threshold as the events occur earlier on. In situations where the ratio of ODSC to T-cells is 1:2 or greater, inhibition of Th17 cells through cell number or percentage begins to decline. If we can still detect IRF4 upregulation however, this may be an ideal system to then determine what the effect of IRF4 upregulation is, regarding cellular phenotype and expression. If we can minimize these other immunomodulators, then a thorough profiling of these IRF4hi cells may provide insights into the significance or IRF4 activation without the effects of other immunomodulators.

**Conclusions**

Activation of omentum demonstrates a broad expansion of MSC-like and MDSC cells in the tissue. The function of this expansion may act as a regulatory mechanism for the peritoneum, an innate immune response that also broadly has immunoregulatory function, and recruitment of MSC-like cells that then are poised to play a role in healing and regeneration following resolution of the foreign insult.

The rapid response of the omentum in this way may be underlying the mechanism for where surgeons have used the omentum for a tolerance-inducing environment for surgical grafts, as physical manipulation of the omentum likely activates some of these same pathways, and transposition of the tissue may permit transfer of these cells to the site of injury. Learning to harness these cells and possibly, activation of omentum prior to transposition could enhance its use for surgery.
BIBLIOGRAPHY


VITA

Nick Huang attended the public magnet school, Hunter College High School in New York, NY. He attended Pitzer College in Claremont, CA in 2002, transferred the following year into Claremont McKenna College in Claremont, CA and graduated with a BA in biology. He worked at City of Hope Cancer Center in Duarte, CA as a research assistant. During that time he was published in PLOS One on alternative non-homologous end joining, a pathway of DNA repair.

Huang was accepted in Loyola University Chicago for the MD/PhD program. He joined the laboratory of Dr. Makio Iwashima and worked in a collaborative effort between the Microbiology / Immunology department and Cardiovascular Surgery. In this lab, he studied the omentum. He has given oral presentations for his work at the International Society for Heart and Lung Transplantation (ISHLT) and for the American Thoracic Society in 2013. Huang has presented abstracts for the Autumn Immunology Conference (2013) and at ATS in 2012.

Huang has authorship in “Cellular Basis of Tissue Regeneration by Omentum” published in PLoS ONE in 2012, and is currently in submission for a manuscript regarding this work. He aspires to work in translational research in the future, with interests in the field of Infectious Disease.